

Increased platelet activation in patients with stable and acute exacerbation of COPD

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ABSTRACT

Rationale Chronic obstructive pulmonary disease (COPD) is associated with systemic inflammation and cardiovascular disease. Interaction between inflammatory cells and activated platelets is important in the pathogenesis of atherothrombosis and may contribute to cardiovascular risk in patients with COPD.

Objectives To assess platelet-monocyte aggregation in patients with COPD and matched controls, and in patients with an acute exacerbation of COPD.

Methods 18 men with COPD and 16 male controls matched for age and cigarette smoke exposure were recruited. A further 12 patients were investigated during and at least 2 weeks after hospitalisation for an acute exacerbation. Platelet-monocyte aggregation and platelet P-selectin expression were determined using flow cytometry.

Results Patients with COPD had increased circulating platelet-monocyte aggregates compared with controls (mean (SD) 25.3 (8.3)% vs 19.5 (4.0)%, $p=0.01$). Platelet-monocyte aggregation was further increased during an acute exacerbation compared with convalescence (32.0 (11.0)% vs 25.5 (6.4)%, $p=0.03$). Platelet P-selectin expression and soluble P-selectin did not differ between groups.

Conclusions Patients with stable COPD have increased circulating platelet-monocyte aggregates compared with well-matched controls. Platelet activation is further increased in patients with COPD during an acute exacerbation. These findings identify a novel mechanism to explain the increased cardiovascular risk in COPD and suggest platelet inhibition as a plausible therapeutic target.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is an independent risk factor for cardiovascular disease,¹ although the mechanisms responsible for this association remain unclear. Systemic inflammation is recognised as an important determinant of atherosclerosis,^{2,3} and COPD is characterised by both pulmonary and systemic inflammation. It has been postulated that low-grade systemic inflammation in patients with COPD may explain this increase in cardiovascular risk.⁴ Inflammatory pathways are upregulated further during an acute exacerbation and may plausibly precipitate an acute cardiovascular event.⁵

Inflammatory cells and cytokines have been implicated in atheromatous plaque formation and coronary thrombosis.⁶ Following vascular injury and endothelial denudation, circulating platelets

Key messages

What is the key question?

- ▶ Is COPD associated with increased platelet activation, a marker of cardiovascular risk, and is this further increased during exacerbations?

What is the bottom line?

- ▶ Platelet-monocyte aggregates are increased in stable COPD and are even higher during exacerbations of COPD.

Why read on?

- ▶ Platelet activation represents a novel mechanism by which cardiovascular risk may be increased in stable COPD and during exacerbations.

become activated, upregulating expression of cell surface receptors such as P-selectin and CD40 ligand to facilitate adhesion to the arterial wall. Activated platelets release inflammatory chemokines and recruit inflammatory cells to form platelet-monocyte aggregates, an early process in atherothrombosis.⁷ Circulating platelet-monocyte aggregates are considered a sensitive measure of platelet activation and are raised in patients with acute coronary syndromes, smokers and in those with rheumatoid arthritis.^{8–10}

We hypothesise that platelet activation will be increased in patients with stable COPD and acute exacerbations, and may represent a link between inflammation and cardiovascular disease in these patients. We therefore measured markers of platelet activation, including platelet-monocyte aggregates, in patients with stable COPD and matched controls, and in patients during an acute exacerbation and in convalescence.

METHODS

We compared measures of platelet activation between patients with COPD and matched controls (study 1) and examined the effect of acute exacerbation by comparing platelet activation in patients during an exacerbation and in convalescence (study 2). These studies were approved by the Lothian regional ethics committee and conducted with the written informed consent of all participants.

Study 1: Patients with COPD and matched controls

Eighteen men with COPD and 16 male controls were recruited from primary care and a hospital

respiratory outpatient clinic at the Royal Infirmary of Edinburgh and matched for age and prior smoking habit. Ex-smokers of at least 6 months with a smoking history of ≥ 10 pack-years were included. Control subjects had normal spirometry and no history of respiratory symptoms. Subjects with COPD had a history consistent with the disease, chronic airflow limitation on spirometry (post-bronchodilator forced expiratory volume in 1 s/forced vital capacity (FEV₁/FVC) ratio ≤ 0.7), stable disease (no exacerbation of COPD within the previous 6 weeks) and were not prescribed regular oral steroid therapy or long-term oxygen therapy. Exclusion criteria in both patients and controls included other respiratory disease, coronary artery disease, diabetes mellitus, hepatic and renal failure, and any systemic inflammatory condition such as rheumatoid arthritis or psoriasis, or use of medication known to affect vascular and platelet function (including statins, ACE inhibitors and clopidogrel), as used in a previous vascular study.¹¹ Our strict inclusion and exclusion criteria were used to allow us to try to separate the effects of COPD on platelet activation from those of comorbid conditions known to influence platelet function.

