Official Journal of the Asian Pacific Society of Respirology
Respirology

ORIGINAL ARTICLE

Low-grade endotoxemia and clotting activation in the early phase of pneumonia

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ABSTRACT

Background and objective: **Community-acquired pneumonia (CAP) is associated with an increased risk of arterial and venous thrombosis but the underlying pathophysiological mechanisms are still unclear. We investigated if, in patients with CAP, a pro-thrombotic state does exist and its relationship with serum levels of endotoxins.**

Methods: **A total of 104 consecutive patients with CAP were prospectively recruited and followed up until discharge. At admission and at discharge, serum endotoxins, systemic markers of clotting activation and zonulin, a marker of gut permeability, were analysed. Hospitalized patients matched for gender, age and comorbidities but without infections were used as control.**

Results: **At admission, CAP patients showed higher plasma levels of F_{1+2}, a marker of thrombin generation ($P = 0.023$), and lower levels of protein C (PC; $P < 0.001$) and activated PC (aPC) ($P < 0.001$) compared with controls. At discharge, plasma levels of both PC and aPC significantly increased while F_{1+2} significantly decreased ($P < 0.001$). Baseline serum endotoxins and zonulin were higher in CAP patients than controls ($P < 0.001$) and significantly decreased at discharge; a significant correlation between serum endotoxins and zonulin was detected ($R = 0.575$; $P < 0.001$)**

Conclusion: **This study provides the first evidence that CAP patients disclose an ongoing pro-thrombotic state and suggests a role for endotoxemia in determining enhanced thrombin generation.**

Clinical trial registration: NCT01773863 at ClinicalTrials.gov

SUMMARY AT A GLANCE

Community-acquired pneumonia (CAP) is associated with an increased risk of arterial and venous thrombosis, but the underlying pathophysiological mechanisms are unclear. This study provides the first evidence that CAP patients disclose an ongoing pro-thrombotic state and suggests a role for endotoxemia in determining enhanced thrombin generation.

Key words: endotoxin, pneumonia, protein C, thrombosis, zonulin.

Abbreviations: ACE, angiotensin-converting enzyme; aPC, activated protein C; BMI, body mass index; CAP, community-acquired pneumonia; CHD, coronary heart disease; CHF, congestive heart failure; COPD, chronic obstructive pulmonary disease; CURB-65, Confusion, Urea, Respiratory rate, Blood pressure and age ≥65; ELISA, enzyme-linked immunosorbent assay; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HRP, horseradish peroxidase; hs-CRP, high-sensitivity C-reactive protein; IQR, interquartile range; LPS, lipopolysaccharide; PAD, peripheral arterial disease; PC, protein C; PCAg, PC antigen; SIXTUS, thromboSIs-related eXTra-pulmonary oUtcomeS in pneumonia; T2DM, type 2 diabetes mellitus; TF, tissue factor; TM, thrombomodulin; WBC, white blood cell.

INTRODUCTION

Community-acquired pneumonia (CAP) is the most common infection-related cause of death in developed countries.[1]

Epidemiological studies indicate that respiratory tract infections are associated with an increased risk for development of arterial thrombosis as shown by an increased incidence of cardiovascular and cerebrovascular events in the early phase of the disease.[2–4] There is also evidence, even if less convincingly, that venous thrombosis may complicate the early phase of

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Received 15 February 2016; invited to revise 29 March 2016; revised 1 April 2016; accepted 8 May 2016 (Associate Editor: Marcos Restrepo).

pneumonia in humans.[5] Together, these data suggest that pneumonia might be associated with a pro-thrombotic state, which eventually leads to arterial and venous thrombosis, but data in this regard are insufficient and elusive. Systemic coagulation abnormalities including activation of the clotting system and inhibition of anticoagulant factors have been observed in patients with severe sepsis;[6–8] however, in patients with CAP, clotting activation has been detected only in the lung compartment.[9] Furthermore, the mechanism potentially accounting for clotting activation has not been defined. Previous studies demonstrated that patients with CAP disclose enhanced levels of endotoxemia,[10] which may account for clotting activation as bacterial lipopolysaccharides (LPS) are able to up-regulate tissue factor (TF), which activates factors VII to VIIa.[5] However, the mechanism eliciting endotoxemia in CAP is unclear.

