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Inflammation, coagulation and their interaction in Chronic Obstructive Pulmonary Disease

Floor Elise Aleva

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Inflammation, coagulation and their interaction in Chronic Obstructive Pulmonary Disease

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CHAPTER 1

General Introduction and aims of the thesis

GENERAL INTRODUCTION

Chronic Obstructive Pulmonary Disease (COPD) is currently defined as '*a common, preventable and treatable disease, which is characterized by persistent airflow limitation that is usually progressive and associated with an enhanced chronic inflammatory response in the airways and lung to noxious particles and gases. Exacerbations and comorbidities contribute to the overall severity in individual patients'*.[1]

COPD is one of the leading causes of morbidity and mortality and accounts for an estimated 3 million deaths a year worldwide.[2] COPD occurs around the globe and the prevalence is still increasing, mostly due to increased life expectancy.[3] In addition to its individual impact, the burden for healthcare facilities is substantial and the costs associated with COPD are estimated to be 50 billion dollars annually in the United States alone.[4] In The Netherlands, around 760.000 people suffer from COPD, which is 4-5% of the overall Dutch population. As COPD develops over years, the reported prevalence among the Dutch adults aged ≥ 40 years is considerably higher and ranges between 11.5% to 24%.[5,6]

In order to increase attention for COPD prevention and management, the Global Initiative for Chronic Obstructive Lung Disease (GOLD) was established in 1998.[7] Among the important objectives of GOLD are to increase awareness of COPD and to help the millions of people who suffer from this disease and die prematurely from it or its complications.[7]

Common symptoms of COPD are breathlessness, excessive sputum production, and a chronic cough, symptoms that generally increase over time and eventually may lead to respiratory failure. In addition to these respiratory complaints, patients often suffer from comorbidities, such as cardiovascular diseases (CVD), which suggests a common denominator in disease pathophysiology.[8,9] This has led to the current understanding of COPD as a multisystem disorder.[8,10,11] Increased attention to comorbidities has emphasized a role for systemic inflammation in its development, as inflammation and coagulation are closely linked biological systems.[8,10-12] Significant research efforts focus on the mechanisms underlying these observations, however, these mechanisms are not well defined so far.[11]

COPD: an inflammatory disease.

COPD develops from an abnormal inflammatory reaction in the lungs in response to prolonged exposure to inhaled noxious particles or gases, such as cigarette smoke or air pollution.[13-16] In response to these irritants, the local immune system initiates an inflammatory response in order to eliminate these foreign substances. Under normal

circumstances this inflammatory response is well regulated and after elimination of the foreign substances, the immune cells will return to a resting state.

In COPD, this inflammatory response is commonly thought to be excessive.[14,17] As a first response to inhaled irritants, the airway epithelial cells and tissue macrophages produce inflammatory mediators, including cytokines.[13,14] The cytokines attract various cells of the immune system to the site of the inflammation, including (but not restricted to) monocytes, neutrophils and dendritic cells. After recruitment of these cells from the blood stream into the lung tissue, an innate inflammatory response is orchestrated and pro-inflammatory cytokines, reactive oxygen species and proteolytic enzymes are released.[14,16,17] In COPD this initial response is not well balanced and results in elaborate damage of the lungs.[14,17]

Accessory release of antigens will subsequently initiate an adaptive immune response. Among these adaptive immune cells are cluster of differentiation (CD) 4 positive- and CD8 positive T cells that proliferate and differentiate into specific subtypes that establish an antigen-specific immune response.[14] This process further aggravates the lung damage and results in thickening of the airway wall and disruption of lung parenchyma.[14,17]

The prolonged exposure to inhaled noxious particles or gases does not only affect inflammation within the lung, but also has a systemic effect on inflammation. Indeed, increased inflammatory cytokines such as interleukin-6 (IL-6), C-reactive protein (CRP), and also endothelial activation markers have been described in COPD and generally increase during acute exacerbations of COPD (AE-COPD).[18-20] In addition, various studies report differences in circulating inflammatory cells in COPD patients compared to control subjects and these cells may contribute to COPD pathophysiology as well as the development of comorbidities.[21,22]

In most cases, COPD develops as a result of prolonged and/or intense tobacco smoke exposure. The effects of smoking on immune activation have been extensively studied, however, these studies vary greatly in their designs. Some studied specific tobacco smoke components,[23] whereas others focused on specific diseases or specific immune cell subtypes, both in human and non-human species.[24-26] Conflicting findings are reported and the exact inflammatory profiles that may eventually progress into COPD are not yet identified.

It should be noted, however, that considerable variability is observed within the clinical course of COPD and in line with this, possibly also in the related local and/or systemic inflammatory responses. Individual variation in immune responses may therefore de-

termine both susceptibility to COPD and to the development of comorbidities. It is well known, for instance, that not all subjects exposed to inhaled noxious particles or gases develop COPD. Around 30-40% of smokers develop COPD,[15] suggesting that a combination of environmental, genetic and intrinsic individual factors determine COPD disease susceptibility and the course of disease.[27-29] Studies that improve insight in the underlying pathophysiolocal mechanisms and provide opportunities for modulation of the immune system are warranted.

Cardiovascular comorbidities in COPD.

In the last two decades, increased interest in the extrapulmonary effects of COPD has identified several comorbidities that are more prevalent in COPD in comparison to healthy smokers and non-smokers.[1,27,30] Among these, cardiovascular comorbidities represent an important group, not at the least for their impact on patient outcome.[31,32] Around 50% of hospital admissions in patients with COPD can be attributed to CVD and 25-27% of deaths.[31,32]

Furthermore, several studies show important associations between respiratory function and the risk for cardiovascular mortality in the general population.[33-36] Hole and colleagues investigated the relation between forced expiratory volume in one second (FEV1) and mortality in 15,411 adults in West Scotland and reported relative hazard ratios of 1.56 (1.26-1.92) in men and 1.88 (1.44-2.47) in women for death due to CVD in the lowest quintile of FEV1.[33] Moreover, in the Baltimore Longitudinal Study of Aging, adults with the most rapid decline in FEV1 had a 3 to 5 times increased risk for cardiovascular death, compared to those with the slowest decline in FEV1, independent of baseline FEV1 and after correction for confounders, such as age, smoking, hypertension and body mass index.[35]

Emerging data suggests that the link between COPD and CVD cannot be fully explained by the risk factor smoking.[33,35,37] Myocardial infarction and, to a lesser extent, stroke have therefore attracted considerable attention in COPD[34,38-41].

Atherosclerosis, a chronic inflammatory condition leading to vascular plaques and vessel damage, plays an important role in the development of these vascular events. Plaque rupture and arterial thrombosis are acute triggers for acute vascular events, especially in a high shear environment.[42,43] Blood platelets do not only play an important role in atherosclerosis, but particularly in thrombus formation[44]. Anti-platelet therapy is therefore the cornerstone of the medication provided to patients with vascular events. Apart from its anti-thrombotic effects, anti-platelet therapy may also have immunomodulatory effects.[45,46]

Blood platelets may therefore also play an important role in the clinical course of disease in COPD. Indeed, thrombocytosis is associated with increased short and long term mortality after acute exacerbations of COPD.[47] Surprisingly, apart from platelet numbers, the function of platelets is scarcely studied in COPD.

Furthermore, it is currently debated whether patients with COPD are also at increased risk for venous thromboembolism, such as pulmonary embolism (PE) and deep venous thrombosis (DVT). Current research efforts show conflicting results that range from an increased risk of venous thromboembolism to no clear association with COPD at all[48-50]. The pathogenesis of PE and DVT is different from myocardial infarction and stroke as venous thrombosis relies more on plasmatic coagulation. Venous thrombi develop in an environment with low shear rates or even blood stasis, that develop over hours to days and are rich in fibrin and contain a high number of red blood cells, whereas arterial thrombi develop in a high shear environment and contain a high number of platelets (Table 1).[42]

Table 1. Pathogenesis of venous thrombi and arterial thrombi

	Venous thrombosis	Arterial thrombosis
Anatomical region	(Large) Veins	Arteries
Environment	Low shear environment	High shear environment
Important trigger(s)	Blood stasis	Atherosclerotic plaque rupture
Content	Fibrin rich, red blood cells	Platelets
Time of development	Hours to days	Minutes to hours
Diseases	PE, DVT	Myocardial infarction, Stroke
Treatment	Anticoagulant treatment	Platelet-inhibitors

(Abbreviations: DVT: Deep Venous Thrombosis; PE: Pulmonary Embolism)

These different pathophysiological mechanisms have important clinical implications and require different therapeutic management.[51,52]

Low-grade systemic inflammation is commonly thought to be responsible for the excess risk for thromboembolic events in COPD.[8,11,34,37,53] This hypothesis is strengthened by the fact that the risk of myocardial infarction and stroke further increases during episodes of increased inflammation, like during or shortly after AE-COPD.[38,40,41]

Interaction between inflammation and coagulation.

