

Impact of acute exacerbations on platelet reactivity in chronic obstructive pulmonary disease patients

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Background: A higher risk of atherothrombotic cardiovascular events, which are platelet-driven processes, has been described during acute exacerbations of chronic obstructive pulmonary disease (AECOPD). However, the relevance of platelet reactivity during AECOPD and whether this is affected by antiplatelet agents are not fully elucidated to date. This study aimed to evaluate whether platelet reactivity is augmented during an exacerbation in COPD patients with and without antiplatelet therapy and its association with systemic inflammatory parameters.

Materials and methods: Prospective, observational, ex vivo investigation was conducted in consecutive patients suffering an exacerbation of COPD. Platelet reactivity was assessed during AECOPD and at stable state. Platelet function assays included: 1) vasodilator-stimulated phosphoprotein assay expressed as P2Y₁₂ reactivity index (PRI), 2) multiple electrode aggregometry and 3) optical aggregometry. Systemic inflammatory parameters such as leukocyte count, interleukin-6 and fibrinogen were also assessed.

Results: Higher platelet reactivity was observed during AECOPD compared to stability measured by vasodilator-stimulated phosphoprotein (PRI: 75.2%±1.9% vs 68.8%±2.4%, $p=0.001$). This augmented platelet aggregability was also observed in the subset of patients on antiplatelet therapy (PRI: 72.8%±3.1% vs 61.7%±7.5%, $p=0.071$). Consistent findings were observed with all other platelet function tests. Patients with greater enhancement of inflammatory markers during AECOPD were more likely to present a higher increase in platelet reactivity.

Conclusion: Platelet reactivity is increased during AECOPD, which may contribute to the augmented cardiovascular risk of these patients. Additionally, the increase in platelet reactivity might be associated with an increment in inflammatory markers during exacerbations.

Keywords: airflow limitation, inflammation, platelet aggregation, antiplatelets agents, thrombosis

Introduction

Cardiovascular disease is the most important comorbidity associated with chronic obstructive pulmonary disease (COPD) due to its impact on overall prognosis including mortality.¹⁻³ Systemic inflammation has been suggested as one of the main factors that play a significant role in the pathogenesis of atherothrombosis in COPD,^{4,5} which is characterized by the presence of low-grade systemic inflammation in the stable state⁶ and, noteworthy, by an acute inflammatory response during exacerbations of the disease, as shown by Thomsen et al.⁷ The increased systemic inflammation associated with acute exacerbations is of particular relevance since it can be an underlying mechanism that may contribute to the impaired cardiovascular outcomes observed in COPD patients. In line with this, atherothrombotic complications such as myocardial

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infarction or stroke are particularly augmented during an acute exacerbation of COPD (AECOPD).^{8,9}

Platelets are key elements in the development of atherosclerosis and its atherothrombotic complications.¹⁰ Several studies have observed that systemic inflammation may contribute to platelet activation and, thus, to an increased cardiovascular risk.^{11,12} In addition, an interesting mechanistic investigation by Maclay et al has shown increased platelet activation in COPD patients compared to non-COPD subjects, and during AECOPD compared to stability.¹³ However, the magnitude of the increment in platelet reactivity during an AECOPD is not fully elucidated to date, and if this is affected by antiplatelet agents has not been previously evaluated. The aim of this study was to evaluate whether platelet reactivity is augmented during an AECOPD compared with stability in COPD patients with and without antiplatelet therapy (APT) and its possible association with systemic inflammatory parameters.