Subjects were fasted overnight and blood sampled between 08.00 h and 10.00 h. Subjects abstained from caffeine and alcohol for 24 h prior to the study and avoided all medications for at least 12 h prior to attendance. Height, weight and post-bronchodilator spirometry were measured (Alpha Spirometer; Vitalograph, Buckingham, UK) according to American Thoracic Society/European Respiratory Society standards following venesection.¹² Arterial stiffness and vascular function were measured in both patients and controls with the findings reported elsewhere.¹¹

Study 2: Acute exacerbations of COPD

Twelve patients admitted to the Royal Infirmary of Edinburgh with an acute exacerbation of COPD were studied within 24 h of admission and at least 2 weeks following discharge from hospital when their condition was considered to be clinically stable. The diagnosis of acute exacerbation of COPD was made by the admitting respiratory physician. All patients had documented chronic airflow limitation on spirometry when stable (post-bronchodilator FEV₁/FVC ratio ≤ 0.7 , FEV₁ percentage predicted $< 80\%$) and a smoking history of ≥ 10 pack years. Subjects with a suspected or proven alternative diagnosis for the acute deterioration in symptoms such as pneumonia, pulmonary embolism or heart failure were excluded. Patients were seen for their follow-up visit at least 2 weeks after treatment for an

exacerbation with improvement in their symptoms. No patients included in study 1 were enrolled in study 2.

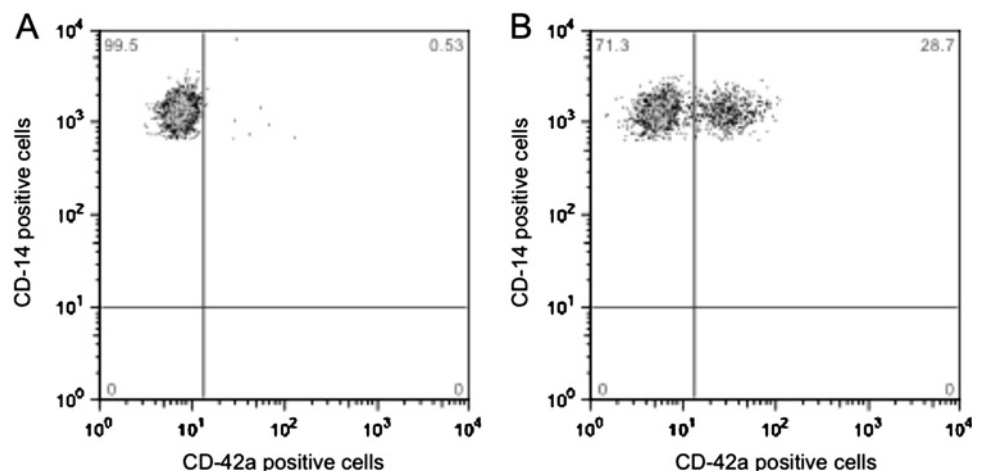
Blood collection

Blood was drawn by clean venepuncture of a large antecubital vein using a 19-gauge needle with care taken to ensure a smooth blood draw. Samples were collected into tubes containing the direct thrombin inhibitor D-phenylalanine-L-prolyl-L-arginine chloromethyl (PPACK, Cambridge Biosciences, Cambridge, UK) as previously described.¹³ Tubes were gently inverted to ensure mixing of whole blood with anticoagulant. Further blood samples were collected for the measurement of haemoglobin, haematocrit and differential leucocyte count (Sysmex, Norderstedt, Germany), and for the measurement of blood glucose (fasting in study 1, random in study 2) and lipid profiles (Olympus Analyzers, Brea, CA, USA) in the regional clinical laboratories at the Royal Infirmary of Edinburgh. Arterial blood gases were measured at rest in study 1 (Bayer Rapidlab, Morristown, NJ, USA), as were D-dimer (bioMerieux, Basingstoke, UK) and fibrinogen levels (ACL TOP analyser, Instrumentation Laboratory, Warrington, UK). Soluble P-selectin was determined by an ELISA (R&D Systems, Abingdon, UK) in platelet-poor plasma. Serum C-reactive protein (CRP) concentrations were measured using a highly sensitive immunonephelometric assay (Behring BN II nephelometer, Hattersheim am Main, Germany).

