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# Glucocorticoids impair platelet thromboxane biosynthesis in community-acquired pneumonia

Roberto Cangemi[1*], Roberto Carnevale[2*], Cristina Nocella[2], Camilla Calvieri[3], Vittoria Cammisotto[1], Marta Novo[1], Valentina Castellani[1], Alessandra D'Amico[2], Chiara Zerbinati[2], Lucia Stefanini[1] and Francesco Violi,[1] in collaboration with the SIXTUS Study Group.

[1]Department of Internal Medicine and Medical Specialties, Sapienza University of Rome, Rome, Italy.

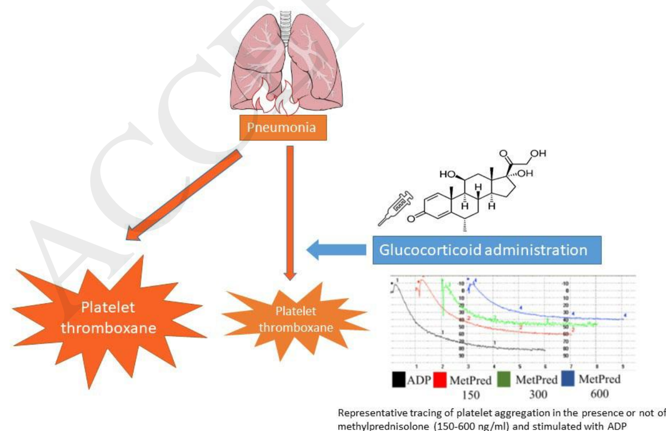
[2]Department of Medical-Surgical Sciences and Biotechnologies, Sapienza University of Rome, Latina, Italy.

[3]Department of Cardiovascular, Respiratory, Nephrology, Anesthesiology and Geriatric Sciences, Sapienza University of Rome, Rome, Italy.

*equally contributed

[*]**Corresponding author**: Prof. Francesco Violi, I Clinica Medica, Department of Internal Medicine and Medical Specialties, Viale del Policlinico 155, Rome, 00161, Italy; phone: +39064461933; fax: +390649970103; e-mail: francesco.violi@uniroma1.it

**Graphical Abstract:**



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**ABSTRACT**

Previous reports suggest that community-acquired pneumonia (CAP) is associated with an enhanced risk of myocardial infarction (MI) and that enhanced platelet activation may play a role.

Aims of this study were to investigate if urinary excretion of 11-dehydro-thromboxane (Tx) $B_2$, a reliable marker of platelet activation in vivo, was elevated in CAP and whether glucocorticoid administration reduced platelet activation.

Three-hundred patients hospitalized for CAP were recruited and followed-up until discharge. Within the first 2 days from admission, urinary 11-dehydro-$TxB_2$ and serum levels of methylprednisolone and betamethasone were measured. 11-dehydro-$TxB_2$ was also measured in a control group of 150 outpatients, matched for age, sex, and comorbidities. Finally, in-vitro studies were performed to assess if glucocorticoids affected platelet activation, at the same range of concentration found in the peripheral circulation of CAP patients treated with glucocorticoids.

Compared to controls, CAP patients showed significantly higher levels of 11-dehydro-$TxB_2$ (110 [69-151] vs. 163 [130-225] pg/mg creatinine; $p<0.001$). During the in-hospital stay, 31 patients experienced MI (10%). A COX regression analysis showed that 11-dehydro-$TxB_2$ independently predicted MI ($p=0.005$). CAP patients treated with glucocorticoids showed significantly lower levels of 11-dehydro-$TxB_2$ compared to untreated ones (147 [120-201] vs. 176 [143-250] pg/mg creatinine; $p<0.001$).

In vitro, glucocorticoids-treated platelets showed a dose-dependent decrease of ADP-induced platelet aggregation, $TxB_2$ production, $cPLA_2$ phosphorylation and arachidonic acid release from the platelet membrane.




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In conclusion, platelet $TxB_2$ is overproduced in CAP patients and may be implicated in MI occurrence. Glucocorticoids reduce platelet release of $TxB_2$ in vitro and urinary excretion of 11-dehydro-$TxB_2$ in vivo and may be a novel tool to decrease platelet activation in this setting.

**ABBREVIATIONS:**

| | |
|---|---|
| AA: | arachidonic acid |
| CAF: | chronic (persistent or permanent) atrial fibrillation |
| CAP: | Community-acquired pneumonia |
| CHD: | coronary heart disease |
| COPD: | chronic obstructive pulmonary disease |
| CVE: | cardiovascular event |
| HF: | heart failure |
| HS: | healthy subjects |
| MI: | myocardial infarction |
| PAD: | peripheral arterial disease |
| PAF: | paroxysmal atrial fibrillation |
| PRP: | platelet-rich plasma |
| PSI: | pneumonia severity index |
| T2DM: | type 2 diabetes mellitus |
| Tx: | Thromboxane |