Materials and methods

Population and study design

This was a prospective, *ex vivo*, observational investigation conducted in consecutive COPD patients attended during an exacerbation at the pulmonology day hospital from a tertiary centre. All patients were between 40 and 80 years of age and had been diagnosed of COPD following Global initiative for chronic Obstructive Lung Disease recommendations.¹ An AECOPD was defined as an acute event characterized by worsening of the patient's respiratory symptoms that is beyond normal day-to-day variations, which leads to a change in medication.¹ Exacerbations were classified as moderate (outpatient treatment requiring antibiotics and/or oral corticosteroids) and severe (requiring hospital admission).¹⁴ Exclusion criteria were the presence of other pulmonary disease, current therapy with anticoagulant agents or glycoprotein IIb/IIIa inhibitors, any active malignancy or hematologic disease, platelet count $<100 \times 10^6/\mu\text{L}$, severe chronic kidney disease (creatinine clearance $<30 \text{ mL/min}$) and pregnant females. Patients could receive APT with aspirin or clopidogrel for concomitant chronic cardiovascular conditions.

The study had a prospective design with paired data, in which assessments were performed at two time points: the first one during AECOPD and the second one after the patient had recovered stability (4–6 weeks after the first visit and no symptoms of exacerbation or administration of antibiotics or oral corticosteroids for at least 2 weeks). In patients receiving APT, blood samples were collected for platelet function testing before the daily maintenance dose of aspirin or

clopidogrel was administered. The study was approved by the local ethics committee "Comitè Ètic d' Investigació Clínica Del Hospital de Bellvitge", No PR077/13, and performed in accordance with the Declaration of Helsinki. All subjects provided written informed consent to the study.

Platelet function assays

Blood samples were collected from antecubital vein, discarding the first 2–4 mL of blood to avoid spontaneous platelet activation. Samples were processed by trained laboratory personnel blinded to the patient status and medication within 2 hours of blood drawing. Several agonists were used in platelet function assessments in order to explore different platelet activation signaling pathways. In patients receiving APT, aspirin-mediated effects were specifically evaluated by using arachidonic acid and collagen as agonists, while clopidogrel-mediated effects were specifically assessed by using adenosine diphosphate (ADP) as an agonist. Platelet function assays included flow cytometric analysis of the phosphorylation status of vasodilator-stimulated phosphoprotein (VASP), light transmission aggregometry (LTA) and multiple electrode aggregometry.

Analysis of the phosphorylation status of VASP

The P2Y₁₂ reactivity index (PRI) measures the functional status of the platelet P2Y₁₂ signaling pathway and was determined according to standard protocols.¹⁵ In brief, VASP phosphorylation (VASP-P) was measured by quantitative flow cytometry using commercially available labeled monoclonal antibodies (Biocytex Inc., Marseille, France). The PRI was calculated after measuring the mean fluorescence intensity (MFI) of VASP-P levels following challenge with prostaglandin E1 (PGE1) and PGE1 + ADP. PGE1 increases the VASP-P levels through stimulation of adenylate cyclase, and ADP binding to purinergic receptors leads to inhibition of adenylate cyclase; thus, the addition of ADP to PGE1-stimulated platelets reduces the levels of PGE1-induced VASP-P. The PRI was calculated as follows: $([\text{MFI PGE1}] - [\text{MFI PGE1} + \text{ADP}]/[\text{MFI PGE1}]) \times 100\%$. An increased PRI is indicative of higher platelet reactivity.

LTA

LTA was performed according to standard protocols as previously described.¹⁶ Briefly, blood-citrate tubes were centrifuged at $100 \times g$ for 10 min to recover platelet-rich plasma (PRP) and further centrifuged at $2,400 \times g$ for 15 min to recover platelet-poor plasma (PPP). Platelet aggregation was assessed using PRP and PPP by the turbidometric method in a two-channel aggregometer (Chrono-Log 490 Model;

Chrono-Log Corp., Havertown, PA, USA). Light transmission was adjusted to 0% for PRP and 100% for PPP for each measurement. Maximal platelet aggregation is reported as a percentage and was measured following stimuli with several agonists: ADP (5 and 20 $\mu\text{mol/L}$), arachidonic acid (1 mmol/L) and collagen (2 $\mu\text{g/mL}$).