Inflammation and coagulation are highly integrated biological systems and extensive crosstalk between these systems exists.[12,54] Dysregulation of one system may result in dysregulation of the other. In critically ill sepsis patients this becomes very evident in the case of disseminated intravascular coagulation or when sepsis is complicated by

acute cardiovascular events.[54] Tissue factor (TF), a type I integrin membrane glycoprotein, plays a pivotal role in the interaction between these systems. Vascular subendothelial cells constitutively express TF, in order to rapidly initiate the coagulation cascade during vascular damage.[55] In contrast, cells in close contact with blood, such as endothelial cells and monocytes, are able to upregulate membrane expression of TF when activated by physical or chemical damage or by inflammatory cytokines.[54,55] The latter is of great interest, as the most important regulator of TF expression seems to be interleukin-6 (IL-6).

Exposure of TF to blood activates the coagulation cascade by complex formation of TF with circulating factor VIIa. This complex catalyzes several steps of conversion of different coagulant factors, resulting in the formation of thrombin (factor IIa).[12] Thrombin enables the conversion from fibrinogen into fibrin which is essential for clot formation and thrombin activates platelets via cleavage of protease activated receptors (PAR) 1 and 4, see figure 1.[12,42] This highlights that the processes of arterial and venous thrombosis are not mutually exclusive, but are connected.

Platelets are key players in interaction between inflammation and coagulation. These small, anucleated cells exhibit important immune functions and can interact both with cells from the innate as with the adaptive immune system.[56] For example, platelets can bind to monocytes to form platelet-monocyte complexes, an early process in atherothrombosis.[44,57] In addition, platelets can promote activation of monocytes and dendritic cells[45], thereby enhancing host response.[46] Also, recent evidence suggests that IL-6 and collagen are able to activate platelets via activation of Signal Transducer and Activator of Transcription 3 (STAT3).[58,59] STAT3 is important for transduction of a variety of cell signals, including signals involved in inflammation.

Another factor that may link inflammation and coagulation is vitamin D. Vitamin D is increasingly appreciated for its inflammatory properties,[60,61] however, its association with coagulation is less investigated. The effects of vitamin D on platelet reactivity are currently unknown, while the occurrence of myocardial infarctions and stroke clearly show seasonal variation, like vitamin D levels.[62-64] Vitamin D is also often prescribed in COPD patients, not only as vitamin D deficiencies and corresponding bone loss are common in COPD patients, but also because of the presumed favourable effects.

Knowledge on these interactions may provide new targets for modulation of inflammation, coagulation and their interaction, as inflammation and coagulation are driven by common pathways. Careful study of these pathways is important as therapeutic interventions are often based on these studies. Anti-inflammatory drugs are the cornerstone of exacerbation management in COPD, while the use of anti-platelet drugs may be considered as well.

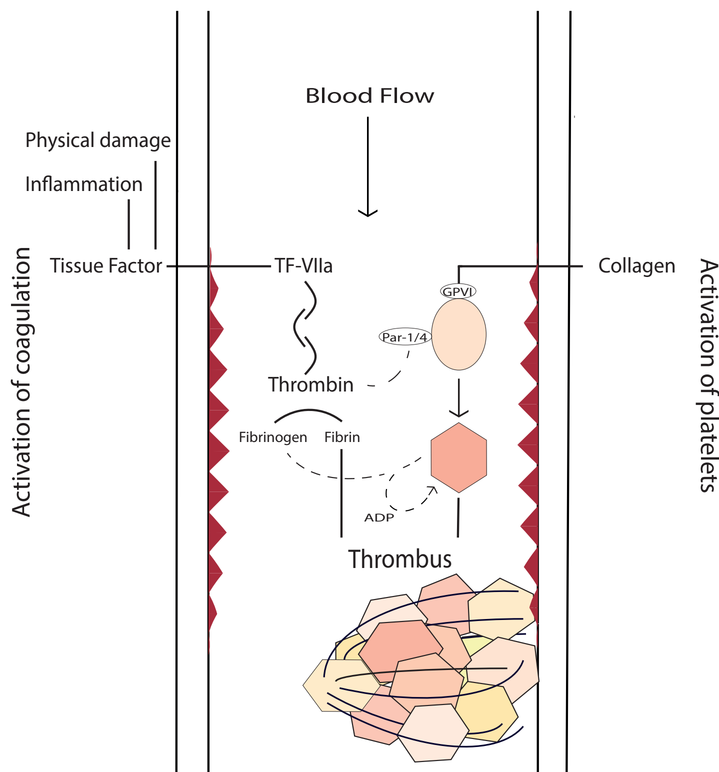

Figure 1. Simplified presentation of the interaction between inflammation and coagulation. During physical damage and/or inflammation tissue factor is released and binds to factor VIIa. Via several steps of conversion thrombin is formed. Thrombin converts fibrinogen into fibrin and is also able to activate platelets via PAR-1 and Par-4, which results in platelet activation and aggregation. These processes are pivotal for thrombus formation. (*Abbreviations: ADP: adenosine di-phosphate; GPVI: glycoprotein VI; PAR: protease activated receptors; TF-VIIa: Tissue factor –factor VIIa complex*)

Aims of the thesis.

The main aim of this doctoral thesis is to investigate the interaction between inflammation and coagulation in COPD and the potential to modulate their interaction. Therefore, the objectives were:

1. To perform a meta-analysis of the current evidence on the prevalence of pulmonary embolism, as an expression of venous thrombosis, in acute exacerbations of COPD
2. To investigate platelet-monocyte interaction and platelet function in COPD as a potential mechanism for arterial thrombosis.
3. To investigate thrombin generation and clotting time in COPD as a potential mechanism for venous thrombosis.
4. To investigate the interaction between inflammation and coagulation in general, with a focus on factors that may play a role in COPD

Outline of the thesis.

The first chapters of this doctoral thesis describe the presence of pulmonary embolism in COPD and the potential mechanisms underlying thromboembolic events in COPD. In **Chapter 2** a systematic review and meta-analysis on the prevalence of pulmonary embolism in patients with unexplained AE-COPD is reported. A thorough literature search of MEDLINE and EMBASE databases was performed and an overview of the current literature is provided. In **Chapter 3,** we describe a case-control study in which platelet activation and thrombin generation potential was measured in 30 patients with moderate to severe stable COPD and in 25 matched control subjects. The patients were recruited at the outpatient clinic of Radboud university medical center and control subjects were recruited among employees. Since cardiovascular events tend to cluster with periods of increased inflammation, platelet function was also assessed in COPD patients with an acute exacerbation. The findings of this study are reported in **Chapter 4.** In this study, we used a before-after model in which COPD patients were recruited during admission for an AE-COPD at the Radboud university medical center. The measurements were performed during the exacerbation and repeated after 6 to 10 weeks post-exacerbation.

The next chapters have a more general focus on the interaction between inflammation and coagulation. First, the effects of different mutations in transcription factor STAT3 on platelets were investigated and the results are described in **Chapter 5.** Two cohorts were investigated, the first cohort concerned patients with autosomal dominant Hyper IgE syndrome, a rare immune deficiency disorder. The specific mutations in these patients result in non-functional STAT3 activity and was therefore used as a model to study the effects on platelets. To determine whether the effects of STAT3 mutations on platelets also occur in the general population, data from the 500FG cohort (a large cohort of healthy human volunteers) were used. The data from the 500FG cohort were also used in the following two chapters. In **Chapter 6** we investigated the association between different vitamin D single nucleotide polymorphisms (SNPs) and platelet function and plasmatic coagulation**.** Finally, to our current knowledge the best preventive measure for COPD is still smoking cessation. Therefore, in **Chapter 7** we highlight the association between cigarette smoking and immunological phenotype and immune function in the 500FG cohort. In this study smokers, former smokers and non-smokers were carefully matched. To conclude, the most important findings of this thesis and the implications are discussed in **Chapter 8.**

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CHAPTER 2

Prevalence and localization of Pulmonary Embolism in Unexplained Acute Exacerbations of COPD: A systematic Review and Meta-analysis

Aleva FE, Voets LWLM, Simons SO, de Mast Q, van der Ven AJAM, Heijdra YF

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ABSTRACT

Background

COPD patients encounter episodes of increased inflammation, so-called acute exacerbations of COPD (AE-COPD). In 30% of AE-COPD no clear etiology is found. Since there is a well-known crosstalk between inflammation and thrombosis, the objectives of this study were to determine the prevalence, embolus localization and clinical relevance, and clinical markers of pulmonary embolism (PE) in unexplained AE-COPD.

Methods

A systematic search was performed using MEDLINE and EMBASE platforms from 1974 – October 2015. Prospective- and cross-sectional studies that included patients with an AE-COPD and used pulmonary CT-angiography for diagnosis of PE were included.

Results

The systematic search resulted in 1650 records. Main reports of 22 articles were reviewed and 7 studies were included. The pooled prevalence of PE in unexplained AE-COPD was 16.1% (95% confidence-interval 8.3%-25.8%) in a total of 880 patients. Sixty-eight percent of the emboli found were located in the main pulmonary arteries, lobar arteries or inter-lobar arteries. Mortality and length of hospital admission seem to be increased in patients with unexplained AE-COPD and PE. Pleuritic chest pain and cardiac failure were more frequently reported in patients with unexplained AE-COPD and PE. In contrast, signs of respiratory tract infection was less frequently related to PE.