Our study hypothesis was that patients with pneumonia disclose an ongoing pro-thrombotic state, which is related to the acute lung infection. To explore this issue, we measured, at admission and immediately before discharge from the hospital, systemic markers of clotting activation and serum levels of endotoxins in patients with CAP.

METHODS

Patient selection

The study was conducted at four clinical centres of the University-Hospital Policlinico Umberto I, Rome. All CAP patients, admitted to the four units through the emergency department from September 2012 to April 2013, were prospectively recruited and followed up until discharge. The institutional review board approved this prospective, observational study, which was registered at ClinicalTrials.gov (Identifier: NCT01773863).

Detailed inclusion and exclusion criteria have been previously published.[3] Patients with any anticoagulation therapy or those needing anticoagulation treatment because of a new-onset atrial fibrillation[11] were also excluded from the study.

A total of 104 consecutive patients who fulfilled the above criteria were enrolled in the study after giving written informed consent.

Data on demographic characteristics and comorbidities were collected. Severity of illness at presentation was quantified by the CURB-65, a validated predictive score of mortality rate in CAP.[11] Type 2 diabetes mellitus, hypertension, history of coronary heart disease, dyslipidaemia, peripheral arterial disease and COPD were defined as previously described.[12–14]

Antibiotic therapy was initiated in the emergency department in accordance with hospital guidelines and/or consultation of an infectious diseases specialist. Daily diet was based on hospital guidelines and was tailored according to age, nutritional status and severity of comorbidities.

Immediately after diagnosis of CAP and at hospital discharge, routine blood laboratory tests including high-sensitivity C-reactive protein (hs-CRP) were performed and systemic markers of clotting activation including protein C (PC) antigen (PCAg), PC activity and F_{1+2} were analysed.

As a control group, we selected 35 hospitalized patients matched for gender, age and comorbidities but without any acute infectious disease. Exclusion criteria were the same as in the CAP patient group. In detail, control subjects were hospitalized for congestive heart failure (CHF) (n = 13), syncope (n = 7), hypertensive crisis (n = 5), decompensated diabetes mellitus (n = 4) and new-onset arrhythmias (n = 6).

The present study was conducted according to the principles stated in the Declaration of Helsinki.

Laboratory analysis

Blood samples were obtained early in the morning, after an overnight fast and a rest period of at least 20 min. Routine biochemical tests were carried out using standard procedures.

Markers of clotting activation

For the measurement of PCAg and soluble thrombomodulin (TM), venous blood was withdrawn from an antecubital vein using a 20-G needle and collected in tubes containing 3.8% sodium citrate (ratio = 9:1). For the measurement of prothrombin fragment 1 + 2 (F_{1+2}) and activated PC (aPC), venous blood (4.5 mL) was collected in tubes containing 0.5 mL of a mixture of sodium citrate and benzamidine–HCl (200 mM). Within 1 h of collection, platelet poor plasma was obtained by centrifugation for 10 min at 2500 g at room temperature and stored in aliquots at –80°C until assay.

F_{1+2} levels were measured by a commercially available enzyme immunoassay (Enzygnost F_{1+2}; Dade-Behring, Marburg, Germany) as previously described.[15] Quantification of aPC levels was performed by a home-made ELISA as described by Liaw *et al.*[16] Briefly, 96-well vinyl micro titre plates (Costar Corning Incorporated, Corning, NY, USA) were coated with the monoclonal antibody HAPC 1555 (5 μg/mL in coating buffer: 20 mM HEPES, pH 7.5, 150 mM NaCl and 5 mM $CaCl_2$). Benzamidine–HCl plasma samples were anticoagulated and recalcified by addition of 2 IU/mL heparin, 20 mM HEPES, pH 7.5 and 10 mM $CaCl_2$ (final concentrations) and incubated (100 μL) at room temperature for 30 min with the coated antibody. Wells were washed thrice and the chromogenic activity of bound aPC was measured by the addition of 100 μL of 0.5 mM S2366 (Chromogenix, DiaPharma, West Chester, Ohio, USA) in coating buffer. Substrate hydrolysis was monitored at 405 nm. A standard curve was generated with increasing amounts of aPC from 0 to 50 ng/mL, spiked into the coating buffer. The monoclonal antibody HAPC 1555 was a gift from Dr Charles T. Esmon (Oklahoma Medical Research Foundation). Plasma concentrations of soluble TM and PCAg were measured by commercial ELISA (Quantikine Human Thrombomodulin, R&D Systems, Minneapolis, MN, USA) and (Asserachrom Protein C, Diagnostica Stago, Asnier sur Seine, France).