Immunolabelling and flow cytometric analysis

Five minutes following sample collection, whole blood was immunolabelled at room temperature for subsequent flow cytometric analysis of platelet-monocyte aggregates using monoclonal antibodies to phycoerythrin (PE)-conjugated CD14 (specifically binds to monocytes), fluorescein isothiocyanate (FITC)-conjugated CD42a (specifically binds to platelets) and isotype matched controls (Biosource, Renfrew, UK). After 20 min of incubation, samples were fixed and red cells lysed with FACS-Lyse solution (Becton Dickinson, Oxford, UK). Samples were analysed using a BD FACScan Flow Cytometer and data analysed using FlowJo software (Treestar, Oregon, USA). A medium flow setting was used to minimise leucocyte-platelet coincident events. Platelet-monocyte aggregates were defined as monocytes positive for CD42a (figure 1). In our laboratory the mean coefficient of variation for the percentage of platelet-monocyte aggregates is 7.8%.¹⁴ In addition, whole blood was immunolabelled with FITC-conjugated CD42a, PE-conjugated CD62P (specifically binds to P-selectin) and isotype-matched controls (Biosource), fixed with paraformaldehyde and analysed

Figure 1 Flow cytometric analysis of platelet-monocyte aggregates in whole blood. (A) A quadrant plot of CD14-positive monocytes with isotype control was used to set the CD42a gate, adjusted for non-specific binding. (B) The upper right quadrant shows complexes positive for CD14 (monocytes) and CD42a (platelets), platelet-monocyte aggregates.



with flow cytometry to determine platelet surface expression of P-selectin.

Data analysis

Results are presented as mean (SD). Unpaired *t* tests were used to compare measures of platelet activation and haematological and biochemical indices between patients and controls (study 1), and paired *t* tests were used for within-subject comparisons (study 2). CRP was log-transformed for positive skewness and the data were presented as median (IQR). There was no evidence of inhomogeneity of variance or departures from normality in any of the other data.

In exploratory analyses, associations between platelet-monocyte aggregates, age, markers of inflammation (blood neutrophils, blood leucocytes and highly sensitive CRP) and markers of disease severity (post-bronchodilator FEV₁ and arterial oxygen tension) were determined using Pearson correlations. Statistical significance was taken at *p*<0.05.

RESULTS

Study 1

Patients with COPD and controls were well matched for age and smoking history with a median pack-year history of 36 and 35, respectively (table 1). Patients with COPD had moderate to severe airflow limitation (Global Initiative in Obstructive Lung Disease (GOLD) stages 2–4) with a mean FEV₁ of 1.5 l and FEV₁/FVC ratio of 0.42 (table 1).

Platelet-monocyte aggregates were increased in patients compared with matched controls (mean (SD) 25.3 (8.3)% vs 19.5 (4.0)%, *p*=0.01; figure 2A, table 2). Platelet expression of P-selectin was higher in patients with COPD than controls, but the difference was not statistically significant (1.6 (1.2)% vs 1.1 (0.8)%, *p*=0.16). Similarly, there was no difference in plasma soluble P-selectin concentrations between patients and matched controls.

Table 1 Baseline characteristics of patients with chronic obstructive pulmonary disease (COPD) and matched controls

	Study 1		Study 2 COPD
	Controls	COPD	
N	16	18	12
Age, years	63 (6)	65 (5)	68 (12)
Male, n (%)	16 (100)	18 (100)	5 (42)
Body mass index, kg/m ²	28 (4)	26 (4)	24 (6)
Smoking history, pack-years*	35 (26–48)	36 (35–51)	45 (39–68)
Current smoking	0	0	3 (25)
Medications when stable			
Short-acting β agonist	0	17 (94%)	8 (67%)
Long-acting β agonist (LABA)	0	3 (17%)	7 (58%)
Inhaled corticosteroids (ICS)	0	1 (6%)	8 (67%)
Oral corticosteroids	0	0	2 (17%)
Combined LABA/ICS	0	13 (72%)	7 (58%)
Anticholinergics	0	11 (61%)	7 (25%)
Oxygen therapy	0	0	1 (8%)
Aspirin	1 (6%)	2 (11%)	1 (8%)
Post-bronchodilator pulmonary function			
FEV ₁ , l	3.4 (0.5)	1.5 (0.7)	0.81 (0.3)
FVC, l	4.2 (0.6)	3.5 (0.6)	2.0 (0.7)
FEV ₁ % predicted	102 (10)	48 (20)	39 (17)
FVC% predicted	100 (11)	85 (15)	90 (22)
FEV ₁ /FVC ratio	0.79 (0.05)	0.42 (0.13)	0.42 (0.12)

Values are mean (SD) or n (%) unless indicated otherwise.