Conclusions

PE is frequently seen in unexplained AE-COPD. Two-thirds of emboli are found at localizations that have a clear indication for anticoagulant treatment. These findings merit clinical attention. PE should receive increased awareness in patients with unexplained AE-COPD, especially when pleuritic chest pain and signs of cardiac failure are present and no clear infectious origin can be identified.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is currently the third cause of mortality and morbidity worldwide.[1-3] Acute exacerbations of COPD (AE-COPD) are an important aspect of disease as they lead to worsening of respiratory symptoms, deterioration of respiratory function and worsening of prognosis.[4-7] The majority of AE-COPD develop in response to infections, however in about 30% of AE-COPD no clear etiology is found.[8]

In AE-COPD, co-existence or development of secondary disorders can significantly determine outcome.[5,9] One of these potentially harmful disorders is pulmonary embolism (PE). Previous studies suggest a high prevalence of PE in AE-COPD, ranging from 18 - 25 percent.[10-12] This prevalence might be explained by the increase of inflammatory markers during AE-COPD.[13-15] Population studies, however, have shown either a modest excess risk for PE in COPD with odds ratios ranging from 2.51 (Confidence interval (CI) 1.62-3.87) to 5.46 (CI 4.25-7.02)[16-18] or no association at all.[19] Since there is a well-known crosstalk between inflammation and coagulation, patients may be particularly at risk for PE during AE-COPD.[13,20-22]

Clinical evaluation of PE in AE-COPD might pose some challenges. First, improvements in resolution of multislice computed tomography pulmonary angiography (CTPA) has led to increased observation of filling defects with a diameter of 2-3mm.[23,24] The clinical relevance of these isolated subsegmental PE is under debate.[25-29] It is unknown how often these isolated subsegmental PE are found in AE-COPD. Another important issue is the lack of clinical markers for identification of PE in AE-COPD. Early recognition of PE is of vital importance, but difficult due to overlap in clinical symptoms.[30] This makes it challenging to recognize co-incidence of PE in AE-COPD.

Since publication of the previous systematic review on PE in AE-COPD in this journal in 2009, several well-performed studies have been published.[10,12,23,24,31] The objectives of this systematic review are 1) to update the pooled prevalence on PE in unexplained AE-COPD, 2) to review the localization and clinical relevance of the filling defects on CTPA and 3) to identify clinical markers of PE in unexplained AE-COPD.

METHODS

Literature search

The systematic review was performed in accordance with PRISMA guidelines.[32] A literature search for articles written in English was performed using the databases MEDLINE

(1946 – October 2015) and EMBASE (1974 – October 2015). The main search terms were "Pulmonary Disease, Chronic Obstructive" and "Pulmonary Embolism". Synonyms of "Pulmonary Disease, Chronic Obstructive" resulted in: "Chronic obstructive pulmonary disease", "Chronic Airflow Obstruction", "COPD", "Chronic obstructive Airway Disease", "COAD", "Chronic Obstructive Lung Disease", "Chronic Airflow Obstruction" , "Obstructive Respiratory Disease" as terms. Synonums of "Pulmonary Embolism" resulted in: "Pulmonary thromboembolism", "PE", "Chronic Lung Embolism", "Lung Embolus", "Lung emboli", "Lung Microembolism", "Lung Microembolus", "Lung Thromboembolism", "Pulmonary emboli", "Pulmonary Embolus", "Pulmonary Microembolism". In addition, search term "Venous Thromboembolism" and its synonyms were used. All terms were searched in title, abstract and as a keyword.

Study selection

We included studies that (1) reported on prevalence of pulmonary embolisms in patients with AE-COPD; (2) provided empirical data; (3) diagnosed PE based on CTPA and excluded studies that (4) were performed in stable COPD patients, (5) were performed retrospectively or (6) used registries for diagnosis of PE. Title screening was performed by one investigator (L.V.). Evaluation of abstracts followed by evaluation of main reports was performed independently by two investigators (F.A. and L.V.). Disagreements were resolved by consultation with a third investigator (Y.H.). Reference lists of all included articles were screened and potentially suitable papers were reviewed.

Study outcomes

The primary outcome of this study was the prevalence of PE in unexplained AE-COPD. Secondary outcome parameters were; prevalence of deep venous thrombosis (DVT), localization of pulmonary embolism, clinical outcome and a set of clinical markers. Clinical markers were divided into patient characteristics, disease characteristics, clinical symptoms, physical examination findings, respiratory function, blood gas analysis, laboratory findings, other diagnostic measurements, clinical prediction scores and treatment. More detailed information on the clinical markers that have been reviewed can be found in e-Table 1.

Data extraction

Two investigators (F.A., L.V.) independently extracted the following data by using a standardized protocol: title, authors, date of publication, study location, inclusion period, number of centers, setting, study design, in- and exclusion criteria, study objectives, diagnostic modality, information on D-dimer testing, number of patients, mean age and range, gender, prevalence of pulmonary embolism, prevalence of deep venous thrombosis (DVT) and localization of pulmonary embolism. Additionally, clinical markers were extracted, these can be found in e-Table 1.

Quality assessment

Two investigators (F.A and L.V.) independently appraised the quality of the included studies using the STROBE-score, a quality measure for observational studies. This score has a maximum of 22 points and is shown in table 1.

Statistical analysis

The primary outcome was the prevalence of PE in patients presenting with an AE-COPD. A double arcsine transformation was performed to stabilize data variance.[33] Heterogeneity among studies was assessed by using the χ^2test, defining a significant heterogeneity as a *P* value < 0.10, whereas inconsistency was quantified using the I^2statistic.[34] As significant heterogeneity was observed, a random-effect model was used to calculate the overall pooled prevalence of PE and DVT in unexplained AE-COPD. To systematically ascertain predictors for study heterogeneity, mixed-effects model meta-regression analyses was performed for the following potential predictors of heterogeneity; 1) age of study population, 2) gender proportions of study population and 3) exclusion of pneumonia (as main difference observed in study design between included studies). All analyses were conducted using the R statistical software, metaphor package.[35,36]

The localization of the filling defects on CTPA was determined and categorized into pulmonary trunk, main pulmonary artery, segmental artery or isolated subsegmental PE. In addition, we reviewed whether the presence and localization of PE affected mortality and the length of hospital admission. Pooling of data regarding clinical markers was not possible as absolute numbers from several studies could not be extracted. Data on clinical markers were synthesized.

RESULTS

Study selection

The literature search resulted in 2997 records in EMBASE- and MEDLINE databases. After limits for articles written in English, exclusion of conference abstracts and deduplication, 1650 unique records remained. Of these, 1551 records were excluded based on title. The remaining 99 abstracts were checked for eligibility, resulting in exclusion of 74 abstracts. Twenty-two studies were selected for evaluation of main reports. Inter-rater reliability was 0.86, rated as 'almost perfect agreement'. Interestingly, 2 studies were performed by Akpinar and colleagues in the same hospital and had the same inclusion period. One of the studies focused on prevalence of PE in AE-COPD and the other focused on D-dimer cut off value in AE-COPD.[31,37] As we were unable to state that these were separate study populations, the study that focused on D-dimer

Table 1. Characteristics of studies included with descriptive statistics on rates of PE and DVT[a].

Study	Year	Country	Patient selection criteria	Definition of AE-COPD	Setting	AE-COPD patients	Age, mean – SD	Gender (% male)	PE (%)	DVT (%)	Mortality in PE	STROBE-score
Shapira-Rootman et al.	2015	Israel	All COPD patients admitted to the hospital for an AE-COPD and COPD confirmed by spirometric data.	Worsening of dyspnea that required admission to the hospital	Inpatients	49	65.5[a]	71.4	18.4	N/A	Not described	19
Akpinar et al.	2014	Turkey	All COPD patients hospitalized for an AE-COPD and COPD confirmed in medical history and medical records	Worsening in respiratory symptoms beyond normal day-to-day variations that led to a change in medication	Inpatients	172	71.31 – 9.6	82.6	29.1	29.1	No significant difference	20
Choi et al.	2013	South Korea	All COPD patients hospitalized for an AE-COPD and COPD confirmed by spirometric data and medical records	Acute deterioration from a stable condition that required admission to the hospital	Inpatients	103	71 – 6.0	70	5.0	6	No significant difference	19
Kamel et al.	2013	Egypt	Not described	Not described	Inpatients	105	49.3 – 8.4	100	28.6	10.5	Not described	18
Gunen et al.	2010	Turkey	All COPD patients admitted to the hospital for an AE-COPD and COPD confirmed by medical history, medical records and medications utilized.	Acute deterioration from a stable condition that required admission to the hospital.	ED	131	67 – 10.1	79.40	13.7	10.6	Increased in-hospital mortality and increased one year mortality	19

Table 1. Characteristics of studies included with descriptive statistics on rates of PE and DVT[a]. (continued)

Study	Year	Country	Patient selection criteria	Definition of AE-COPD	Setting	AE-COPD patients	Age, mean – SD	Gender (% male)	PE (%)	DVT (%)	Mortality in PE	STROBE-score
Rutschmann et al.	2007	Switzerland	All patients admitted to the emergency department for an AE-COPD and moderate to severe COPD according to GOLD definition.	Worsening of dyspnea that required admission to the emergency department.	Inpatients	123	71 – 8.0	68	3.3	2.2	Not described	20
Tillie-Leblond et al.	2006	France	All COPD patients admitted to the hospital for an AE-COPD and COPD diagnosed according to ATS criteria.	Acute deterioration from a stable condition that required admission to the hospital.	inpatients	197	60.5 – 12.1	83.6	25.0	12.7	Not described	21

a. Range 43-92 years (*Abbreviations: AE-COPD: acute exacerbations of COPD; ATS: American thoracic society; GOLD: global initiative for obstructive lung disease; DVT: deep venous thrombosis; ED: emergency department; N/A, not available; PE, pulmonary embolism; SD: standard deviation; STROBE: strengthening the reporting of observational studies in epidemiology)*

cut off value in AE-COPD was excluded.[37] After evaluation of the other main reports, investigators agreed on definitive inclusion of 7 studies.[11,12,31,38-41] Study selection and main reasons for exclusion are depicted in figure 1.