Endotoxin serum levels

Serum levels of endotoxins (LPS) were measured by a commercial ELISA method (human lipopolysaccharides, Cusabio, Tema Ricerca, Italy). Briefly, 100 μL of

serum sample were plated for 2 h at room temperature. After incubation, samples were read at 450 nm. Values were expressed as pg/mL; detection range was 6.25–400 pg/mL; and intra-assay and inter-assay coefficients of variation were 8% and 10%, respectively.

Zonulin serum levels

Serum zonulin measurement was performed by an ELISA Kit (Elabscience Biotechnology Co., Ltd, Wuhan, China). Antibodies specific for zonulin were pre-coated onto a microplate and 100 μL of standards and patient sera samples were added and incubated for 90 min at 37°C. Then, a biotinylated detection antibody specific for zonulin and avidin-horseradish peroxidase (HRP) conjugate was added. The amount of zonulin was measured with a microplate auto-reader at 450 nm. Values were expressed as ng/mL and coefficient of variation was <10%.

Statistical analysis

All continuous variables were tested for normality with the Shapiro–Wilk test. Variables with normal distribution were expressed as means and SD, and tested for differences using a *t*-test. Non-parametric variables were expressed as median and interquartile range (IQR) and differences tested using the Mann–Whitney *U*-test. Categorical variables were expressed as percentages and analysed by chi-square test. Bivariate analysis was performed with Pearson's linear correlation or with Spearman rank correlation test. A multivariable linear regression model was performed to identify significant factors associated with the combined biomarker of systemic clotting activation (aPC/F_{1+2}). Skewed continuous variables were log transformed. Stochastic level of entry into the model was set at a *P*-value of 0.10, and interaction terms were explored for all the variables in the final model.

The differences between baseline and end of hospitalization biomarker's values were analysed with the Wilcoxon signed-rank test. Percent changes were calculated on each patient with the following formula: 100 × (discharge value − baseline value)/baseline value.

Only *P*-values <0.05 were regarded as statistically significant. All tests were two-tailed and analyses were performed using computer software packages (SPSS-22.0, IBM, NY, USA or R version 2.15.2, R Development Core Team, Vienna, Austria).

Sample size calculation

We computed the minimum sample considering (i) a relevant difference in serum F_{1+2} from 150 pmol/L in the control group to 190 pmol/L in the CAP patients, (ii) SD of the paired differences of 50 pmol/L and (iii) type I error probability $\alpha = 0.05$ and power $1 - \beta = 0.90$. This resulted in $n = 33$ for each group.

RESULTS

Baseline clinical characteristics of patients with CAP ($n = 104$) are summarized in Table 1. Patients were aged 68.7 ± 15.1 years. Sixty-four percent of patients were males; 31% of patients had a history of coronary artery disease, 12% had stroke and 22% had heart failure. Six percent of patients had peripheral arterial disease and 22% had type 2 diabetes mellitus. Most of the patients had arterial hypertension and were treated with ACE inhibitors or angiotensin II receptor blockers. Thirty-eight percent of patients were treated with antiplatelet agents and 35% of CAP patients received statins. During the intra-hospital stay, all CAP patients were treated with antibiotics (as monotherapy or combination regimes). In detail, macrolides were used in 60% of patients, cephalosporins in 52%, fluoroquinolones in 24%; piperacillin/tazobactam in 24%, carbapenems in 3% and aminopenicillins in 1%. Sixty-six percent of patients received supplemental oxygen by nasal cannula (28%), Venturi-mask (32%) or non-invasive ventilation (6%).