*Median (IQR).

FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity.

Platelet-monocyte aggregates were compared with markers of inflammation and disease severity across all subjects in study 1. Platelet-monocyte aggregates correlated with total blood leucocyte count (*r*=0.45, *p*=0.007) and neutrophil count (*r*=0.36, *p*=0.03). There was a weak association with FEV₁ (*r*=0.31, *p*=0.07). There were no significant associations between platelet-monocyte aggregates and age, arterial oxygen or serum CRP concentration.

Study 2

Patients with an acute exacerbation had a mean age of 68 years, with lung function measured when stable similar to patients in study 1 (table 1). However, other indicators of disease severity such as the long-term use of nebuliser therapy, oxygen therapy and oral corticosteroid were more prevalent in this group. During acute exacerbations, all subjects received controlled oxygen, nebulised bronchodilators, oral corticosteroids and prophylactic low molecular weight heparin (enoxaparin 40 mg) for a median of 6 days following their first symptom. Clinical parameters were consistent with an acute exacerbation and had returned to normal values by the follow-up visit.

Platelet-monocyte aggregates were increased during the acute exacerbation compared with follow-up (mean (SD) 32.0 (11.0)% vs 25.5 (6.4)%, *p*=0.03; figure 2B, table 2). Platelet expression of P-selectin was higher during the acute exacerbation than during follow-up but the difference was not statistically significant (1.4 (2.1)% vs 0.8 (1.2)%, *p*=0.40), while there was no difference in soluble P-selectin concentrations.

DISCUSSION

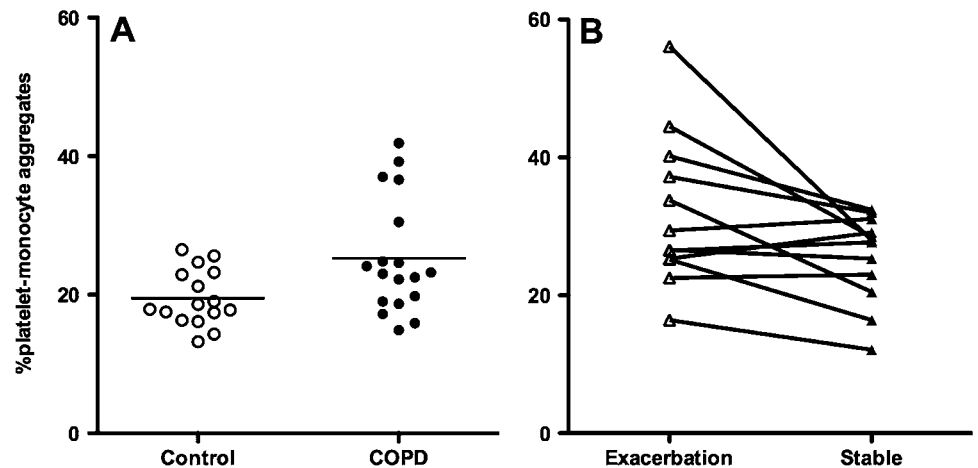
Using a highly sensitive marker of platelet function, we have shown that platelet activation is increased in patients with stable COPD compared with controls matched for age and previous cigarette smoke exposure. Moreover, platelet activation is further increased in patients with COPD during an acute exacerbation. Taken together, these findings suggest that platelet function may be modified as a consequence of COPD.

We suggest that platelet activation represents a novel mechanism linking COPD, inflammation and cardiovascular disease. Platelet activation is known to predict an adverse outcome in patients with stable coronary disease¹⁵ and to identify patients likely to have recurrent cardiovascular events following percutaneous coronary intervention.¹⁶ The interaction between platelets and inflammatory cells stimulates release of chemokines and further recruitment of immune mediators which are central to the development of atherosclerotic plaque.

Platelet activation has also been implicated in structural remodelling of the pulmonary vasculature, and is thought to play a role in the pathogenesis of all forms of pulmonary arterial hypertension.¹⁷ Our findings are therefore potentially of relevance to the pulmonary vascular—as well as systemic vascular—features of COPD. We have previously reported abnormal systemic vascular function in COPD. More specifically, patients with COPD have increased arterial stiffness in comparison with controls.¹¹ In this study we have looked at a distinct but equally important aspect of atherothrombosis and cardiovascular risk—namely, platelet activation.