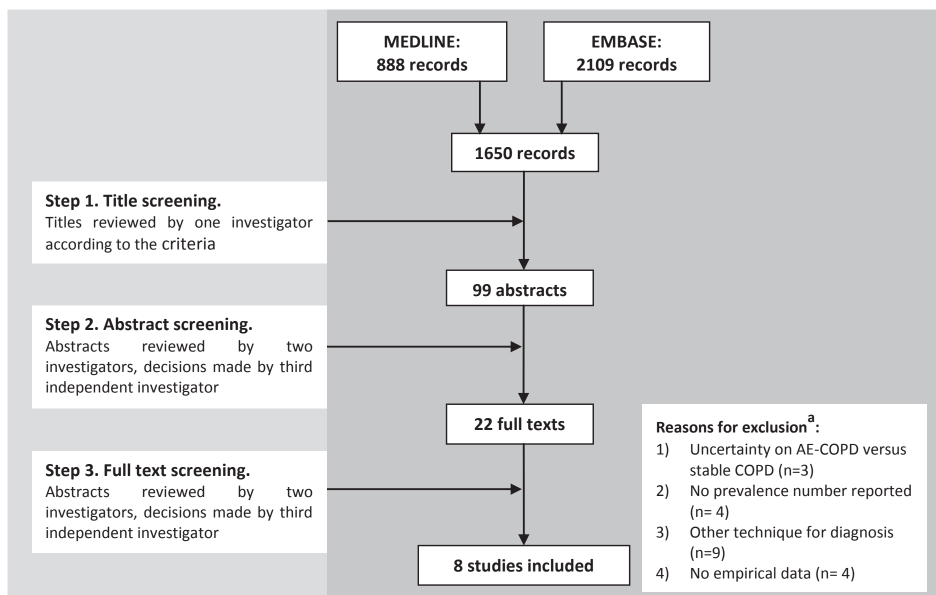

Figure 1. Flow diagram of the study selection process and numbers of studies identified.
a. *studies could have fulfilled more than one criterion. (Abbreviations: AE-COPD: acute exacerbation of COPD)*

Study characteristics

A total of 880 patients were included in this systematic review. The 7 included studies were performed in 6 different countries and 2 studies originated from Turkey. One study recruited patients at the emergency department, while the other studies included hospitalized patients. The mean age and percentage of male patients of included studies varied from 49.3 to 71.3 years and 68% to 100%, respectively. Characteristics of included studies can be found in Table 1.

Pooled prevalence of pulmonary embolism in acute exacerbations of COPD

Overall, 16.1% (95% confidence-interval 8.3%-25.8%) of patients with AE-COPD had PE (Figure 2). Variation between included studies was large, with a prevalence ranging from 3.3% to 29.1%. Six studies reported on the prevalence of deep venous thrombosis (DVT) in AE-COPD.

The pooled prevalence of DVT in AE-COPD was 10.5% (95% confidence interval 4.3%-19.0%) (Figure 3). Mixed-model meta-regression analyses of age, gender differences and exclusion of pneumonia as main difference in study design could not determine the main predictors for heterogeneity observed in included studies.

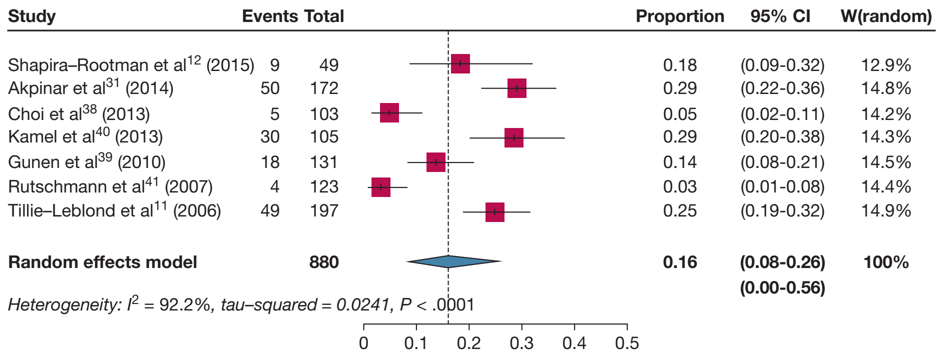

Figure 2. Prevalence of PE in patients with AE-COPD.
(Abbreviations: AE-COPD: acute exacerbations of COPD; PE: pulmonary embolism; W: weight)

Localization and clinical relevance of PE in AE-COPD

Five included studies documented embolism localization, including a total of 726 COPD patients of which 120 patients presented with PE in unexplained AE-COPD.[11,31,38,39,41] Of PE cases, 39 (32.5%) had isolated subsegmental PE. Other localizations were one of the main pulmonary arteries in 42 cases (35.0%), lobar and inter-lobar arteries in 38 cases (31.67%), and one patient had an embolism in the pulmonary trunk (0.83%) (Table2).

The relation between isolated subsegmental PE and more proximal localizations of PE and clinical outcome was not reported. Three studies assessed length of hospital admission, two studies found an increase in PE, whereas one could not detect a significant difference.[31,38,39] In terms of clinical outcome, in-hospital mortality and one-year mortal-

Table 2. Localization of PE found in AE-COPD.

Anatomical region	Cases	Percent (%)
Pulmonary trunk	1	0.8
Main pulmonary arteries	42	35.0
Lobar- and inter-lobar arteries	38	31.7
Isolated subsegmental arteries	39	32.5
Total	120	100%

(Abbreviations: AE-COPD: acute exacerbations of COPD; PE: pulmonary embolism)

ity was increased in patients with PE in AE-COPD in one study,[39] but not in another (p= 0.26).[38] Gunen and colleagues additionally performed a Cox regression analysis and found that presence of venous thromboembolism was the only factor that increased one-year mortality.[39]

Clinical markers of PE in AE-COPD

Included studies considered a broad range of clinical symptoms, biochemical parameters and patient- and disease characteristics (e.g. age, gender, weight, GOLD stage). An overview of all clinical markers that have been reviewed is provided in e-Table 1. Clinical markers that show significant differences in AE-COPD patients in presence or absence of PE are shown in table 3. Studies that report upon the same clinical marker but did not detect a statistically significant difference are also included in the table.

Overall, pleuritic chest pain was reported more frequently in AE-COPD with PE compared to AE-COPD without PE.[31,39] Akpinar and Gunen et al. found pleuritic chest pain in 81.0% in the PE group versus 40.0% in non-PE patients, and 24.0% in the PE group versus 11.5% in non-PE patients, respectively.[31,39] However, Tillie-Leblond et al. could not show a difference (p-value 0.73).[11] Signs of cardiac failure like hypotension, syncope and acute right failure on ultrasonography were diagnostic clues associated with PE, whereas signs of a respiratory tract infection was seen less frequent in PE cases.[31,38,39]

In general, age, gender, presence of comorbidities (hypertension, diabetes mellitus and coronary artery disease), smoking habits and BMI did not show a relation with risk for PE in AE-COPD, as shown in e-Table 1. None of included studies related findings to exacerbation frequency, severity of exacerbation or treatment with glucocorticoids in included patients.

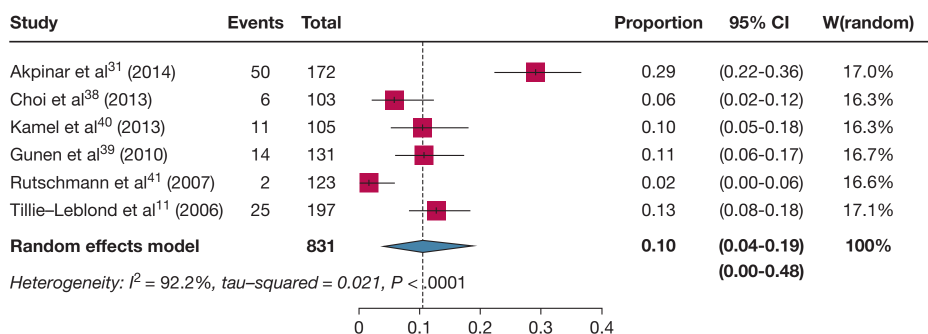

Figure 3. Prevalence of DVT in patients with AE-COPD.
(Abbreviations: AE-COPD: acute exacerbations of COPD; DVT: deep venous thrombosis; W: weight)

Table 3. Markers for identification of PE in AE-COPD.