**Keywords**: Glucocorticoids, Myocardial infarction, Platelet activation, Pneumonia, Thromboxane B2

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## 1. INTRODUCTION

Community-acquired pneumonia (CAP) is one of the most common causes of hospitalization in medical wards and represents a serious social issue. In fact, the incidence of CAP steadily increases with age and is associated with a higher rate of morbidity and mortality, particularly in the elderly [1]. Among the reasons for the increased risk of morbidity and mortality, thrombotic complications occurring in the coronary and cerebral districts seem to play an important role. In a recent multicenter study conducted in 1,182 patients hospitalized for CAP, 32.2% experienced cardiovascular disease including heart failure (HF), myocardial infarction (MI), atrial fibrillation (AF) and stroke during the in-hospital stay [2]. The occurrence of such complications has a relevant clinical impact since CAP patients experiencing cardiovascular events (CVEs) during hospitalization are at higher risk of mortality and cardiovascular recurrences in short- and long-term follow-up [2, 3]. Among the factors influencing the occurrence of in-hospital CVEs, the severity of pneumonia has a prominent role as indicated by the independent association between pneumonia severity index (PSI) score and cardiovascular events [2]. Other factors contributing to in-hospital CVEs could include platelet activation, since ex-vivo markers of platelet activation, such as soluble CD40L, soluble P-selectin and serum thromboxane (Tx) $B_2$, are elevated during the acute phase of the disease and independently predict MI [4]. However, neither these soluble serum markers nor serum $TxB_2$ adequately reflect platelet activation in vivo [5], thus further study is necessary to assess if platelets are really overactivated in CAP patients. Furthermore, CAP patients treated with aspirin did not display complete COX1 inhibition [4], suggesting that other pathways of platelet activation could be targeted to prevent platelet activation in this clinical setting [6-8].

Previous studies investigated whether glucocorticoids could improve clinical outcomes such as mortality in CAP patients, but the results were equivocal [9, 10]. Experiments in vitro and in vivo

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demonstrated that glucocorticoids inhibit platelet aggregation and platelet-related arterial thrombosis [11-14]; conversely, interventional studies with variable glucocorticoid doses showed no effect on ex-vivo platelet aggregation and urinary excretion of 11-dehydro-$TxB_2$ [15-17], a reliable marker of platelet activation in vivo [18]. To further explore this issue, we investigated 1) if platelet activation assessed by urinary excretion of 11-dehydro-$TxB_2$ is elevated in a large population affected by CAP compared to controls, matched for demographic and clinical characteristics, 2) if elevated levels of urinary excretion of 11-dehydro-$TxB_2$ are associated with MI risk in this cohort of CAP patients, 3) if glucocorticoid administration reduces platelet activation of CAP patients in vivo and 4) if glucocorticoids affect platelet activation in vitro, at the same range of concentration found in the peripheral circulation of CAP patients treated with glucocorticoids.

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## 2. MATERIALS AND METHODS

### 2.1. Study Population

This cohort study recruited patients from January 2012 to July 2017 at three medical centers of the University-Hospital Policlinico Umberto I, Rome, Italy (Department of Internal Medicine and Medical Specialties, Department of Clinical Medicine, Department of Public Health and Infectious Diseases).

After they gave written informed consent, we enrolled 300 consecutive patients who fulfilled the following criteria in the study: (1) age ≥18 years; (2) clinical presentation of an acute illness with at least two or more of the following signs or symptoms of CAP: presence of rales, rhonchi, bronchial breath sounds, dullness, increased fremitus and egophony, fever (>38.0˚C), tachycardia, chills, dyspnea, coughing (productive or unproductive cough), chest pain; and (3) presence of new consolidation(s) on chest X-ray. Pneumonia was considered as CAP if it was diagnosed upon hospitalization and the patient had not been discharged from an acute care facility within 14 days preceding the clinical presentation [2].

Patients were excluded from the study if any of the following criteria applied: radiographic evidence of pre-existing infiltrates; immunosuppression (HIV infection, chemotherapy, high dose of immunosuppressive agents to prevent the rejection of transplanted organs and tissues or to treat autoimmune diseases); presence of malignancy; pregnancy or breastfeeding; documented severe allergy to antibiotics; healthcare-associated pneumonia [19].

One-hundred and fifty controls were selected among outpatients referring to the Day Service of Internal Medicine and to the center for monitoring and management of antithrombotic therapies of the Department of Internal Medicine and Medical Specialties, Sapienza University

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Hospital. They were matched for age, sex, smoking habit, and comorbidities, including hypertension, diabetes, CHD, HF, dyslipidemia and chronic renal failure.

The present study was conducted according to the principles stated in the Declaration of Helsinki. The institutional review boards at each Institution approved this study, which was registered at ClinicalTrials.gov (Identifier: NCT01773863).

**2.2 Baseline assessment**

Data on demographic characteristics, comorbidities and concomitant therapy were collected. The severity of illness at presentation was quantified by the Pneumonia Severity Index (PSI), a validated prediction score for 30-day mortality [20].

**The PSI score is comprised of several variables: age, gender, co-morbid illnesses, vital sign abnormalities, plus laboratory, blood gas, and radiographic parameters. The score was calculated as presented in the study by Fine et al. [20] and divided in five classes. Patients in the first three classes are considered at low risk, patients in the fourth class at moderate risk and patients in the fifth class at high risk.**

Pre-existence of T2DM, hypertension, dyslipidemia, history of CHD, COPD, PAD, HF, and AF were defined as previously described [21-25]. Baseline treatments were defined according to the patients' pharmacological history.

Immediately after diagnosis of CAP, routine blood laboratory tests including serum high-sensitivity cardiac troponin T (hs-cTnT), a 12-lead electrocardiography and arterial blood gas test were performed.

**2.3 Assessment of myocardial infarction during the in-hospital stay.**

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The occurrence of an MI was diagnosed according to the "Third Universal Definition of Myocardial Infarction" [26], ST-elevation MI (STEMI) and non-ST-elevation MI (NSTEMI) were defined as previously reported [26] and were confirmed by cardiologists.

**2.4 Urinary $TxB_2$ assay**

Urinary 11-dehydro $TxB_2$ was analyzed by ELISA commercial kit (Cayman, USA). The absorbance was measured with a microplate reader at 405 nm and expressed as pg/mg creatinine. Intra-assay and inter-assay coefficients of variation were 3.6% and 7.6%.