Previous studies have suggested that COPD is associated with a prothrombotic and hypercoagulable state.^{18–19} Few studies have measured platelet activation and none has employed a direct measure of platelet function. Soluble P-selectin was increased in patients with COPD²⁰ and inversely related to FEV₁.²¹ However, soluble P-selectin is not a direct measure of platelet activation and may reflect P-selectin release from the

Figure 2 (A) Patients with chronic obstructive pulmonary disease (COPD) have increased platelet-monocyte aggregates in comparison with matched controls ($p=0.01$). (B) Patients with an exacerbation of COPD have increased platelet-monocyte aggregation compared with stable disease ($p=0.03$). Lines represent the median values of the groups.



endothelium and platelet surface.²² Additionally, concentrations of soluble P-selectin may not reflect platelet surface P-selectin expression.^{23 24} Our study adds to the literature by demonstrating increased platelet activation using whole blood flow cytometry, which is both a sensitive and specific technique, in patients with COPD and controls well-matched for both age and, importantly, for smoking history. Furthermore, our findings are consistent across two separate cohorts, with levels of platelet-monocyte aggregation identical in patients with stable COPD from both studies.

The effects of cigarette smoking on markers of platelet activation and platelet-monocyte aggregates are well established.⁹ In our case-control study, all patients and controls were ex-smokers with normal exhaled carbon monoxide levels and were matched for smoking history, yet we found that platelet-monocyte aggregates were increased in patients with COPD by approximately one-third compared with controls. The magnitude of this difference was comparable to differences previously reported between smokers and non-smokers.⁹ This implies that platelet function may be modified as a direct consequence of

COPD, and that this potentially prothrombotic manifestation may be as important as cigarette smoking in determining cardiovascular risk in these patients. Our results are consistent with the differences seen in other inflammatory conditions associated with increased cardiovascular risk, such as rheumatoid arthritis, where platelet-monocyte aggregate levels were around 20% higher than matched controls.¹⁰ Higher platelet-monocyte aggregation is found in acute coronary syndromes, with a 30–50% increase in platelet-monocyte aggregation in comparison with patients with non-cardiac chest pain, but these levels were found during acute arterial thrombotic events.^{8 25}

We have not identified the precise mechanism of platelet activation in patients with COPD, but a number of variables—such as increased systemic inflammation, hypoxaemia and haemodynamic stress—that differed between patients and controls or were enhanced during acute exacerbation may be implicated.

Platelet activation is inextricably linked to local vascular inflammation, with activated platelets causing release of chemokines together with upregulation of cell surface adhesion

Table 2 Haematological and biochemical indices in patients with chronic obstructive pulmonary disease (COPD) and controls

	Study 1			Study 2		
	Controls	COPD	p Value	Stable	Exacerbation	p Value
Measures of platelet activation						
Platelet-monocyte aggregates, %	19.5 (4.0)	25.3 (8.3)	0.01	25.5 (6.4)	32.0 (11.0)	0.03
P-selectin expression, %	1.1 (0.8)	1.6 (1.2)	0.16	0.8 (1.2)	1.4 (2.1)	0.40
Soluble P-selectin, ng/ml	53.0 (22.2)	56.5 (20.3)	0.65	53.5 (17.8)	61.5 (20.8)	0.56
Haematological indices						
Leucocytes, cells, $\times 10^9/l$	5.3 (1.3)	7.0 (1.4)	0.23	7.9 (1.6)	14.8 (3.2)	<0.001
Monocytes, $\times 10^9/l$	0.5 (0.2)	0.6 (0.2)	0.001	0.7 (0.3)	1.0 (0.3)	0.001
Haemoglobin, g/l	139 (11)	141 (13)	0.03	136 (9)	138 (12)	0.40
Haematocrit	0.4 (0.03)	0.4 (0.03)	0.004	0.4 (0.02)	0.4 (0.04)	0.41
Platelets, $\times 10^9/l$	196 (31)	262 (68)	0.001	290 (54)	333 (127)	0.09
D-dimer, ng/ml	341 (202)	373 (137)	0.63			
Fibrinogen, mg/dl	2.7 (0.5)	2.8 (0.5)	0.49			
Biochemistry						
Total cholesterol, mmol/l	4.9 (0.9)	5.5 (0.7)	0.03	5.4 (1.4)		
Glucose, mmol/l	5.2 (0.6)	5.1 (0.6)	0.71	5.5 (1.7)		
C-reactive protein, mg/l*	1.0 (0.5–3.2)	2.1 (1.3–5.4)	0.03	2.8 (1.6–8.2)	41 (4–103)	0.01
Arterial oxygenation, kPa	12.3 (1.6)	10.5 (1.7)	0.004			

Values are mean (SD) unless indicated otherwise.

p Values calculated using independent samples t test in study 1 and paired t tests in study 2. C-reactive protein was log-transformed for analysis.