Multiple electrode aggregometry

Blood was collected in hirudin-treated tubes. Multiple electrode aggregometry was performed in whole blood with the Multiplate™ analyzer (Hoffman-La Roche Ltd., Basel, Switzerland) as previously described.¹⁷ This instrument assesses the change in impedance caused by the adhesion of platelets onto sensor units formed by silver-covered electrodes. In this investigation, the following agonists were used: ADP (6.4 $\mu\text{mol/L}$), arachidonic acid (0.5 mmol/L), collagen (3.2 $\mu\text{g/mL}$) and thrombin receptor activating peptide (32 $\mu\text{mol/L}$). Aggregation curves were recorded for 6 min and platelet aggregation was determined as the area under the curve of arbitrary aggregation units ($\text{AU} \times \text{min}$).

Systemic inflammatory parameters

Three inflammatory parameters involved in COPD exacerbations were assessed: leukocyte count, interleukin-6 (IL-6) and fibrinogen.⁷ Leukocytes were measured as part of complete cell blood counts performed at the time points described above. To quantify the IL-6 (expressed in pg/mL) and fibrinogen (expressed in g/L) serum levels, individual enzyme-linked immunosorbent assay tests were used following the manufacturer's instructions. The lower limit of detection for IL-6 was 0.156 pg/mL (HS600B; R&D Systems, Inc., Minneapolis, MN, USA) and for fibrinogen was 1.56 ng/mL (ab208036; Abcam, Cambridge, UK).

Study endpoints and sample size

The primary endpoint of this study was the difference in platelet reactivity measured as PRI of COPD patients during the AECOPD compared to stable state. Assuming an SD of 20%, a sample size of 36 subjects was needed to detect a minimal difference between time points of 10%, with 85% power and a two-tailed p -value <0.05 for a paired data comparison. Considering an approximate 10% dropout rate (eg, inadequate samples or loss in the follow-up), the inclusion of 40 subjects was allowed to ensure that data from 36 patients were available for analysis. The secondary endpoints included: 1) differences in platelet reactivity measured with the other platelet function assays during the AECOPD compared to stable state and 2) changes in inflammatory

parameters during the AECOPD compared to stable state and their association with platelet reactivity.

Statistical analysis

Conformity to the normal distribution was evaluated for continuous variables with the Kolmogorov–Smirnov and the Shapiro–Wilk tests. For baseline characteristics, categorical variables were expressed as frequencies and percentages, whereas continuous variables were summarized by mean $\pm$ SD or by median (interquartile range) if a normal distribution could be assumed or not, respectively. A repeated-measures analysis of variance (ANOVA) model was used to evaluate the primary endpoint and to make all other intragroup comparisons between the AECOPD compared to the stable state. In order to evaluate the association between inflammation and platelet aggregability, exploratory analyses comparing the change in platelet reactivity measured by VASP (ΔPRI : PRI during exacerbation–PRI during stability) according to tertiles of the difference (Δ) of each inflammatory marker (levels during exacerbation–levels during stability) were performed using an ANOVA method with a general linear model. Results are reported as least squares mean $\pm$ standard error of the mean for the above-detailed ANOVA analyses. A p -value <0.05 was considered statistically significant for all comparisons. Statistical analysis was performed using SPSSv18.0 software (SPSS Inc., Chicago, IL, USA).

Results

Forty consecutive severe COPD patients consulting with an AECOPD were prospectively included. Two patients were excluded because they were lost in the follow-up, and one patient was excluded because anticoagulant therapy for atrial fibrillation was prescribed before visit 2 occurred. Therefore, a total of 37 patients were included in this analysis: 29 (78.4%) with a moderate AECOPD and 8 (21.6%) with a severe AECOPD, thus requiring admission. In this cohort, 10 patients receiving APT were included: 8 on aspirin and 2 on clopidogrel. Demographic and clinical characteristics of the overall population are summarized in Tables 1 and 2. Characteristics of the AECOPD as well as treatment approach are listed in Table 3.