**2.5 HPLC determination of Methylprednisolone and Betamethasone in serum**

**Serum sample was collected in BD Vacutainer (Franklin Lakes, New Jersey, USA) without anticoagulant (code 367615) by centrifugation at 300g for 10 min at room temperature. The supernatant serum was divided into aliquots and stored at −80°C for analyses.** Stock solutions of methylprednisolone and betamethasone were prepared at a concentration of 1 mg/ml in methanol and diluted to 100 µg/ml in methanol for working stock solution. A stock solution of internal standard (IS, 17α-Hydroxyprogesterone; Sigma Aldrich, Saint Luis, USA) in methanol was prepared and diluted to 150 µg/ml. Standard solutions of methylprednisolone and betamethasone were prepared in the mobile phase and IS was spiked to achieve a constant concentration of 5 µg/ml.

500 µl of 5% acetic acid solution containing IS and 5 ml of hexane were added to 500 µl of serum into a test tube and centrifuged for 5 minutes at 1400g. After extraction with ethyl acetate, samples were evaporated to dryness. The residue was dissolved in 500 µl of mobile phase and 25 µl were injected into the column and analyzed using an Agilent 1200 Infinity series high-performance liquid

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chromatography system equipped with an Eclipse Plus C18 column (4.6 x100 mm). The mobile phase was prepared by mixing acetonitrile-water-glacial acetic acid (35:62:2% v/v) with a flow rate of 1.2 ml/min and peaks were analyzed at 254 nm.

**2.6 In vitro study**

*2.6.1 Platelet Aggregation*

Venous blood was drawn in trisodium citrate (3.8%, 1/10 (v: v)) from healthy subjects (HS, n=5) who had fasted for at least 12 hours. To obtain platelet-rich plasma (PRP), blood was centrifuged for 15 min at 180 g at room temperature and the supernatant PRP was separated **(2 x $10^5$ platelets/ µl).** To avoid leukocyte contamination, only the top 75% of the PRP was collected. Within thirty minutes after venipuncture, PRP samples (250 µl) were pre-incubated (20 minutes at 37°C) with scalar concentration (150-300-600 ng/ml) of methylprednisolone (Sigma Aldrich, Saint Luis, USA) or betamethasone (Sigma Aldrich, Saint Luis, USA) or equal volumes of DMSO (5%). After incubation, samples were stimulated with collagen (2 µg/ml; Mascia Brunelli) or adenosine diphosphate (ADP, 10 µM; Sigma Aldrich, Saint Luis, USA) for 10 minutes at 37°C. Platelet aggregation was performed on a Bio/Data 8-channel platelet aggregometer (PAP-8E BioData) according to the method of Born [27]. After stimulation with agonists, samples were centrifuged for 3 minutes at 3000 rpm. Supernatants were stored at -80°C for analysis of TxB2 and pellets were stored at -80°C for analysis of cPLA2 phosphorylation and arachidonic acid production.

*2.6.2 Western blot analysis of $cPLA_2$ protein phosphorylation*

Platelet pellets were suspended in a 2X Lysis buffer (5 mM EDTA, 0.15 mol NaCl, 0.1 mol Tris pH

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8.0, 1% Triton and 10 µg/ml of protease and phosphatase inhibitors cocktail). The protein concentration of each lysate was determined by Bradford assay. Equal amounts of protein (30 µg/lane) were solubilized in a 2X Leammli sample buffer containing 20% of 2-mercaptoethanol and were electrophoretically separated on a 10% SDS-polyacrylamide gel and then electrotransferred to nitrocellulose membranes. After blocking with Bovine Serum Albumin (BSA) 5% (Sigma Aldrich, Saint Luis, USA) the membranes were incubated overnight at 4°C with a polyclonal anti-p-cPLA$_2$ antibody or a polyclonal anti-cPLA$_2$ antibody (both Santa Cruz Biotechnology). 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.

*2.6.3 Platelet TxB$_2$ assay*

Platelet TxA$_2$ generation was analyzed as previously described [28] by evaluating its stable metabolite TxB$_2$ by ELISA commercial kit (Cusabio, USA) and expressed as pg/ml x10$^8$ cells. Intra- and inter-assay coefficients of variation for TxB$_2$ were 4.0% and 3.6%, respectively.

*2.6.4 Platelet arachidonic acid assay*

Arachidonic acid was analyzed in platelets by gas chromatography as previously described [29, 30]. Briefly, Arachidonic acid was processed for direct transesterification with acetyl chloride and evaluated using mixtures of authentic methylated Fatty Acid standards and a control plasma pool as previously described [31]. Analyses were performed on an Agilent 7820A Plus Gas Chromatograph (Agilent Technologies) equipped with a G4513A automatic liquid sampler and a

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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).

**2.7 Statistical analysis**

*2.7.1 Sample size*

The minimum sample size was computed considering a relevant difference in urinary 11-dehydro-$TxB_2$ levels to be detected between groups $|\delta|\geq 20$ pg/mg/creatinine, (ii) SD of the paired differences SD=30 pg/mg creatinine (iii) a type-I error probability $\alpha=0.05$ and power $1-\beta=0.90$. This resulted in a minimum of 48 patients per group.

*2.7.2 Statistical methods*

All continuous variables were tested for normality with the Shapiro-Wilk test. Variables with normal distribution were expressed as means and standard deviations (SD) and tested for differences using a t-test. Appropriate nonparametric tests were employed for all the other variables. Non-parametric variables were expressed as median and interquartile range (IQR). Categorical variables were expressed as percentages and analyzed by chi-squared test.