Table 1 Clinical characteristics of patients with community-acquired pneumonia

	Patients	Controls	*P*
n	104	35	
Age†	68.7 ± 15.1	69.4 ± 11.6	0.809
Gender (males)	64%	63%	0.867
BMI†	25.1 ± 3.0	25.6 ± 3.7	0.404
CURB-65 I Class	24%	n.a.	n.a.
CURB-65 II Class	57%	n.a.	n.a.
CURB-65 III Class	19%	n.a.	n.a.
Arterial hypertension	62%	69%	0.517
T2DM	22%	17%	0.531
History of CHD	31%	37%	0.486
COPD	33%	29%	0.650
Previous stroke	12%	14%	0.785
Heart failure	22%	29%	0.437
Dyslipidaemia	29%	31%	0.772
PAD	6%	9%	0.560

†Data are expressed as mean ± SD.

CHD, coronary heart disease; n.a., not applicable; PAD, peripheral arterial disease; SD, standard deviation; T2DM, type 2 diabetes mellitus.

In the early phase of pneumonia, patients disclosed increased levels of F_{1+2}, a systemic marker of in vivo thrombin generation,[17] along with a reduction in both PC zymogen and aPC levels. Thus, compared with a control group, CAP patients showed higher plasma levels of F_{1+2} (237 (165–388) vs 163 (136–313) pmol/L; $P = 0.023$) and lower levels of PCAg (83% (63–97) vs 98% (89–106), $P < 0.001$) and aPC (36.3 (19.7–52.4) vs 49.9 (43.7–56.0) pmol/L; $P < 0.001$). Moreover, CAP patients disclosed lower aPc/F_{1+2} ratio (0.13 (0.07–0.26) vs 0.28 (0.16–0.37); $P < 0.001$), a global marker of imbalance between thrombin generation and the anticoagulation system.[18]

The median length of hospital stay was 8 days (IQR: 7–10 days). At discharge from hospitalization, levels of PCAg, aPC, F_{1+2} and aPC/F_{1+2} returned to values comparable to those of control subjects. Thus, compared with baseline values, PCAg and aPC plasma levels and aPC/F_{1+2} significantly increased, while F_{1+2} significantly decreased at discharge ($P < 0.001$) (Fig. 1; Table 2). The percentage increase in PCAg correlated with a