Platelet reactivity during an AECOPD

Higher platelet reactivity was observed during AECOPD compared to stability, as measured by VASP (PRI: $75.2\% \pm 1.9\%$ vs $68.8\% \pm 2.4\%$, $p=0.001$) in the overall sample (Figure 1). Consistent results were obtained with the other platelet function assays performed and all agonists used, which showed a numerically greater platelet reactivity

Table 1 Demographic and clinical characteristics

	N=37
Age (years), mean $\pm$ SD	69.8 $\pm$ 5.7
Male, n (%)	35 (94.6)
Body mass index (kg/m ²), mean $\pm$ SD	27.7 $\pm$ 5.1
Risk factors and medical history	
Current smoking, n (%)	4 (10.8)
Pack-years, median (IQR)	45.0 (37.0–68.5)
Hypertension, n (%)	22 (59.5)
Diabetes mellitus, n (%)	4 (10.8)
Dyslipidemia, n (%)	16 (43.2)
Coronary heart disease, n (%)	5 (13.5)
Chronic heart failure, n (%)	6 (16.2)
OSAS, n (%)	7 (18.9)
Previous stroke, n (%)	2 (5.4)
Peripheral vascular disease, n (%)	2 (5.4)

Abbreviations: IQR, interquartile range; OSAS, obstructive sleep apnea syndrome.

during exacerbations, although statistical significance was not reached in all of them (Table 4).

The increase in platelet reactivity during exacerbations was consistent in patients with or without concomitant APT. In patients not receiving APT, a statistically significant greater PRI was observed during AECOPDs compared to stability (76.0% $\pm$ 1.5% vs 71.2% $\pm$ 2.0%, $p=0.007$; Figure 1), with parallel findings obtained with the other platelet function tests used (Table 4).

Table 2 Baseline pulmonary function parameters, laboratory data and medical therapy

	N=37
Pulmonary function test	
FEV ₁ post-bronchodilator % predicted, median (IQR)	38.6 (30.0–45.5)
FEV ₁ /FVC post-bronchodilator, median (IQR)	38.5 (35.4–45.7)
Medical therapy	
Aspirin, n (%)	8 (21.6)
Clopidogrel, n (%)	2 (5.4)
Statins, n (%)	14 (37.8)
PPI, n (%)	24 (64.9)
Oral antidiabetic agents/insulin, n (%)	4 (10.8)/2 (5.4)
ACEIs/ARBs, n (%)	15 (40.5)
Beta-blockers, n (%)	4 (10.8)
Calcium antagonists, n (%)	6 (16.2)
CPAP, n (%)	6 (16.2)
Oxygen at home, n (%)	15 (40.5)
Laboratory data	
Hemoglobin (g/L)	137 (126–150)
Platelet count ($\times 10^9$ /L)	246.0 (186.5–301.0)
Glucose (mmol/L)	5.6 (5.1–7.2)
Creatinine (μ mol/L)	72.0 (62.0–91.0)

Abbreviations: ACEIs, angiotensin converting enzyme inhibitors; ARBs, angiotensin II receptor blockers; CPAP, continuous positive airway pressure; FEV₁, forced expiratory volume in 1 second; FVC, forced vital capacity; IQR, interquartile range; PPIs, proton pump inhibitors.

Table 3 Characteristics of the acute exacerbations

	N=37
Severity of current exacerbation	
Moderate, n (%)	29 (78.4)
Severe, n (%)	8 (21.6)
Days of worsening symptoms, median (IQR)	4.0 (3.0–7.0)
pH, median (IQR)	7.41 (7.39–7.42)
PaO ₂ (mmHg), median (IQR)	69.0 (64.8–74.3)
PaCO ₂ (mmHg), median (IQR)	44.0 (39.0–50.5)
Positive culture to bacterial infection, n (%)	26 (70.3)
Virus isolation, n (%)	1 (2.7)
Requirements of systemic corticosteroids, n (%)	36 (97.3)
Requirements of antibiotics, n (%)	34 (91.9)
Requirements of hospitalization, n (%)	8 (21.6)

Abbreviation: IQR, interquartile range.