Markers	Significant Studies	Not significant Studies
Patient characteristics		
Gender	Gunen	Akpinar, Choi, Tillie-Leblond
Obesity	Akpinar	Choi, Gunen, Tillie-Leblond
Clinical symptoms		
No symptoms of RTI	Choi	
Increased sputum	Choi	Akpinar
Pleuritic chest pain	Akpinar, Gunen	Tillie-Leblond
Physical examination		
Hypotension	Gunen	
Lower leg asymmetry	Akpinar	Tillie-Leblond
Syncope	Gunen	
Respiratory function		
FEV1	Shapira-Rootman	Choi
Arterial blood gas		
PaCO2 lower in PE	Akpinar	Choi, Gunen, Tillie-Leblond
pH value higher in PE	Akpinar	
Decrease in PaCO2	Tillie-Leblond	
Laboratory findings		
NT-proBNP	Akpinar	Choi
Polycytemia	Kamel	
Diagnostics		
ECG		
AF	Gunen	
Ultrasonography		
Cardiac failure	Gunen	Tillie-Leblond
Chest radiograph		
Atelectasis	Shapira-Rootman	
Pulmonary artery enlargement	Shapira-Rootman	
Wells score		
Previous VTE	Tillie-Leblond	Akpinar, Gunen
Malignancy	Akpinar, Gunen	Tillie-Leblond
Immobility	Gunen	
Outcome measures		
Length of stay	Akpinar, Gunen	Choi
Mortality	Gunen [a]	Choi
Need for ICU treatment		Akpinar

[a] Cox regression analyses revealed that VTE was the only factor that significantly increased mortality.

(Abbreviations: AF: atrial fibrillation; NT-proBNP: N-terminal prohormone of brain natriuretic peptide; RTI: respiratory tract infection)

DISCUSSION

Our review shows that PE is common in unexplained AE-COPD with an estimated prevalence of 16% percent. Moreover, two-thirds of these emboli are located in the main pulmonary arteries, lobar arteries or inter-lobar arteries, suggesting that the majority of these embolisms have important clinical consequences. Included studies reported conflicting results regarding clinical markers. Overall, patients with PE in AE-COPD more frequently experienced pleuritic chest pain and more frequently had signs of cardiac failure. On the contrary, symptoms suggestive of a respiratory tract infection is reported less often in patients with PE in AE-COPD. Although clinical outcome could not be related to embolus localization, patients with PE in unexplained AE-COPD seem to have increased mortality and increased length of hospital admission.

This review shows a slightly lower prevalence of PE in unexplained AE-COPD compared to previous systematic review that reported an overall prevalence of 19.9%.[10] Since publication of this systematic review 5 relevant studies have been published. Two studies from the previous systematic review were excluded since our review solely included patients with AE-COPD.[42,43] Inclusion of these 2 studies to the analyses would slightly increase PE prevalence in AE-COPD (18.5%, 95% CI 10.1%-27.5%). The risk for PE is strongly increased in unexplained AE-COPD, especially compared to other inpatient populations that show PE prevalence rates of 5.7-6.0%.[44,45] PE may be seen in particular in exacerbations as systemic inflammation and thrombosis are closely related.[13,14,19]

Markers of systemic inflammation, like acute-phase reactants C-reactive protein (CRP) and fibrinogen, contribute to development of thrombotic events.[13,20,21] Also, tumor necrosis factor-α (TNF-α), a pro-inflammatory cytokine, contributes to a procoagulant state by induction of tissue factor and inhibition of anticoagulant protein C.[20,22] In AE-COPD these mediators are markedly increased systemically, while in stable COPD only a subgroup of patients show low-grade systemic inflammation.[46-48] This may explain lower risk estimates found in population studies.[16,18,19] Therefore, exacerbation susceptibility and exacerbation frequency could be important determinants for development of PE. Second, patients with AE-COPD are often treated with glucocorticoids, that increase risk for venous thromboembolism, especially after initiation of treatment.[49] Others have suggested that PE could be a trigger for AE-COPD or mimick exacerbation-like symptoms in COPD patients, since vascular occlusion leads to bronchoconstriction[30,50-52] This could be another potential explanation for the high prevalence found specifically in unexplained AE-COPD.[11,39,53] In order to fully understand the exact mechanisms involved in development of PE in unexplained AE-COPD further mechanistic studies are needed.

Two thirds of the emboli were clinically relevant PE, located in more proximal than subsegmental pulmonary arteries. These more proximally located emboli have a clear indication for anticoagulant treatment according to the new ACCP guideline.[28] Pulmonary embolism has a general 28-day case fatality rate of 15.1%, which increases to 32.5% in patients requiring hospitalization for another condition.[19,54] Also, we found increased hospital stay and mortality in patients with PE. One third of PE in AE-COPD is limited to isolated subsegmental pulmonary arteries. Unfortunately, these outcomes could not be related to embolus localization. This would have been of great interest, as the clinical relevance of these smaller filling defects found on CTPA is not well defined.[25,55] In isolated subsegmental PE anticoagulant treatment may be withheld and clinical surveillance is preferred under certain circumstances.[28]

Several clinical markers seem to be related to PE in AE-COPD, in particular pleuritic chest pain and signs of cardiac failure. Similarly, symptoms suggestive of a respiratory tract infection would argue against PE in AE-COPD. Though formal logistic regression analysis was not possible owing to the lack of raw data, our study suggests that these findings merit clinical attention and should be taken into account in patients with unexplained AE-COPD. It might add to the clinical decision making in patients with an AE-COPD, because it would be undesirable to perform a CTPA in every patient with an AE-COPD. Unintended adverse effects such as renal failure and allergic reactions to iodinated contrast agents should be taken into account. Also, the liberal use of CTPA in AE-COPD poses the risk of unnecessary radiation exposure and could increase the costs of the health care system. Currently, efforts are made to safely withhold CTPA.[55] Very recent work by van Es and colleagues shows that age-adjustment of D-dimer may improve exclusion of PE in order to safely withhold imaging in suspected cases.[56] These findings need further confirmation, but may have important potential to address this issue in COPD patients.

The present study has several limitations. First and most important limitation is the heterogeneity of findings observed between included studies, with prevalence rates of PE ranging from 3.3% to 29.1%. This makes the mean estimate of 16.1% less stable and this estimate should be seen in conjunction with the CI of 8.3%-25.8%. The low prevalence found by Choi and colleagues might be explained by ethnicity as Asians have a lower risk for development of venous thromboembolism (Risk Ratio=0.2, (95% CI 0.1-0.5)).[57] Yet, the study with the lowest prevalence was performed in a predominantly white population.[41] PE was excluded in patients with a negative D-dimer, which is generally accepted as safe.[58-60] Also, small sample sizes may influence the overall estimate and although the random-effects model takes sample size into account, findings of individual studies may be skewed. A meta-regression analysis of potential predictors; age, gender and exclusion of pneumonia (as difference observed in study design), was

unable to determine the main sources for heterogeneity. Since all included studies applied comparable criteria for acute exacerbations and used CTPA for detection of PE we cannot completely explain differences observed between included studies. This heterogeneity requires that the findings, and mean prevalence in particular, should be interpreted with caution.

Second limitation is the risk of publication bias. Studies with findings near the extremes, such as a very high or very low prevalence, might be easier published due to newsworthiness.[61] Also in this review very low and relatively high prevalence rates are found in included studies. Although publication bias seems less relevant for prevalence studies, since findings are not criticized based on p-value, the potential influence publication bias should not be underestimated.[62] Additionally, studies that have not been included in MEDLINE and EMBASE databases may be overlooked.

Last, all studies in this systematic review included a relatively large proportion of male subjects. One of the included studies found that male gender is associated with PE in their study, while 3 other studies did not detect significant differences.[11,31,38,39] Male gender has been shown to be an independent risk factor for venous thromboembolism in the general population, however our meta-regression analysis could not identify gender as potential predictor of the findings.[63]

CONCLUSIONS

PE is seen in 16% of patients with AE-COPD and two-thirds of these emboli are found at localizations that have a clear indication for anticoagulant treatment. These findings merit clinical attention. PE should receive increased awareness in patients with unexplained AE-COPD, especially when pleuritic chest pain and signs of cardiac failure are present and no clear infectious origin can be identified.

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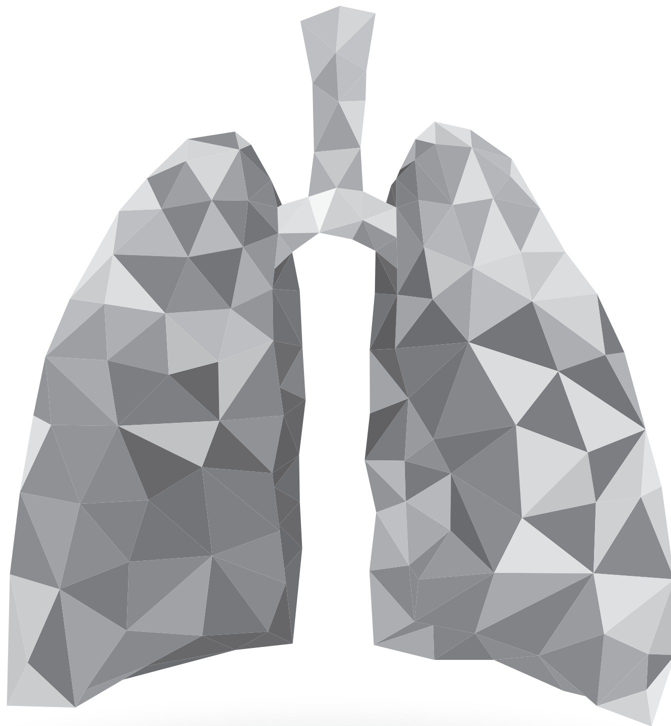
Online supplement to

Prevalence and localization of Pulmonary Embolism in Unexplained Acute Exacerbations of COPD: A systematic Review and Meta-analysis

Aleva FE, Voets LWLM, Simons SO, de Mast Q, van der Ven AJAM, Heijdra YF



Chest. 2017. Mar;151(3):544-554

e-Table 1. Clinical markers associated with PE in AE-COPD.