Cox proportional hazards analysis was used to calculate the adjusted relative hazards of MI by each clinical variable.

In addition to urinary 11-dehydro $TxB_2$, possible independent variables considered were age, sex, body mass index (BMI), PSI score, smoking habit, history of CHD and stroke, dyslipidemia, T2DM, hypertension, HF, chronic kidney disease, COPD, PAD, PAF, and CAF. Stochastic level of entry into the model was set at a p-value=0.10, and interaction terms were explored for all the variables in the final model. For multivariate models, model selection was performed using forward stepwise



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regression. To avoid collinearity issues, age did not enter the multivariate model, as it is a fundamental component of the PSI score [20].

Nonparametric tests were employed in the analysis of the in vitro study. As an overall non-parametric ANOVA, the Friedman test for the analysis of intragroup variations was used. In cases of significance, we compared pair related samples using the Wilcoxon test.

Only p values <0.05 were regarded as statistically significant. All tests were 2-tailed and analyses were performed using computer software packages (IBM SPSS Statistics 24.0; R version 2.15.2, R Development Core Team, Vienna, Austria).

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## 3. RESULTS

### 3.1 Urinary 11-dehydro-TxB2 in CAP patients and controls.

Clinical characteristics of CAP patients and controls are reported in table 1.

Three-hundred patients hospitalized for CAP (201 males; 99 females; age: 74.3 ± 14.1 years) were recruited. Most of the patients had arterial hypertension (74%). A history of coronary heart disease (CHD) was present in 41% of patients, previous stroke in 13%, type 2 diabetes mellitus (T2DM) in 36%, chronic obstructive pulmonary disease (COPD) in 35%, peripheral arterial disease (PAD) in 7%, dyslipidemia in 29% and chronic kidney disease in 16%. A history of paroxysmal AF (PAF) was present in 17% of patients, while 12% were affected by chronic (persistent or permanent) AF (CAF).

No significant differences in age, gender, BMI, smoking habit, hypertension, dyslipidemia, history of CHD, HF, history of stroke, chronic kidney disease, PAD, PAF, and CAF were found between CAP and controls (n=150); conversely, CAP patients were more frequently affected by COPD and treated with heparins while controls were more frequently treated with oral anticoagulants (tab.1).

Compared to controls, CAP patients showed higher levels of urinary 11-dehydro-$TxB_2$ (110 [69-151] vs. 163 [130-225] pg/mg creatinine; $p<0.001$).

During the in-hospital stay, 31 patients experienced MI (10.3%), most of them were NSTEMI (n=29), while 2 were STEMI. Most of the MI occurred within the first days from hospitalization: 52% within the first day, 81% within 2 days and 94% within 4 days.

CAP patients who experienced MI during hospitalization had higher levels of urinary 11-dehydro-$TxB_2$ than patients who did not (175 [150-271] vs. 163 [130-222] pg/mg creatinine;

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p=0.042). A COX regression analysis showed that urinary 11-dehydro-$TxB_2$ and PSI score independently predicted MI occurrence during the in-hospital stay, after adjusting for possible confounding factors (Table 2).

**3.2 Corticosteroids and urinary 11-dehydro-$TxB_2$.**

One-hundred and one CAP patients (33%) were treated with i.v. corticosteroids during the first day from hospitalization.

The most common glucocorticoids used were methylprednisolone (n=56; 20-80 mg/day); followed by betamethasone (n=28; 4-8 mg/day) and prednisone (n=15; 25-50 mg/day).

Overall, urinary 11-dehydro-$TxB_2$ was lower in CAP patients treated with glucocorticoids compared to untreated ones (147 [120-201] vs. 176 [143-250] pg/mg creatinine; $p<0.001$)

Urinary 11-dehydro-$TxB_2$ was also lower in patients treated with glucocorticoids and aspirin (100 mg/day) compared to patients treated with aspirin alone (125 [115-140] vs. 140 [130-162] pg/mg creatinine; $p<0.001$). During the in-hospital stay, MI was detected in 7 (6.9%) glucocorticoid-treated patients and in 24 (12.1%) untreated ones.

HPLC analyses in randomly selected serum of 63 out of 101 CAP patients treated with glucocorticoids showed that serum betamethasone (n=21) and methylprednisolone (n=42) levels were 527±366 ng/ml and 348±302 ng/ml, respectively. In 23 randomly selected out of 199 patients untreated with glucocorticoids serum levels were undetectable (not shown).

**3.3 In vitro study**

*3.3.1 Glucocorticoids and agonist-induced platelet aggregation.*

Platelets from HS (n=5, males 3, females 2, age 40.2±3.4 years) were incubated with ADP (10 μM) or collagen (2 μg/ml) in presence or absence of scalar concentrations of betamethasone or

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methylprednisolone (150-600 ng/ml), which corresponded to the levels detected in serum of patients upon glucocorticoid administration. Betamethasone- and methylprednisolone-treated platelets showed a dose-dependent decrease of ADP-induced platelet aggregation compared to untreated ones (Fig. 1, panels A-D). Conversely, no changes of platelet aggregation were observed in betamethasone and methylprednisolone-treated platelets after stimulation with collagen (Fig. 1, panels E and F).

*3.3.2 Glucocorticoids and TxB2-mediated positive feedback signaling*

Betamethasone-treated platelets stimulated with ADP showed a dose-dependent decrease of $TxB_2$ production compared to untreated ones (Fig. 2, panel A). Similar results were observed with methylprednisolone -treated platelets stimulated with ADP (Fig. 2, panel B).