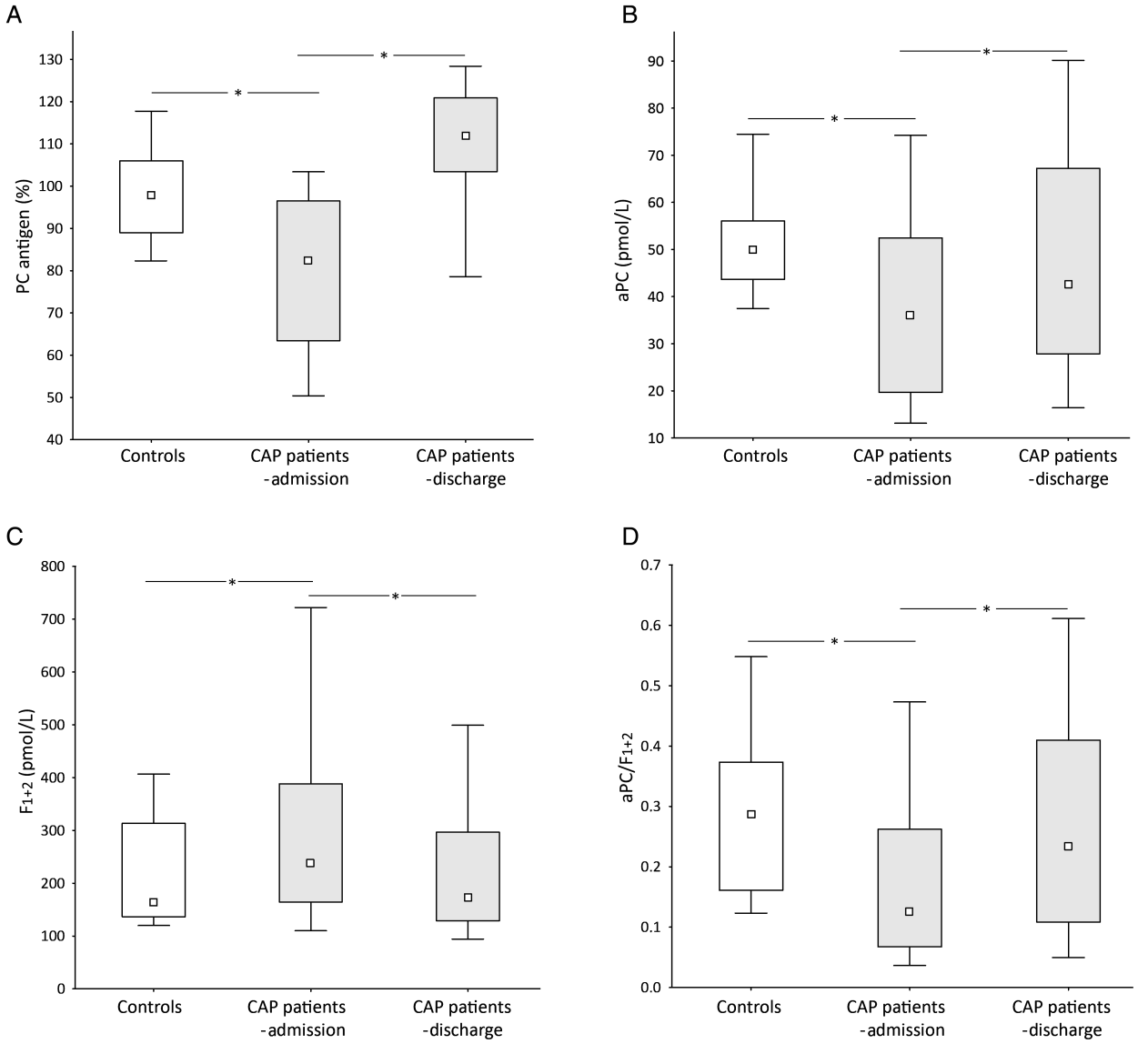

Figure 1 Coagulation biomarker profile in controls and in community-acquired pneumonia (CAP) patients at admission and at discharge. $*P < 0.05$; $**P < 0.01$.

Table 2 Inflammation and coagulation biomarker profile at admission and at discharge

	Baseline	At the end of hospitalization	Median change (%)[†]	*P*-value
hs-CRP (mg/L)	60 (25–169)	10 (4–22)	–83	<0.001
WBC ($\times 10^3/mm^3$)	12.4 ± 5.3	9.6 ± 3.9	–22	<0.001
PCAg (%)	83 (63–97)	112 (103–121)	+32	<0.001
TM (pg/mL)	2413 (2017–2876)	2716 (2196–3434)	+7	0.078
aPC (pmol/L)	36.3 (19.7–52.4)	42.6 (27.9–68.0)	+33	0.040
F_{1+2} (pmol/L)	237 (165–388)	174 (127–298)	–23	0.019
aPC/F_{1+2}	0.13 (0.07–0.26)	0.23 (0.11–0.42)	+58	0.002

Data are expressed as median (IQR) or mean ± SD.

[†]Median of percent changes calculated for each patient.

aPC, activated protein C; hs-CRP, high-sensitivity C-reactive protein; IQR, interquartile range; PCAg, protein C antigen; SD, standard deviation; TM, thrombomodulin; WBC, white blood cells.

percentage increase in aPC (R = 0.308, $P = 0.024$). There was no significant change in plasma soluble TM levels (Table 2).

To evaluate the relationship between endotoxemia and systemic markers of clotting activation, serum endotoxin (LPS) concentration was evaluated at admission and at discharge. Baseline values of endotoxemia were significantly higher in CAP patients compared with controls (230 ± 34 vs 27 ± 22 pg/mL; $P < 0.001$). In CAP patients, endotoxemia significantly decreased at discharge compared with baseline values (36 ± 21 vs 230 ± 34 pg/mL; $P < 0.001$).