Clinical Markers		Significant studies ($p < 0.05$)	Not significant studies ($p > 0.05$)
Patient characteristics			
	Age		Akpinar, Choi, Gunen, Kamel, Tillie-Leblond
	Gender (male)	Gunen	Akpinar, Choi, Tillie-Leblond
	Body Mass Index		Choi, Gunen
	Obesity	Akpinar	Tillie-Leblond
	Comorbidities		Akpinar, Choi
	Smoking Status		Choi
	Pack Years		Choi, Gunen
Disease characteristics			
	Gold classification		Akpinar, Gunen, Tillie-Leblond
Clinical symptoms			
	Lower extremity complaints		Gunen
	Absence of symptoms of respiratory tract infection	Choi	
	Increased sputum	Choi	Akpinar
	Purrulent sputum		Choi
	Pleuritic chest pain	Akpinar, Gunen	Tillie-Leblond
	Cough		Akpinar, Tillie-Leblond
	Hemoptysis		Akpinar, Gunen, Tillie-Leblond
	Palpitations		Gunen, Tillie-Leblond
	Dyspnea		Tillie-Leblond, Akpinar
Physical examination			
	Fever		Choi, Akpinar
	Tachycardia		Akpinar, Tillie-Leblond
	Hypotension	Gunen	
	Lower leg asymmetry	Akpinar	Tillie-Leblond
	Signs congestive heart faillure		Gunen
	Syncope	Gunen	
Respiratory function			
	FEV1	Shapira-Rootman	Choi
	FEV1/VC		Choi
Arterial blood gas			
	PaO2		Choi, Gunen, Tillie-Leblond
	PaCO2 lower in PE	Akpinar	Choi, Gunen, Tillie-Leblond
	Pa(A-a)O2		Choi

e-Table 1. Clinical markers associated with PE in AE-COPD. (continued)

Clinical Markers		**Significant studies ($p < 0.05$)**	**Not significant studies ($p > 0.05$)**
	pH value higher in PE	Akpinar	
	Decrease in paCO2 of > 5mmHg from baseline	Tillie-Leblond	
Laboratory findings			
	NT-proBNP	Akpinar	Choi
	White blood cell count		Choi, Gunen
	Erythrocyte sedimentation rate		Choi
	C-reactive protein		Choi
	procalcitonin		Choi
	Creatine kinase - MB		Choi
	Troponines		Choi
	serum albumin		Choi, Gunen
	Glucose		Gunen
	Urea		Gunen
	Creatinine		Gunen
	Polycytemia	Kamel	
Diagnostics			
	ECG		
	Atrial fibrillation	Gunen	
	S1Q3T3 pattern		Gunen
	Ultrasonography		
	signs of acute right heart failure	Gunen	Tillie-Leblond
	CTPA findings		
	Atelectasis	Shapira-Rootman	
	Pulmonary artery enlargement	Shapira-Rootman	
	Emphysema		Shapira-Rootman
	Bronciectasis		Choi, Shapira-Rootman
	Ground glass opacities / Consolidations		Choi, Shapira-Rootman
	Noduli		Shapira-Rootman
	Interstitial changes		Shapira-Rootman
	Fibrosis		Choi, Shapira-Rootman
	Pleural effussion		Choi, Shapira-Rootman
	Pulmonary edema		Shapira-Rootman
	Peribronchial cuffing		Shapira-Rootman
	Granulomas		Shapira-Rootman

e-Table 1. Clinical markers associated with PE in AE-COPD. (continued)

Clinical Markers		Significant studies ($p < 0.05$)	Not significant studies ($p > 0.05$)
Wells criteria			
	Previous venous thromboembolism	Tillie-Leblond	Akpinar, Gunen
	Surgery <4 weeks		Akpinar, Tillie-Leblond
	Malignancy	Akpinar, Gunen	Tillie-Leblond
	Immobility	Gunen	
	Travel < 3 days		
	Hemoptysis		Akpinar, Gunen, Tillie-Leblond
Treatment			
	Long term oxygen treatment		Gunen
Outcome measures			
	Length of hospital admission	Akpinar , Gunen	Choi
	Mortality		Choi
	Need for ICU treatment		Akpinar
	In-hospital mortality	Gunen	
	One-year mortality	Gunen	
	3-month mortality		Akpinar
Predefined clinical markers that have been reviewed, but have not been reported by included studies			
	Treatment		
	Use of medication		
	All medication		
	Specific use of glucocorticoids		
	Exacerbation frequency		
	Exacerbation severity		

(Abbreviations: AE-COPD: acute exacerbations of COPD; CTPA: computed tomography pulmonary angiography; FEV1: forced expiratory volume in 1 second; FEV1/VC: forced expiratory volume in 1 second divided by vital capacity; PE: pulmonary embolism; NT-proBNP, N-terminal pro b-type natriuretic peptide)

CHAPTER 3

Increased platelet-monocyte interaction in stable COPD in absence of platelet hyper-reactivity

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ABSTRACT

Background

COPD is well known for its cardiovascular co-morbidities. Increased platelet-monocyte interaction may reflect altered platelet function and a potential role for anti-platelet therapy.

Objectives

The objectives were to investigate platelet-monocyte interaction, platelet activation and reactivity and plasmatic coagulation in stable COPD.

Methods

Platelet-monocyte interaction and platelet activation were determined by flow cytometry in 30 stable COPD patients and 25 controls. Platelet activation was measured by binding of fibrinogen to the activated fibrinogen receptor and platelet P-selectin expression at baseline and after platelet stimulation with platelet agonists. Plasmatic coagulation was measured by D-dimer and thrombin generation.

Results

Platelet-monocyte interaction was increased in stable COPD (median fluorescence intensity of platelet CD61 was 19.8 (IQR 14.0 – 33.2) versus 10.0 (IQR 8.7 – 16.7), p=0.002). In contrast, platelet activation and reactivity, reflected by fibrinogen binding and P-selectin expression, was equal between groups. Plasma P-selectin and Interleukin-6 were increased in COPD (p=0.01 and p=0.02, respectively), whereas soluble fibrinogen, D-dimer and thrombin generation were similar.

Conclusions

Increased platelet-monocyte interaction was found in absence of platelet hyperreactivity and activation of plasmatic coagulation in stable COPD. Future clinical evaluation of the effects of different anti-platelet drugs in COPD is warranted, as anti-platelet therapy may interfere with platelet-monocyte interaction.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is not only recognized as the third leading cause of death worldwide, but also known for development of cardiovascular co-morbidities.[1-5] Most cardiovascular diseases (CVD) in COPD result from arterial thrombosis.[2-4] However, the risk for venous thrombosis should not be underestimated.[6,7] A further increase in thrombotic complications is seen during or shortly after episodes of acute exacerbations of COPD (AE-COPD).[8,9] A recent meta-analysis indicates a higher risk of myocardial infarction during AE-COPD compared to stable disease,[9] and similar results have been reported for stroke.[10] Low-grade systemic inflammation in COPD is commonly thought to play an important role, both in COPD pathophysiology, as in the development of its cardiovascular complications.[4,11,12]

Extensive crosstalk between inflammation and coagulation exists, and platelets, monocytes and their interaction play a pivotal role in chronic inflammation and development of CVD.[13,14] When activated, platelets secrete a variety of biologically active substances including cytokines and chemokines. Platelet degranulation leads to increased expression of P-selectin on the platelet membrane, whereas a conformational change in the integrin αIIbβ3 results in increased fibrinogen binding.[15] P-selectin facilitates binding to P-selectin glycoprotein ligand-1 (PSGL-1) expressed on monocytes, resulting platelet-monocyte complexes (PMCs), an early process in atherothrombosis[13,16]. Fibrinogen binding results in platelet aggregation, clot formation and adhesion of platelets to the vascular endothelial surface.[13,14] Compelling evidence shows that platelet hyper-reactivity and increased PMC formation contribute to development of CVD.[13,17,18] Anti-platelet therapy is the cornerstone of treatment to prevent thrombotic cardiovascular morbidity.