To further investigate the mechanism accounting for glucocorticoid-dependent platelet inhibition, we studied $cPLA_2$ phosphorylation, a key enzyme for the release of eicosanoids from the platelet membrane that has already been shown to be inhibited by glucocorticoids in other cell types [32, 33]. Indeed, betamethasone (Fig. 3, panels A and B) and methylprednisolone dose-dependently (Fig. 3, panels C and D) reduced ADP-induced $cPLA_2$ phosphorylation (Fig. 3, panels A and D). Furthermore, we found that both betamethasone and methylprednisolone-treated platelets stimulated with ADP reduced AA release from platelet membrane compared to control (Fig. 3, panels E and F).

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## 4. DISCUSSION

The present study shows that in vivo platelet activation, as assessed by urinary excretion of 11-dehydro-$TxB_2$, is enhanced and associated with MI in CAP patients, thus reinforcing the hypothesis that platelet activation might play a role in precipitating acute coronary syndrome in this setting. We also show that glucocorticoids exert an antiplatelet activity via $cPLA_2$ inhibition, an effect that may become useful in CAP patients, in whom inflammation and platelet activation coexist.

Previous studies have outlined the importance of an early recognition of CVEs occurring in the acute phase of CAP, as CVEs increase the risk of uneventful outcomes in short- and long-term follow-up [2, 3]. Among cardiovascular events, MI might occur in approximately 10% of individuals, which agrees with the data of the present cohort [2, 34]; consistent with our previous data, most MI were NSTEMI while the rate of STEMI was negligible [4]. The present study supports and extends a previous report which suggested that platelet activation may be a potential mechanism leading to MI in CAP [4, 35]. In accordance with this, we found that urinary excretion of 11-dehydro$TxB_2$ was elevated in CAP and associated with MI, suggesting that platelet over-production of this eicosanoid could contribute to platelet-related coronary thrombosis and/or coronary dysfunction as a consequence of the vasoconstrictor property of $TXA_2$. This last possibility relies on the fact that most CAP-related MI were NSTEMI, which may occur because of a mismatch between myocardial oxygen “supply and demand” according to the general definition of type 2 MI [26, 36]. However, the implication of platelet activation in the pathogenesis of CAP-related MI is still a matter of discussion as it is unclear if platelet inhibition results in lowered MI occurrence. Also, preliminary data suggest a sort of “aspirin resistance” of CAP patients as platelet $TxB_2$ was not fully inhibited by aspirin [4]. Since glucocorticoids can be administered to CAP patients to improve clinical outcomes (despite data are still inconsistent [9]) we took advantage of this fact to assess if glucocorticoids also possess

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antiplatelet activity in vivo. Comparing CAP patients who were given or not given glucocorticoids, we found a significant decrease of urinary excretion of 11-dehydro-$TxB_2$ in glucocorticoid-treated patients, indicating that these molecules possess an antiplatelet activity. **It is also noteworthy to point out that the combined administration of glucocorticoids and aspirin rendered lower 11-dehydro-TxB levels than either of the monotherapies.**

We also investigated if this antiplatelet effect was reproducible in vitro and explored the underlying mechanism. Thus, we first measured the circulating levels of glucocorticoids in CAP-treated patients. Then we used the range of glucocorticoid concentrations detected in human blood to assess the response to agonists of platelets incubated with glucocorticoids. These experiments showed that glucocorticoids reduce ADP-induced platelet aggregation coincidentally with an impaired production of platelet $TxB_2$. At the same time no effect was seen in collagen-stimulated platelets, which is consistent with a previous in vitro study on this topic [12]. We hypothesized that the reason why glucocorticoids reduce the aggregation response to weak agonists, e.g. ADP, and not to strong agonists, e.g. collagen, is that they interfere with the positive feedback signaling mediated by $TxA_2$. For this purpose, we performed further in vitro experiments to see if glucocorticoids would lower $cPLA_2$ phosphorylation, which initiates arachidonic acid (AA) metabolism via its release from the platelet membrane [37]. Our hypothesis was supported by the fact that dose-dependently glucocorticoids reduced $cPLA_2$ phosphorylation and AA release from the platelet membrane, suggesting that glucocorticoids could impair platelet activation by down-regulating AA metabolism. This mechanism also provides insight into the more powerful reduction of urinary 11-dehydro-$TxB_2$ in patients in whom glucocorticoids were combined with aspirin compared to patients given aspirin alone.

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Our data are in line with previous in vitro studies showing that agonist-induced platelet aggregation and $TxB_2$ formation were inhibited by prednisolone [11, 12]. We also found an inhibitory effect by betamethasone, while previous data regarding other glucocorticoids provided equivocal results [11, 12, 38, 39]. Moreover, clinical studies consistently failed to observe an effect on urinary $TxB_2$ or platelet aggregation [15-17] though we found an inhibitory effect. Some of these previous studies were performed on healthy subjects [15, 16] in whom the inhibitory effect was difficult to appreciate because of normal basal levels of $TxB_2$. Conversely, in patients with high basal levels of $TxB_2$, such as those with unstable angina, a very high dosage of glucocorticoids was employed (1 mg/Kg b.id.) and could have masked the antiplatelet effect [17]. Experimental studies demonstrated that the inhibitory effect of glucocorticoids on platelets is preferentially observed with low/ intermediate doses of glucocorticoids [13]. In the present study, glucocorticoids were used in patients with basal high values of $TxB_2$ and the glucocorticoid dose was on an average of 0.5 mg/Kg per day.