A numerically higher platelet reactivity was seen during exacerbations in the subset of patients with APT, although not achieving statistical significance (PRI: 72.8% $\pm$ 3.1% vs 61.7% $\pm$ 7.5%, $p=0.071$; Figure 1). Among those receiving aspirin ($n=8$), a higher collagen-induced platelet aggregation measured with LTA was observed during AECOPD (56.2% $\pm$ 6.1% vs 43.8% $\pm$ 6.4%, $p=0.022$), whereas the numerical differences did not reach statistical significance with the other assays sensitive to aspirin therapy (data not shown). In the two patients on clopidogrel, no differences in platelet reactivity were observed during exacerbations compared to stability (data not shown).

Systemic inflammatory parameters

The levels of all inflammatory parameters assessed were augmented during AECOPD compared to the stable state (Figure 2): leukocytes (8.8 $\pm$ 0.5 vs 7.3 $\pm$ 0.4 $\times 10^9$ /L, $p=0.001$), IL-6 (5.7 $\pm$ 0.8 vs 3.0 $\pm$ 0.4 pg/mL, $p=0.002$) and fibrinogen (5.5 $\pm$ 0.7 vs 4.7 $\pm$ 0.8 g/L, $p=0.021$).

The change in platelet reactivity measured by VASP was numerically higher in those patients in whom a greater difference in the levels of inflammatory markers between exacerbation and stability was observed. The most evident and almost statistically significant association was found between Δ PRI and Δ leukocyte count split into tertiles (lower tertile: $-0.5\%\pm 3.4\%$, mid tertile: $7.2\%\pm 3.2\%$, upper tertile: $9.0\%\pm 3.2\%$; p for trend 0.051; Figure 3), while the association was less marked for IL-6 and fibrinogen (p for trend 0.072 and 0.227, respectively; Figure 3).

Discussion

In this investigation, we evaluated the impact of an acute exacerbation on platelet reactivity in COPD patients with a comprehensive panel of assays in order to explore different

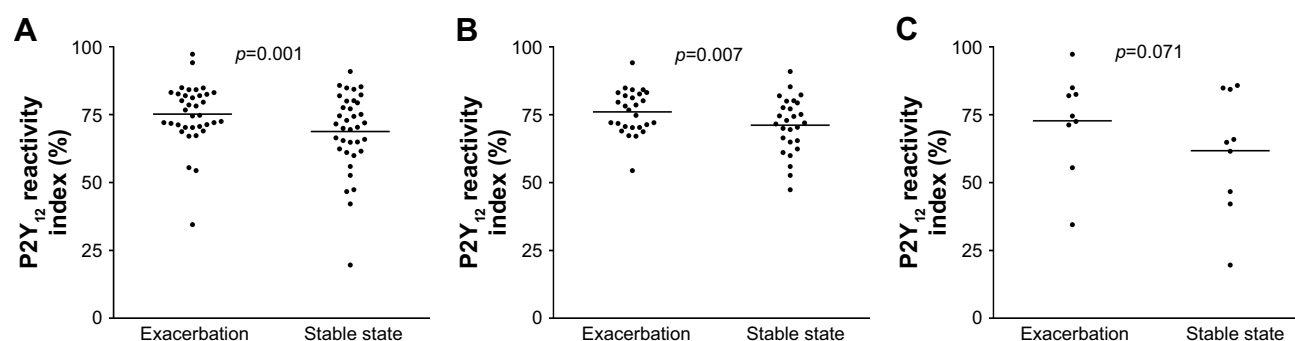


Figure 1 Platelet reactivity measured by VASP assay.

Notes: Black lines represent the least squares mean of the groups. (A) Overall population, (B) subset of patients without antiplatelet therapy, (C) subset of patients under antiplatelet therapy.

Abbreviation: VASP, vasodilator-stimulated phosphoprotein.

platelet signaling pathways. Noteworthy, patients receiving APT were not excluded, expanding upon prior studies by evaluating the impact of these agents on the results. The main findings of this study can be summarized as follows: 1) platelet reactivity is increased during AECOPD; 2) this augmented platelet aggregability is also observed in patients on APT and 3) those patients with greater enhancement of inflammatory markers during an AECOPD are more likely to present a higher increase in platelet reactivity.