Baseline serum LPS levels correlated with F_{1+2} (R = 0.412; $P < 0.001$; Fig. 2) and inversely correlated to aPC/F_{1+2} (R = –0.303; $P = 0.002$). In addition to serum LPS, baseline values of the combined biomarker aPC/F_{1+2} were also inversely associated with age (R = –0.317; $P = 0.001$) and CURB-65 (R = –0.431; $P < 0.001$).

A multivariable regression analysis showed that CURB-65 (β = –0.434; $P < 0.001$) and serum LPS (β = –0.262; $P = 0.008$) remained significantly associated with aPC/F_{1+2}.

To investigate the potential relationship between serum LPS and gut permeability, we measured the circulating levels of zonulin in a subgroup of 45 consecutive CAP patients and in the control subjects. Clinical characteristics of this group of CAP patients were similar to those of the whole cohort (data not shown).

Serum zonulin was higher in CAP patients compared with controls (3.07 ± 0.74 vs 1.97 ± 0.74; $P < 0.001$) and significantly decreased at discharge (2.30 ± 0.80 ng/mL; $P < 0.001$). In CAP patients, the decrease of serum zonulin paralleled those of LPS (Fig. 3A,B) and a significant overall correlation was detected between serum levels of zonulin and LPS (R = 0.575; $P < 0.001$) (Fig. 3).

DISCUSSION

This study provides evidence that patients with CAP disclose an enhanced clotting activation as shown by the significant increase of in vivo thrombin generation and lowered circulating levels of aPC in the early phase of disease. Such changes are associated with infection severity as suggested by the significant correlation between serum endotoxins and clotting activation and by the reduction of thrombin generation in the remission phase of CAP.

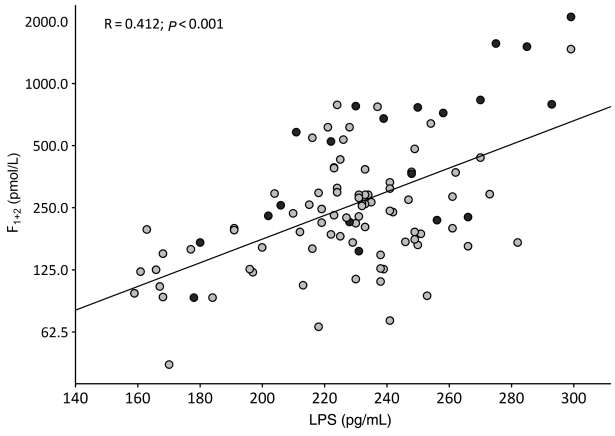

Figure 2 Relationship between endotoxins and F_{1+2} in community-acquired pneumonia (CAP) patients. Closed circles represent patients with CURB-65 class ≥ 3.

While activation of the clotting system has been reported in the lung alveolar cells of CAP patients,[9] data regarding systemic clotting activation as well the mechanism potentially accounting for it have scarcely been investigated. Yende *et al.*[19] showed that in CAP patients markers of clotting activation such as thrombin–antithrombin complexes and D-dimer may be elevated at the time of hospital discharge and predict long-term mortality; however, clinical characteristics as well as predictors of clotting activation were not investigated. Herewith, we provide the first evidence that the early phase of disease is associated with systemic signs of enhanced thrombin generation along with a reduction of the natural anticoagulant PC, suggesting that CAP and clotting activation are closely associated. In order to investigate if clotting activation was related to the severity of lung infection, we scored our patients with CURB-65, which is a reliable marker of pneumonia severity and is associated with survival.[11] About two-third of our population had moderate-severe CAP with only 24% being of class I, which is characterized by low mortality risk.[11] Multiple regression analysis demonstrated that CURB-65 was independently associated with clotting activation suggesting that pneumonia severity was an important pathophysiological factor triggering thrombin generation. To investigate the reason for such association, we focused on the possibility that bacterial endotoxin may favour clotting activation. Thus, there is good experimental and clinical evidence that bacterial-derived endotoxins such as LPS predispose to thrombosis by increasing thrombin generation via TF up-regulation of endothelial cells and monocytes, triggering factor VII activation and the coagulation cascade.[20,21] Based on this, we measured serum endotoxemia in CAP patients and found significantly higher values compared with controls. In accordance with the hypothesis that endotoxemia may be a trigger for clotting activation, serum endotoxins significantly correlated with plasma levels of the prothrombin fragment F_{1+2}; the potential interplay between endotoxemia and clotting activation was reinforced by the coincident decrease of thrombin generation and endotoxemia in the remission phase of pneumonia.