The study has implications and limitations. **The results of the present study further contribute to the hypothesis that platelet activation might be implicated in CAP-related MI but do not establish a cause-effect relationship because trials with antiplatelet drugs are still lacking in this setting. The mechanisms accounting for platelet activation in CAP have not been fully elucidated.** Experimental data demonstrated a virus- or bacteria-dependent platelet interaction which may result in platelet activation [40, 41], but it cannot be excluded that systemic inflammation could also play a role as suggested by the independent association between PSI and platelet activation [4]. In this context, the novel data regarding the antiplatelet activity of glucocorticoids may be useful because, in addition to the anti-inflammatory properties, glucocorticoids possess an antiplatelet effect. This aspect is of potential interest in a clinical setting where the disease severity is another

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important factor associated with MI [4]. It is of note that glucocorticoid-treated patients showed less incidence of MI compared to untreated ones, but the small sample size limits any conclusion. **Thus, randomized clinical trials with glucocorticoids are warranted to assess the potential clinical usefulness in CAP in reducing the potential cardiovascular events associated to pneumonia. At this regard, the potential usefulness of corticosteroids during CAP was recently supported by a meta-analysis, showing that CAP patients treated with glucocorticoids had lower clinical failure rates, a shorter hospital stay, and fewer complications [42]. Among the possible adverse events investigated (hyperglycemia, neuropsychiatric events, gastrointestinal bleeding and adverse cardiac events), only hyperglycemia was significantly more common among corticosteroids-treated patients, but the harm did not seem to outweigh the benefits.**

In conclusion, we report that platelet $TxB_2$ is overproduced in CAP and may be implicated in MI occurrence. Glucocorticoids lower urinary excretion of 11-dehydro-$TxB_2$ and **might** be a novel tool to modulate platelet activation in clinical settings associated with $TxA_2$ over-production.

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**SIXTUS (thromboSIs-related eXTra-pulmonary oUtcomeS in pneumonia) study group:** Simona Battaglia,[a] Giuliano Bertazzoni,[a] Cinzia Myriam Calabrese,[a] Marco Casciaro,[a] Maurizio De Angelis,[c] Paolo De Marzio,[a] Rozenn Esvan,[c] Marco Falcone,[b] Lucia Fazi,[a] Domenico Ferro,[a] Lucia Fontanelli Sulekova,[c] Cristiana Franchi,[c] Laura Giordo,[a] Sergio Morelli,[a] Giulia Naspi Catassi,[a] Paolo Palange,[b] Daniele Pastori,[a] Anna Prosperi,[a] Valeria Raparelli,[a] Marco Rivano Capparuccia,[c] Giulio Francesco Romiti,[a] Elisabetta Rossi,[a] Maria Gabriella Scarpellini,[a] Gloria Taliani,[c] Giusy Tiseo,[b] Stefano Trapè,[a] Filippo Toriello,[a] Marco Vano,[a] Mario Venditti.[b]

[a] Department of Internal Medicine and Medical Specialties, Sapienza University of Rome, Italy.

[b] Department of Public Health and Infectious Diseases, Sapienza University of Rome, Italy.

[c] Infectious and Tropical Diseases Unit, Department of Clinical Medicine, Sapienza University of Rome, Italy.

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**CONFLICT OF INTEREST**

The authors have reported that they have no relationships relevant to the contents of this paper to disclose.



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## FIGURE LEGENDS

### Figure 1. Glucocorticoids and platelet aggregation

Platelet aggregation was evaluated in PRP incubated with scalar concentrations (150-600 ng/ml) of betamethasone (A) or methylprednisolone (C) and stimulated with ADP (10 µM) (A and C) or collagen (Coll, 2µg/mL) (E and F) (n=5 experiments) (*p<0.05).

Representative tracing of platelet aggregation in the presence or not of betamethasone or methylprednisolone (150-600 ng/ml) and stimulated with ADP (B and D).

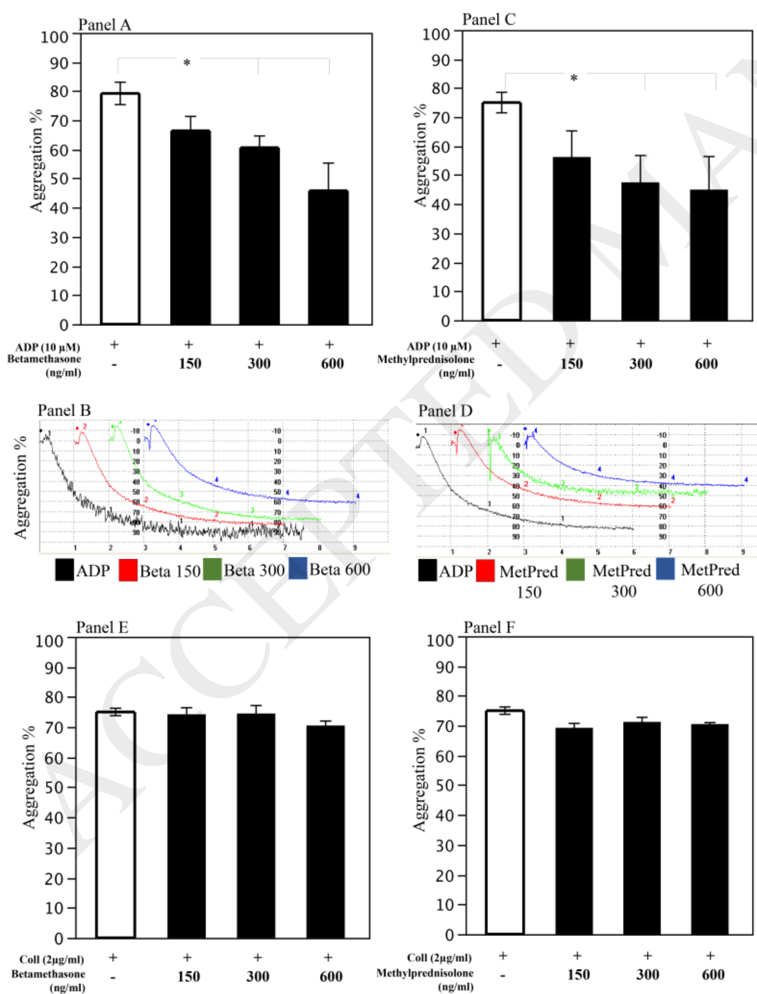

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**Figure 2. Glucocorticoids and platelet $TxB_2$ production**