Cardiovascular disease is the leading cause of mortality in COPD patients when respiratory failure is excluded.¹⁸ In fact, prior investigations have observed that COPD subjects have an increased risk of ischemic heart disease irrespective of age, gender or smoking status.^{19–22} Remarkably, a 2–3-fold increased risk of myocardial infarction has been described during the first 5 days following an AECOPD,⁸

which underscores the need for a better understanding of the mechanisms linking AECOPD, inflammation and cardiovascular atherothrombotic complications. Few studies have previously evaluated and observed augmented platelet activation in COPD patients compared to control subjects.^{23–26} Further, the impact of an AECOPD in platelet reactivity has been only evaluated in a single experience by Maclay et al who demonstrated increased circulating platelet–monocyte aggregates and, thus, higher platelet reactivity during exacerbations compared to stable COPD.¹³ In line with this, our investigation confirmed that exacerbations of COPD lead to a hyperreactive platelet phenotype, showing upregulation of multiple signaling pathways. Noteworthy, this is the first study evaluating the impact of AECOPD on platelet reactivity with platelet function assays with proven ability to predict clinical outcomes in patients with ischemic heart disease.²⁷

Table 4 Platelet reactivity during the acute exacerbation and stable state

Test	Overall population, N=37			No antiplatelet therapy, N=27		
	Exacerbation	Stable state	p-value	Exacerbation	Stable state	p-value
VASP						
PRI (%)	75.2±1.9	68.8±2.4	0.001	76.0±1.5	71.2±2.0	0.007
LTA						
MPA ADP-5 µmol/L (%)	64.2±3.4	60.6±3.6	0.076	68.7±3.5	64.1±4.2	0.069
MPA ADP-20 µmol/L (%)	69.0±2.9	67.4±2.9	0.461	72.5±2.6	69.1±2.8	0.177
MPA AA-1 µmol/L (%)	54.9±6.2	54.0±6.0	0.459	72.8±1.2	71.2±1.7	0.328
MPA Coll-2 µmol/L (%)	72.6±2.5	66.4±3.2	<0.001	78.3±1.3	73.9±1.8	0.009
MEA						
ADP (AU × min)	749.6±41.3	636.4±39.3	<0.001	824.5±33.5	707.6±41.5	0.007
Coll (AU × min)	755.4±40.5	672.51±41.6	0.028	1,049.9±60.5	1,005.3±65.1	0.393
AA (AU × min)	786.0±65.4	731.28±64.9	0.124	948.3±49.0	871.6±48.4	0.067
TRAP (AU × min)	1,048.9±55.8	1,006.3±57.5	0.383	767.4±44.3	648.7±42.7	<0.001

Notes: Platelet reactivity was measured with different platelet function assays in the overall population and in the cohort of patients not receiving antiplatelet therapy. Values are expressed as least squares mean ± SEM. The p-values were obtained using repeated-measures analysis of variance.

Abbreviations: AA, arachidonic acid; ADP, adenosine diphosphate; AU, area under the curve; Coll, collagen; LTA, light transmission aggregometry; MEA, multiple electrode aggregometry; MPA, maximal platelet aggregation; PRI, P2Y₁₂ reactivity index; SEM, standard error of the mean; TRAP, thrombin receptor activating peptide; VASP, vasodilator-stimulated phosphoprotein.