A reduction in PC during the acute phase of pneumonia in the systemic circulation was another picture of CAP indicating that an imbalance between clotting activation and anticoagulant system is an early phenomenon of pneumonia. In baboons injected with lethal doses of *Escherichia coli*, the reduction in PC zymogen levels was exclusively due to PC activation[22]; however additional mechanisms may have played in our patient series, such as PC leakage into the extravascular spaces and/or reduced biosynthesis.

The activation rate of PC is linearly related to its concentration, and in our patients both PCAg and aPC levels increased at discharge from the hospital. The reduction in aPC levels at admission is most probably explained by the reduced PC zymogen levels, but increased inhibition of aPC by α1-antitrypsin may have also played a role.[18]

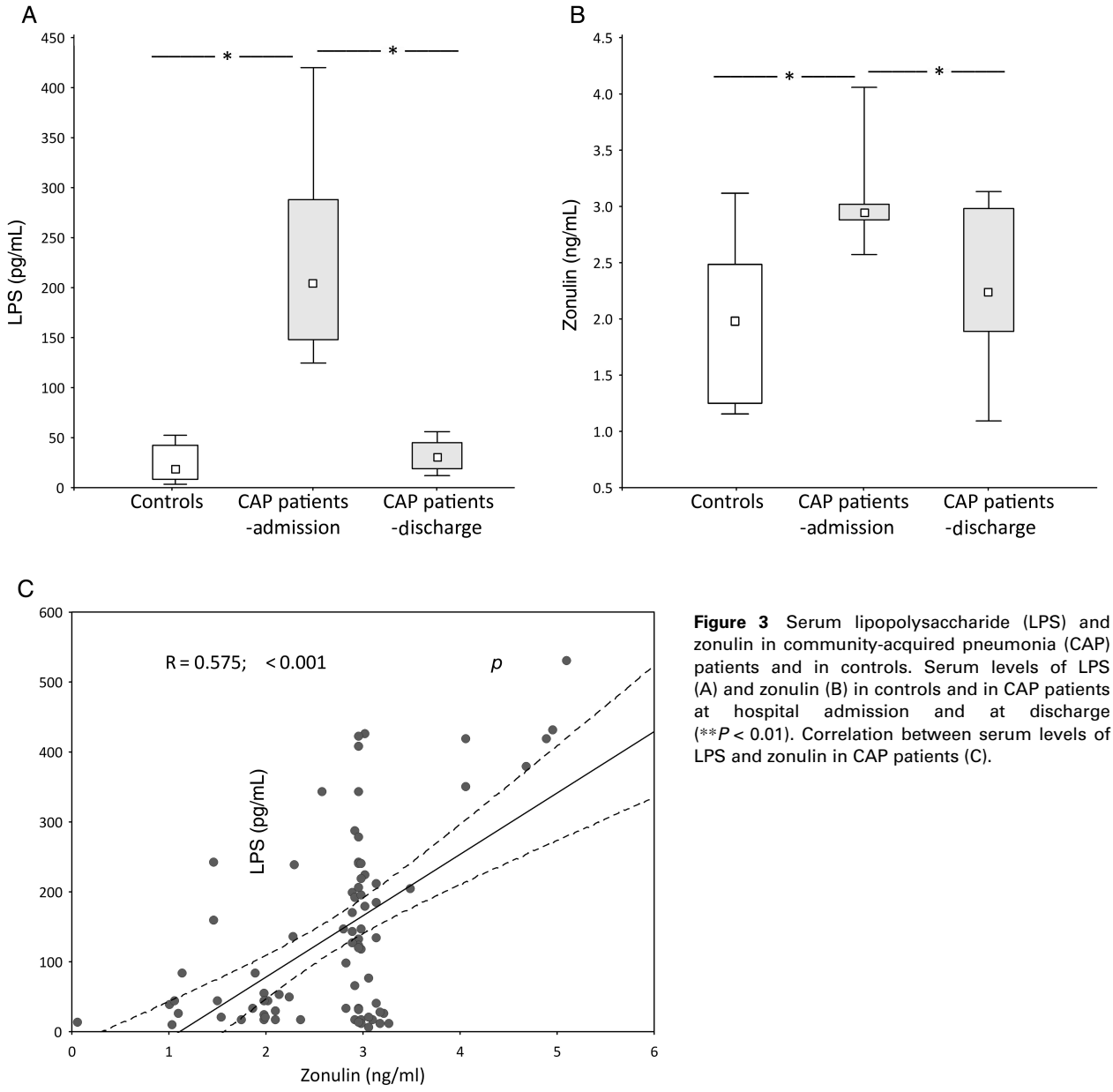

Figure 3 Serum lipopolysaccharide (LPS) and zonulin in community-acquired pneumonia (CAP) patients and in controls. Serum levels of LPS (A) and zonulin (B) in controls and in CAP patients at hospital admission and at discharge (**$P < 0.01$). Correlation between serum levels of LPS and zonulin in CAP patients (C).

A previous study demonstrated that CAP patients disclose enhanced circulating levels of LPS and suggested that gut permeability may account for it.[23] To specifically address this issue, we measured the circulating levels of zonulin, which modulates gut permeability by disassembling the intercellular tight junctions.[24] Experimental and clinical studies demonstrated that zonulin up-regulation plays a role in increasing gut permeability and that serum levels of zonulin correlate with enhanced intestinal permeability.[25] The increased serum levels of zonulin in our CAP population and its correlation with serum LPS provide the first evidence that gut permeability is enhanced in CAP and may be responsible for the high circulating levels of LPS.