Surprisingly, platelet function is scarcely studied in COPD. Some evidence indicates that platelet function may be altered.[19-22] Maclay and colleagues showed that stable COPD patients have increased circulating PMCs that increase further during AE-COPD.[21] Increased PMC formation is often considered to be a result of platelet hyper-reactivity, however, platelets and monocytes can interact in various ways.[15] To the best of our knowledge, no direct assessment of platelet function in stable COPD has been performed to date. These data add to the current debate on whether COPD patients may benefit from anti-platelet therapy in order to prevent CVD.[20,21,23]

We hypothesized that COPD patients have increased platelet-monocyte interaction due to increased platelet activation and hyper-reactivity upon platelet stimulation. Therefore, we determined: 1) platelet-monocyte interaction 2) platelet activation and reactivity, and 3) plasmatic coagulation in stable COPD patients and control subjects.

METHODS

Patients selection

Thirty stable COPD patients and 25 controls were included in the study. The inclusion criteria for COPD patients were irreversible expiratory airway obstruction according to the ATS/ERS criteria[24] (FEV1/FVC ratio < 70% and post-bronchodilator FEV1<80% (COPD Gold II-IV)) and clinical diagnosis of COPD confirmed by a pulmonologist. General exclusion criteria were use of platelet function inhibitors, asthma, malignancies or chronic inflammatory diseases, such as rheumatoid arthritis and inflammatory bowel disease. Patients were considered stable when they had not experienced an AE-COPD, as defined by the Anthonisen criteria,[25] in the preceding 6 months. Patients were recruited between October 2015 and February 2016 at the outpatient clinic of our respiratory department and controls were recruited among employees at Radboud University Medical Center. Spirometry was performed in the control group to exclude underlying lung disease. This study was approved by the Ethical Committee of Radboud University Nijmegen and was conducted according to the principles of the Declaration of Helsinki (version Oct 2008) and in accordance with the Dutch Medical Research Council working under the Human Subjects Act. All participants gave written informed consent. Blood was collected in EDTA and 3.2% sodium citrate vacutainer tubes (Becton Dickinson, Plymouth, UK) and processed immediately. Blood counts were measured with a haematology analyzer (Sysmex XN-450, Kobe, Japan).

Platelet reactivity and platelet-monocyte interaction

Platelet activation and reactivity were measured by a flow cytometry-based assay that has previously been described and that is able detect both platelet hyper-reactivity and thrombopathie in different clinical settings.[26-28] In short, whole blood samples were stained with antibodies for the platelet identification marker CD61 and for two platelet activation markers. The platelet activation markers used were the binding of fibrinogen to the activated fibrinogen receptor αIIbβ3 (GPIIb/IIIa complex) and the expression of alpha-granule protein P-selectin (CD62P) on the platelet surface, representing platelet aggregation and degranulation, respectively. Platelet reactivity was defined as the expression of these markers after stimulation of the samples with platelet agonists.[27] The samples were measured unstimulated (at baseline) and after *ex vivo* stimulation with the following agonists; adenosine 5' diphosphate (ADP) (Sigma-Aldrich, Saint Louis, Missouri, USA), cross linked collagen-related peptide (CRP-XL) (kind gift from Prof. Dr. R. Farndale, Cambridge, UK) and thrombin receptor-activating peptide-6 (TRAP) (Sigma-Aldrich, Saint Louis, Missouri, USA). The staining of the samples was performed with antibodies for CD61 (PC7-conjugated) (Beckman Coulter, Brea, California, USA), anti-human fibrinogen (fluorescein isothiocynate (FITC)-conjugated) (Dako, Santa Clara,

California, USA) and P-selectin (CD62P, phycoerythrin (PE)-conjugated) (Biolegend, Biolegend, San Diego, California, USA) and the samples were co-incubated with the platelet agonists or medium control for 20 minutes. After stimulation, the samples were fixated with 0.2% paraformaldehyde and measured by flow cytometry (FC500 flow cytometer, Beckman Coulter, Brea, California, USA). Gating of platelets was performed based on forward and sideward scatter and additionally for of CD61 positivity. The mean fluorescence intensity (MFI) of the human fibrinogen and human P-selectin antibodies on the platelet surface was used as quantification of the platelet activation marker signal. The MFI is a measure for the mean intensity of the fluorescence signal of the labeled antibody that is detected in the corresponding flow cytometry fluorescence channel.

For measurement of platelet-monocyte interaction, samples were incubated with monoclonal antibodies to CD14 (PE-conjugated) (Becton Dickinson, Franklin Lakes, New Jersey, USA) and CD61 (PC7-conjugated) (Beckman Coulter, Brea, California, USA) for 20 minutes. Erythrocytes were lysed with optilyse B (Beckman Coulter, Brea, California, USA) and the reaction was stopped by dilution with distilled water. Gating of monocyes was performed based on forward and sideward scatter and additionally for of CD14 positivity. The degree of platelet-monocyte interaction was quantified by the MFI of platelet marker CD61 on monocytes (CD14 positive cells).

Plasmatic coagulation

Tissue factor (TF) triggered thrombin generation was measured in platelet-poor plasma using the automated thrombogram method as described previously.[29] In this assay, the thrombin generation curve was evaluated based on parameters that describe the initiation, propagation and termination phases of thrombin generation expressed as lag time, endogenous thrombin potential (ETP) and thrombin peak, respectively. Plasma was obtained after centrifugation of blood tubes at 3800 RPM for 10 minutes. Samples were measured unstimulated and after stimulation with tissue factor (1pM).

Soluble markers of platelet activation, coagulation and inflammation

Soluble fibrinogen and soluble P-selectin levels were determined in plasma using the human Fibrinogen ELISA kit and human P-selectin/CD62P duoset ELISA kit (R&D systems, Minneapolis, Minnesota, USA), respectively. D-dimer and high-sensitive Interleukin-6 (hsIL-6) and C-reactive protein were determined with the human D-dimer ELISA kit (Abcam, Europe, Cambridge, UK),human high sensitive IL-6 Quantikine ELISA kit (R&D systems, Minneapolis, Minnesota, USA) and human C-Reactive Protein ELISA kit (R&D systems, Minneapolis, Minnesota, USA), respectively. For platelet content whole blood was centrifugated for 15 minutes at 156 g without break to obtain platelet-rich-plasma (PRP). Platelet concentration was adjusted to $300x10^9$/L by addition of autologous

plasma. Samples were freeze-thawed for 3 cycles to fragment the platelets, followed by ultra-centrifugation for 5 minutes at 5000 RPM to spin down large particles and supernatants were taken for ELISA. Platelet fibrinogen and P-selectin were determined with the ELISA kits described above.

Data analyses

Statistical analysis was performed with Graphpad Prism 5.0 (San Diego, California, USA) and IBM SPSS statistics 22.0 (New York, New York, USA). Non-normally distributed data was analyzed with Mann-Whitney U tests and results are presented as median with the interquartile range (IQR). Normally distributed data was analyzed by Student's T tests (two-sided) and results are presented as mean with the standard error of the mean (SEM). Two-way ANOVA analyses were performed for repeated measures. Haematological data were analyzed using Student's T test for continuous variables and presented as mean with the standard deviation (SD). For expected counts less than 5 a Fisher's Exact test was performed. Associations between inflammation and platelet markers were determined using Spearman's rank correlation coefficient. The differences were considered significant if p value < 0.05.

RESULTS

The study population demographics can be found in table 1. Control subjects were slightly younger and had less tobacco smoke exposure, as reflected by pack years. Haematological parameters were not different, including platelet counts and platelet characteristics (table 2). Notably, mean platelet volume (MPV), a commonly used measure for platelet activation, was not different between groups.

Interaction between platelet and monocytes was increased in stable COPD patients compared to controls, median MFI for platelet marker CD61 on CD14 positive cells was 19.8 (IQR 14.0 – 33.2) in COPD patients versus 10.0 (IQR 8.7 – 16.7) in controls, p=0.002 (Figure 1A). In addition, in the population of CD14/CD61 double positive cells, the intensity of the CD61 fluorescence signal was significantly higher in COPD patients, median MFI 24.6 (IQR 19.6 – 37.7) versus 19.3 (IQR 16.6 - 24.2), p=0.006 (Figure 1B), suggesting that more platelets were bound to the monocyte surface. After exclusion of outliers both parameters were still statistically significant (p=0.005 and p=0.01, respectively).

Next, we investigated platelet activation by measurement of fibrinogen binding to the activated fibrinogen receptor and expression of P-selectin both in unstimulated samples and after *ex vivo* stimulation by ADP, CRP-XL and TRAP. In unstimulated samples,

Table 1. Demographics.

	COPD patients	Control subjects
Number of participants	30	25
Age, years	61.6 ± 10.0	53.0 ± 11.7
Male	14 (46.7%)	9 (36.0%)
Current smokers	6 (20.0%)	2 (8.0%)
Smoking history, pack years	50.7 ± 27.8	1.6 ± 4.3
GOLD classification		N/A
I	1 (3.3%)	
II	11 (36.7%)	
III	15 (50.0%)	
IV	3 (10.0%)	
Respiratory function		
FEV1, L	1.36 ± 0.6	3.12 ± 0.89
FEV1% predicted	48.9 ± 17.5	98.0 ± 13.9
FVC, L	3.31 ± 0.73	3.94 ± 1.08
FEV1/FVC ratio	42.9 ± 12.9	80.4 ± 5.5

Values are mean ± SD or n (%). (*Abbreviations: COPD: Chronic Obstructive Pulmonary Disease; FEV1: forced expiratory volume in 1 second; FVC: forced vital capacity; GOLD: Global Initiative for Chronic Obstructive Lung Disease; N/A: not applicable; SD: Standard deviation)*

Table 2. Haematological Parameters.