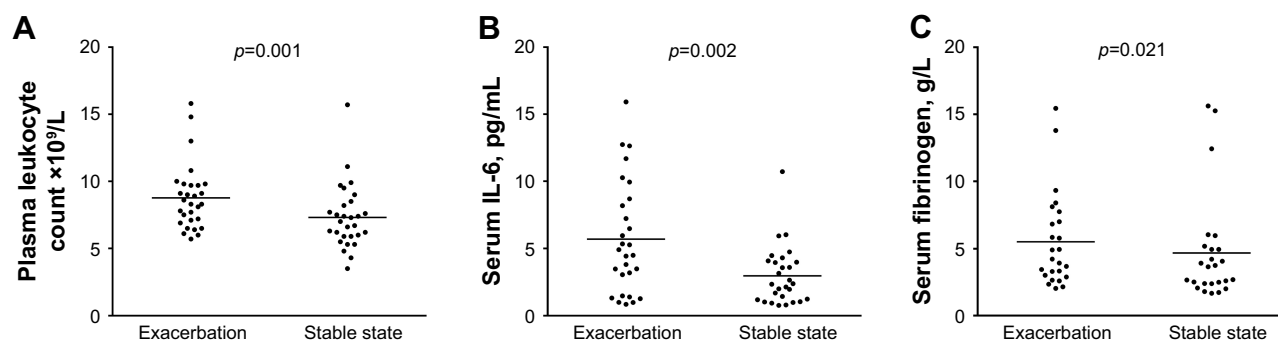


Figure 2 Inflammatory parameters assessed during AECOPD compared to the stable state.

Notes: Black lines represent the least squares mean of the groups. (A) Plasma leukocyte count, (B) serum IL-6 and (C) serum fibrinogen.

Abbreviations: AECOPD, acute exacerbations of chronic obstructive pulmonary disease; IL-6, interleukin-6.

It has been reported that COPD patients suffering a myocardial infarction have worse prognosis than non-COPD patients.²⁸ This can be partially explained by the fact that COPD patients are less likely to receive guideline-recommended secondary preventive medications with proven benefit, such as antiplatelet agents.²⁸ Another important feature of our investigation is that patients receiving APT were also included in the study, whereas they were specifically excluded in prior experiences. The finding that patients receiving APT with aspirin or clopidogrel also displayed increased platelet reactivity during an AECOPD raises questions about the efficacy of these two agents in this setting and suggests that a more potent antiplatelet drug could be of help for COPD patients in certain scenarios. Moreover, the results of a recent post hoc analysis of a randomized large-scale trial showed that ticagrelor, a very potent platelet P2Y₁₂ inhibitor, was more effective in terms of reducing the risk of ischemic events than clopidogrel (both in addition to aspirin) in COPD patients with an acute coronary syndrome.²⁹ However, further clinical

studies are required to consider if potent antiplatelet treatment must be used during exacerbations in COPD patients with concomitant ischemic cardiovascular diseases.

The exact underlying mechanisms of platelet activation in COPD patients have not been identified, although several factors have been suggested to contribute, such as increased systemic inflammation, hypoxemia and oxidative stress.³⁰ In particular, COPD is characterized by the presence of low-grade systemic inflammation and an acute inflammatory response during exacerbations of the disease.^{7,31} Moreover, the role of inflammation in the formation of atherosclerotic lesions and the occurrence of atherothrombotic complications, where platelets also play a key role, is undisputed.^{11,12} In line with this idea, an association between leukocyte count and platelet reactivity has been previously reported.³² In our investigation, those patients with greater increment of inflammatory markers such as leukocyte count had a numerical trend towards larger increases in platelet reactivity without reaching statistical significance. This finding might support