*Median (IQR).

molecules that drive monocyte recruitment, platelet-monocyte interaction and adherence to denuded endothelium.²⁶ Furthermore, leucocytes recruited by chemotaxis cause activation of platelets.²⁷ We identified increases in the number of peripheral blood leucocytes and neutrophils as well as higher serum concentrations of CRP in patients with COPD compared with control subjects. Circulating leucocyte and neutrophil concentrations were associated with platelet-monocyte aggregates, supporting the hypothesis that systemic inflammation in COPD may contribute to platelet activation in this condition. This mechanism is thought to be important in other chronic inflammatory conditions with increased platelet-monocyte aggregates such as rheumatoid arthritis and type 1 diabetes mellitus.^{10 28} Although there was not a significant relationship between CRP and platelet-monocyte aggregates, there was a positive association and this may have been revealed in a study of larger numbers.

Alternative mechanisms through which platelet activation may occur in patients with COPD include hypoxia, tachycardia and hyperglycaemia.^{29 30} These factors may cause further platelet activation during an acute exacerbation and explain the association between lower respiratory tract infection and acute myocardial infarction.³¹ Further studies using cellular and animal models are needed to elucidate the relative importance of these mechanisms.

Interestingly, patients with COPD had higher platelet counts than controls although levels remained within the normal range. An increase in platelet count per se has been associated with adverse cardiovascular outcomes in both healthy persons and patients with acute myocardial infarction.^{32 33} Previous studies have suggested that anaemia is associated with an increased morbidity and mortality in patients with COPD independent of disease severity,^{34 35} but the relationship between platelet count and clinical outcome has not been examined. Thus, in comparison with controls, patients with COPD not only have greater platelet activation but also increased numbers of platelets, and both may increase cardiovascular risk.

Platelet counts and platelet-monocyte aggregates may be more than simply markers of cardiovascular risk, with platelet activation a potential target for therapy. Platelet-monocyte aggregates form independently of the cyclo-oxygenase pathway and thus are not modified by aspirin therapy.³⁶ Population studies and controlled trials are needed to determine whether aspirin is an effective antiplatelet therapy in COPD and whether the regular use of antiplatelet agents could prevent cardiovascular events in patients with COPD.

Limitations of the study

Although selection bias is possible in all case-control studies, the groups in study 1 were well-matched for age, smoking and other clinical characteristics and, as such, we do not think this can explain the reported differences in platelet activation. We did not match on weight or body mass index as we were interested in the systemic effects of COPD and, since reduced weight is a systemic effect of COPD, we did not want to over-match patients and controls. In study 2 we examined platelet activation within 24 h of admission with an exacerbation of COPD and at least 2 weeks post-exacerbation, while there is some evidence that the effects of acute exacerbations may persist beyond 90 days.³⁷ However, this would probably cause an underestimation of an effect of exacerbation on platelet activation. Additionally, we were unable to impose the same restrictions on patients with acute exacerbations as we could in the stable condition, so differences in medication, dietary intake or

other environmental factors may have contributed to the platelet-monocyte aggregation observed during exacerbations. However, imposing such restrictions is impractical in these patients, and medications used during acute exacerbations—such as steroids and prophylactic low molecular weight heparin—do not influence platelet activation.^{38 39}

CONCLUSION

Using a highly sensitive marker of platelet activation, we have shown that platelet-monocyte aggregates are increased in patients with stable COPD independent of cigarette smoke exposure. Platelet activation was further increased in patients during an acute exacerbation. Our findings suggest that platelet function may be modified as a direct consequence of COPD, and identify platelet activation as an important prothrombotic manifestation of the disease which may be a useful therapeutic target in COPD.

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Competing interests None.

Ethics approval This study was conducted with the approval of the Lothian regional ethics committee.

Contributors JDM and DAMc contributed equally to this manuscript.

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