$TxB_2$ production was evaluated in platelets incubated with scalar concentrations (150-600 ng/ml) of betamethasone (A and C) or methylprednisolone (B and D) and stimulated with ADP (10 µM) (A and B) or collagen (Coll, 2 µg/mL) (C and D) (n=5 experiments) (*p<0.05).

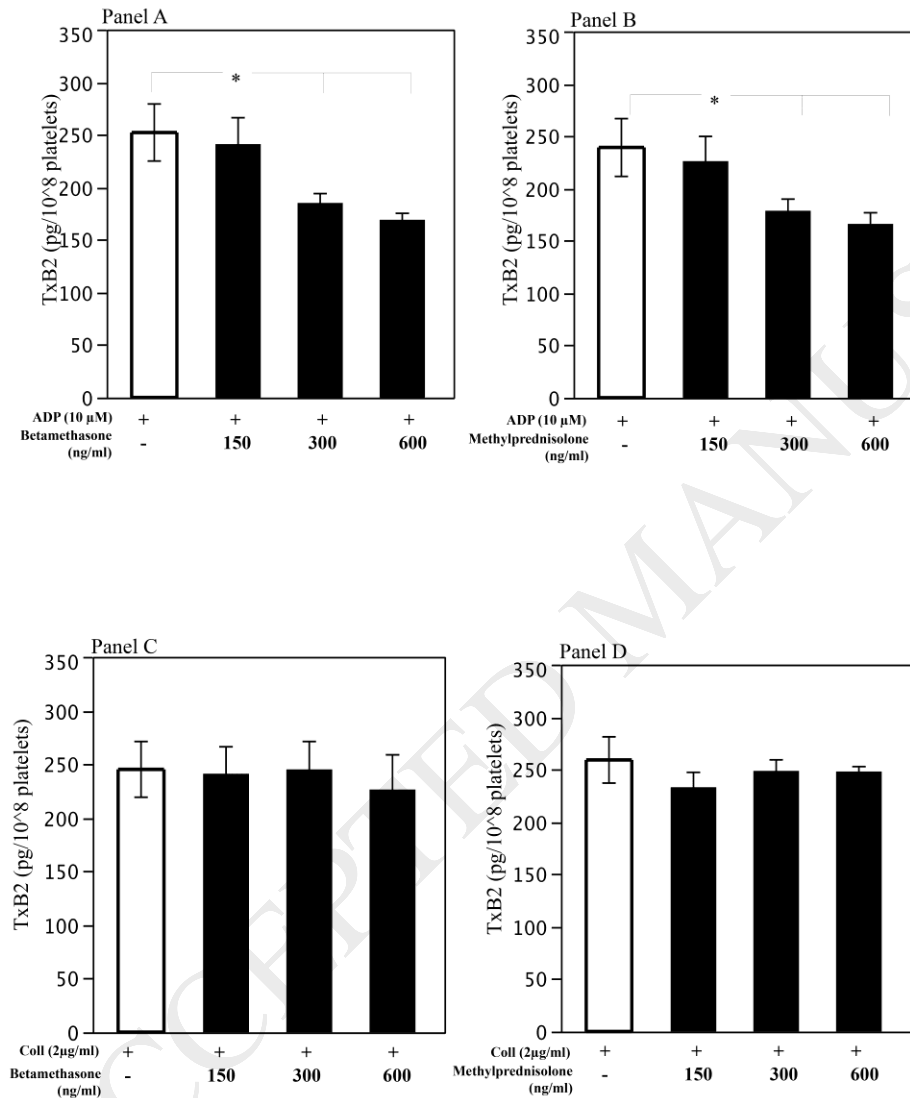

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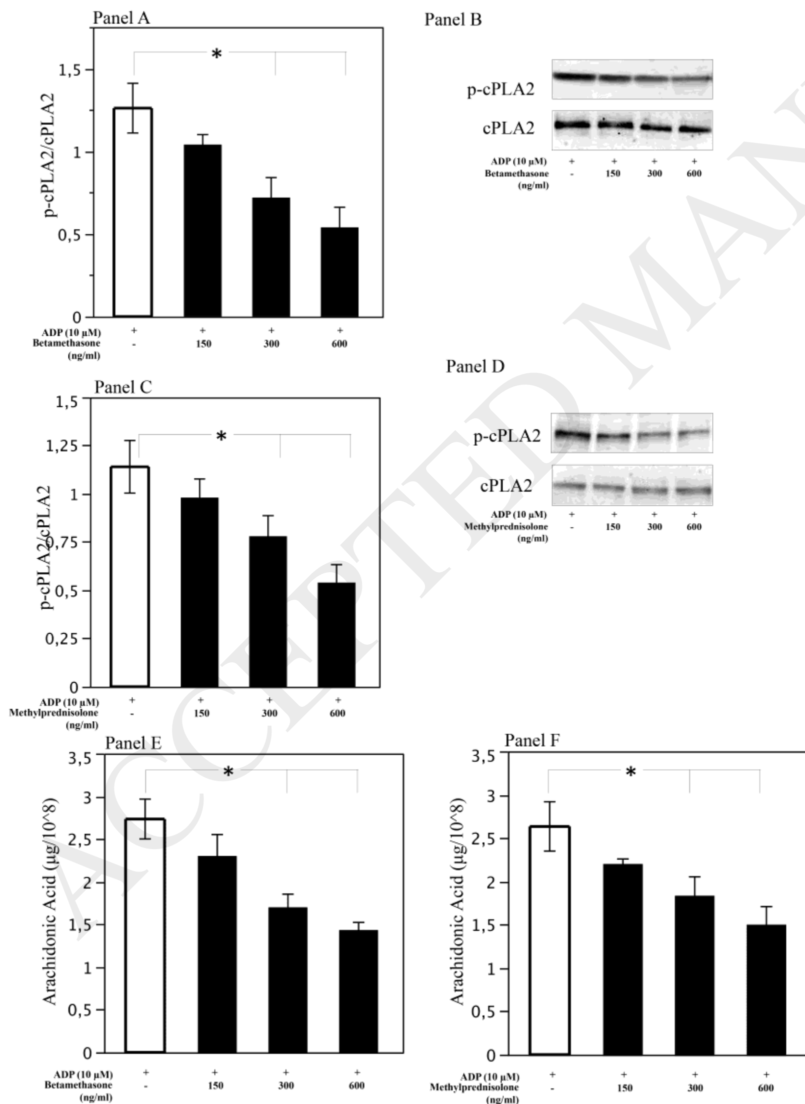
**Figure 3. Glucocorticoids and platelet $cPLA_2$ and Arachidonic Acid (AA) production**

$cPLA_2$ phosphorylation was analyzed in platelets incubated with scalar concentrations (150-600 ng/ml) of betamethasone (panel A) or methylprednisolone (panel C) and stimulated with ADP (10 µM) (*p<0.05, n=3 experiments). A representative western blot of c-$PLA_2$ phosphorylation in the presence or not of betamethasone or methylprednisolone (150-600 ng/ml) (panels B and D). AA release in platelets incubated with scalar concentrations (150-600 ng/ml) of betamethasone (E) or methylprednisolone (panel F) and stimulated with ADP (10 µM) (E and F) (*p<0.05, n=3 experiments).

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**Tables:**

**Table 1. Characteristics of CAP patients and controls.**

| Variables | CAP patients | Control patients | p |
|---|---|---|---|
| | n=300 | n=150 | |
| Age (years)* | 74.4 ±14.1 | 73.4±12.2 | 0.467 |
| Male sex (%) | 66% | 69% | 0.968 |
| BMI (kg/m²)* | 26.3± 4.4 | 26.0±4.8 | 0.476 |
| Current smokers (%) | 23 | 19 | 0.418 |
| Former smokers (%) | 37 | 34 | 0.602 |
| Arterial Hypertension | 74 | 77 | 0.617 |
| Coronary heart disease (%) | 41 | 36 | 0.349 |
| Cerebrovascular disease (%) | 13 | 11 | 0.644 |
| Diabetes (%) | 36 | 30 | 0.275 |