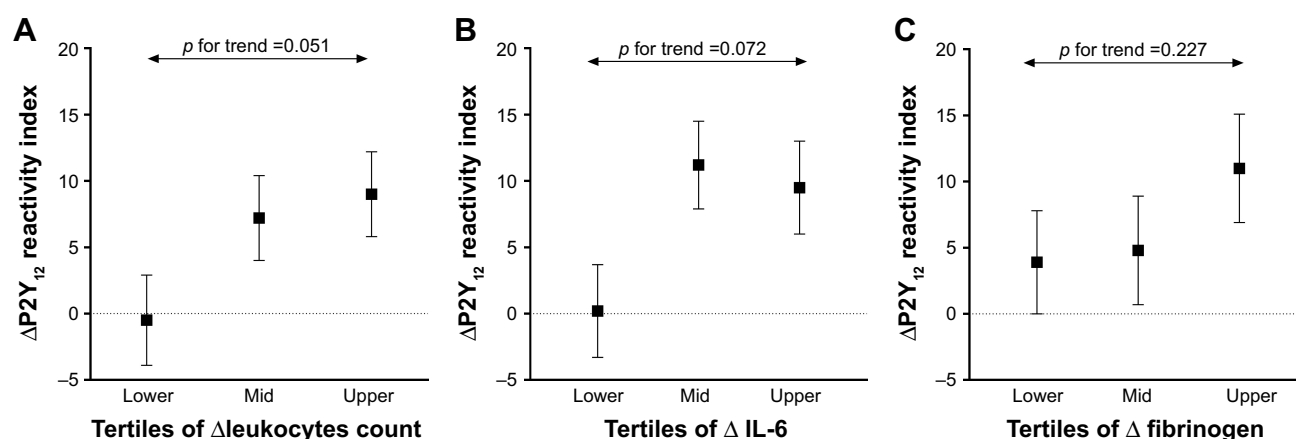


Figure 3 Association between the PRI and the inflammatory parameters.

Notes: The change in PRI was evaluated as the difference: PRI during AECOPD–PRI during stable state. The difference in the inflammatory parameters during AECOPD and stable state was divided into tertiles. (A) Difference (Δ) in plasma leukocyte count, (B) difference (Δ) in serum IL-6 and (C) difference (Δ) in serum fibrinogen.

Abbreviations: AECOPD, acute exacerbations of chronic obstructive pulmonary disease; IL-6, interleukin-6; PRI, P2Y₁₂ reactivity index.

the idea of an association between inflammation and platelet activation in COPD patients.

This study has several inherent limitations due to its observational design and relatively small sample size. However, the consistent findings obtained using a variety of assays and agonists are supportive of our study conclusions. The subset of patients receiving APT is particularly small, making the study underpowered to detect differences in this subgroup; therefore, results obtained in this subgroup should be considered exploratory and hypothesis generating. In addition, all patients included in our study had severe and very severe airway limitation, representing an advanced stage of the disease. Therefore, whether the findings of our study can be extrapolated to mild or moderate COPD patients cannot be ascertained.

Conclusion

Platelet reactivity is augmented during acute exacerbations of COPD, irrespective of treatment with antiplatelet agents. This platelet hyperreactivity may contribute to the augmented cardiovascular risk of these patients. In addition, the increase in platelet reactivity could be directly associated with an increment in systemic inflammatory markers during exacerbations.

Acknowledgments

The authors would like to thank E Aliagas and M Gracida for their contributions to acquisition and analysis of data. Similarly, the authors would like to thank MJ Manuel, M Navarro and G Sosa for their assistance in sample collection and performing laboratory techniques. The study was funded by a grant from The Spanish Society of Pulmonology and Thoracic Surgery (SEPAR-116/2013) awarded to Dr Muñoz-Esquerre and by a grant from the Bellvitge Biomedical Research Institute (IDIBELL) as part of a Pre-doctoral Research Fellowship awarded to Dr Muñoz-Esquerre. The Catalan Foundation of Pulmonology (FUCAP 2013) supported this study with a grant awarded to Dr Santos. JL Ferreira reports receiving honoraria for lectures from Eli Lilly Co, Daiichi Sankyo Inc., Astra Zeneca, Pfizer; consulting fees from AstraZeneca, Eli Lilly Co, Daiichi Sankyo Inc.; and research grants from AstraZeneca. This work is solely the responsibility of the authors.

Author contributions

MME is the guarantor of the paper, taking responsibility for the integrity of the work as a whole, from inception to the published article. MME and JLF contributed substantially to

the study concept and design, data collection, data analysis and interpretation, and the writing of the manuscript. DH, ALM, MLS, GR, JAGH, JD, AC and SS contributed substantially to the study design, data analysis and interpretation, and the writing of the manuscript. All authors have read and approved the final version of the manuscript.

Disclosure

The authors report no conflicts of interest in this work.

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