This study has implications and limitations. We cannot exclude that clotting activation may occur via pathogens such as viruses, which may worsen outcome of pneumonia via platelet activation.[26,27] Also, we did not explore if clotting activation is associated with artery or venous thrombosis; therefore, it is still premature to consider the activation of clotting system as a mechanism potentially accounting for the thrombotic disorders, which characterize the early phase of pneumonia. Finally, the exact mechanism accounting for enhanced gut permeability has not been evaluated. In this context, it could be interesting to perform an interventional trial to explore if non-absorbable antibiotics are capable of hampering clotting activation.

In conclusion, CAP patients are characterized by a significant increase in thrombin generation rate along with reduced PC zymogen and enzyme. Changes of clotting activation are significantly associated with serum endotoxins, suggesting endotoxemia as a key

factor in determining enhanced thrombin generation in CAP patients. Enhanced gut permeability seems to be implicated in inducing enhanced circulating levels of endotoxins in CAP patients.

Acknowledgments

The SIXTUS (thromboSIs-related eXTra-pulmonary oUtcomeS in pneumonia) study group consists of the following researchers: Simona Battaglia, Giuliano Bertazzoni, Elisa Biliotti, Cinzia Myriam Calabrese, Marco Casciaro, Paolo De Marzio, Lucia Fazi, Domenico Ferro, Laura Giordo, Elisa Manzini, Sergio Morelli, Daniele Pastori, Pasquale Pignatelli, Giulio Francesco Romiti, Elisabetta Rossi, Eleonora Ruscio, Maria Gabriella Scarpellini and Filippo Toriello (Department of Internal Medicine and Medical Specialties, Sapienza University of Rome, Italy); Maurizio De Angelis, Rozenn Esvan, Lucia Fontanelli Sulekova, Cristiana Franchi, Marco Rivano Capparuccia and Stefania Grieco (Infectious and Tropical Diseases Unit, Department of Clinical Medicine, Sapienza University of Rome, Italy); and Paolo Palange and Alessandro Russo (Department of Public Health and Infectious Diseases, Sapienza University of Rome, Italy).

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