| Dyslipidemia (%) | 29 | 35 | 0.233 |
| Chronic kidney disease (%) | 17 | 19 | 0.692 |
| COPD (%) | 35 | 16 | <0.001 |
| Peripheral artery disease (%) | 9 | 11 | 0.779 |
| Heart failure (%) | 29 | 26 | 0.629 |
| Paroxysmal atrial fibrillation (%) | 16 | 22 | 0.152 |
| Chronic atrial fibrillation (%) | 14 | 20 | 0.161 |
| PSI* | 105.1±35.3 | n.a. | |
| PSI class II (%) | 15 | n.a. | |
| PSI class III (%) | 22 | n.a. | |
| PSI class IV (%) | 40 | n.a. | |
| PSI class V (%) | 23 | n.a. | |
| | | | |
| ASA (%) | 34 | 32 | 0.698 |
| VKA (oral anticoagulants) (%) | 17 | 26 | 0.042 |
| Heparins (%) | 9 | 2 | 0.012 |
| ACE inhibitors/ARBs (%) | 58 | 60 | 0.813 |
| Statins (%) | 34 | 31 | 0.594 |

ACE: angiotensin converting enzyme; ARBs: Angiotensin receptor blockade; ASA: aspirin; CAP: community-acquired pneumonia; BMI: body mass index; COPD: chronic obstructive pulmonary disease; PSI: pneumonia severity index, n.a.: not applicable, VKA: vitamin K antagonists; *Data are expressed as mean±SD

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**Table 2**

**Adjusted hazard ratios (HR), based on a Cox Proportional Hazards model, of in-hospital MI according to selected variables.**

| **Variables** | **HR*** | **P** | **CI 95%** | |
|---|---|---|---|---|
| | | | **Lower** | **Upper** |
| **Urinary 11-dehydro-$TxB_2$** | 1.002 | 0.005 | 1.001 | 1.004 |
| **PSI score** | 1.015 | 0.003 | 1.005 | 1.025 |

After adjusting for sex, BMI, PSI score, smoking habit, history of CHD, stroke, dyslipidemia, T2DM, hypertension, HF, renal failure, COPD, PAD, paroxysmal and chronic atrial fibrillation.

* HR for an increasing unit change in the independent factor