	COPD patients	Control subjects	p-value
Leukocytes (x 10^9/L)	7.0 ± 2.3	6.5 ± 1.2	0.399
Platelets (x 10^9/L)	273 ± 103	261 ± 66	0.876
Mean platelet volume (fL)	10.2 ± 0.8	10.2 ± 0.7	0.958
Platelet distribution width (fL)	11.7 ± 1.8	12.0 ± 1.6	0.526
Platelet-large cell ratio (%)	26.6 ± 6.7	26.8 ± 5.9	0.931

Values are mean ± SD. (*Abbreviations: COPD: Chronic Obstructive Pulmonary Disease; SD: Standard deviation)*

no differences in these two parameters were observed between stable COPD patients and controls (the median MFI for fibrinogen was 5.11 (IQR 3.8 – 6.2) versus 5.68 (IQR 5.2 – 6.3), p=0.20 and the mean MFI for P-selectin was 7.018 ± 0.26 versus 7.194 ± 0.24, p=0.71)(Figure 2).

In stimulated samples, platelet reactivity was similar for ADP, CRP-XL and TRAP in COPD patients and controls, for both markers (Figure 3). Soluble P-selectin and soluble fibrinogen, markers for platelet degranulation, were measured in plasma. Plasma P-selectin concentrations were found to be increased in COPD patients (median 31.07 ng/ml (IQR 26.3 – 40.5) versus 25.25 ng/ml (IQR 24.3 – 32.3), p=0.03) in controls, whereas

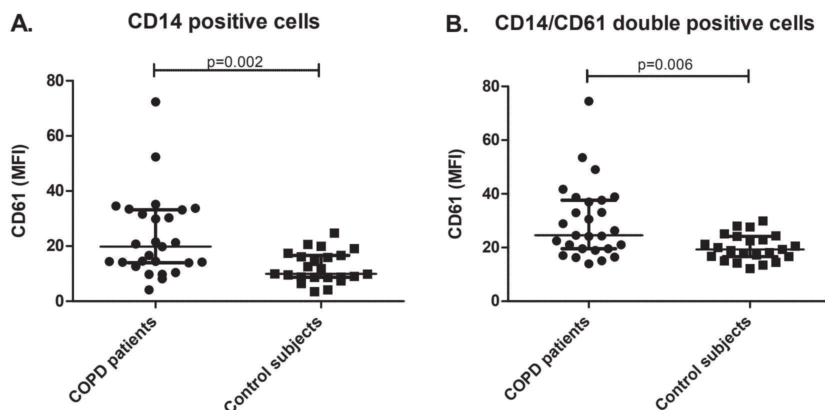

Figure 1. Platelet-monocyte interaction.
Patients with stable COPD show **A.** increased platelet-monocytes complexes compared to controls, median MFI for platelet marker CD61 on CD14 positive cells 19.8 (IQR 14.0 – 33.2) in stable COPD patients versus 10.0 (IQR 8.7 – 16.7) in controls, p=0.002. **B.** In addition, in CD14/CD61 double positive cells, CD61 expression was higher in COPD patients compared to controls, median MFI 24.6 (IQR 19.6 – 37.7) versus 19.3 (IQR 16.6 - 24.2), p=0.006. Lines represent medians with IQR. (*Abbreviations: COPD: Chronic Obstructive Pulmonary Disease; IQR: Interquartile Range; MFI: Mean Fluorescence Intensity)*

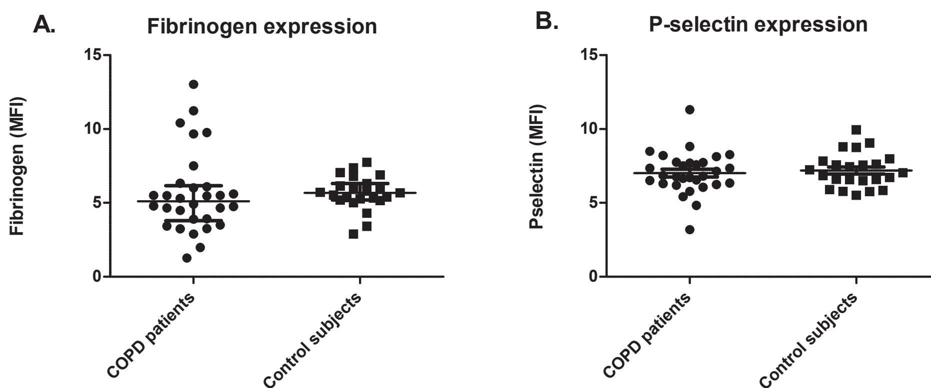

Figure 2. Platelet activation in COPD patients and control subjects.
Flow cytometric analyses of platelet fibrinogen binding and platelet P-selectin expression. **A.** The median MFI of fibrinogen binding in COPD was 5.11 (IQR 3.8 – 6.2) versus 5.68 (IQR 5.2 – 6.3) in controls, p=0.20 and **B.** The mean* MFI of P-selectin expression in COPD was 7.018 ± 0.26 versus 7.194 ± 0.24 in controls, p=0.71. Lines represent median with IQR (A) and mean with SEM (B). (Abbreviations: *COPD: Chronic Obstructive Pulmonary Disease; IQR: Interquartile Range; MFI: Mean Fluorescence Intensity; SEM: Standard error of the mean*) *normally distributed data is presented as mean ± SEM

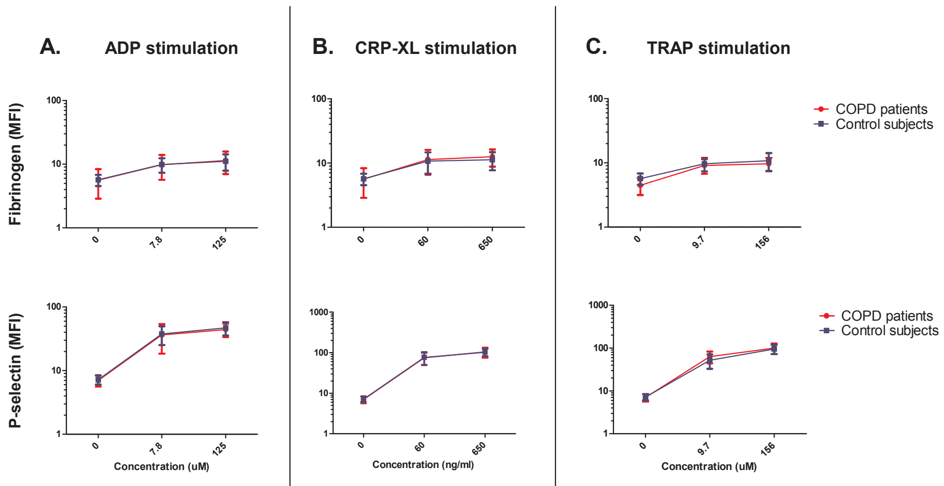

Figure 3. Platelet reactivity in COPD patients and controls.
Platelet reactivity was measured after stimulation of with two concentrations of different platelet agonists. Data are presented as mean ± SD. **A.** Reactivity after stimulation with ADP was not different, fibrinogen binding p=0.91, P-selectin expression p=0.56, **B.** The same applies to stimulation with CRP-XL, fibrinogen binding p=0.39, P-selectin expression p=0.95, and **C.** stimulation with TRAP, fibrinogen binding p=0.39, P-selectin expression p=0.26. (*Abbreviations: ADP: adenosine 5' diphosphate; COPD: Chronic Obstructive Pulmonary Disease; CRP-XL: Cross linked Collagen-Related Peptide; SD: Standard deviation; TRAP: thrombin receptor-activating peptide; MFI: Mean Fluorescence Intensity*)

plasma fibrinogen levels were similar (mean 3314 µg/ml ± 259 versus 3051 µg/ml ± 245, p=0.53) (Figure 4).

Since fibrinogen is rapidly stored intracellular after binding to the receptor, we measured platelet fibrinogen content to investigate fibrinogen internalization. However, no differences in both platelet fibrinogen and platelet P-selectin content could be detected (data not shown).

In summary, increased platelet-monocyte interaction was observed, in absence of increased platelet activation and platelet hyper-reactivity. Since considerable variation in platelet fibrinogen binding (Figure 2A) and soluble P-selectin (Figure 4) was observed between patients, a correlation with systemic inflammation was explored. Plasma levels of high sensitive interleukin-6 (hsIL-6) were determined and were found to be higher overall in stable COPD patients compared to controls (median 313.5 pg/ml (IQR 265 – 465) versus 258.3 pg/ml (IQR 310 – 316), p=0.02)(Figure 5). No correlations could be detected between hsIL-6 and platelet activation and platelet reactivity, neither for platelet-monocyte interaction, in stable COPD patients (data not shown).

Additionally, we determined CRP and similarly, no significant correlations could be detected with the platelet parameters. A trend towards a positive correlation was seen for CRP and platelet-monocyte interaction, although not statistically significant (Spearman's rho 0.264, p=0.095) when examining the entire cohort (supplementary figure).

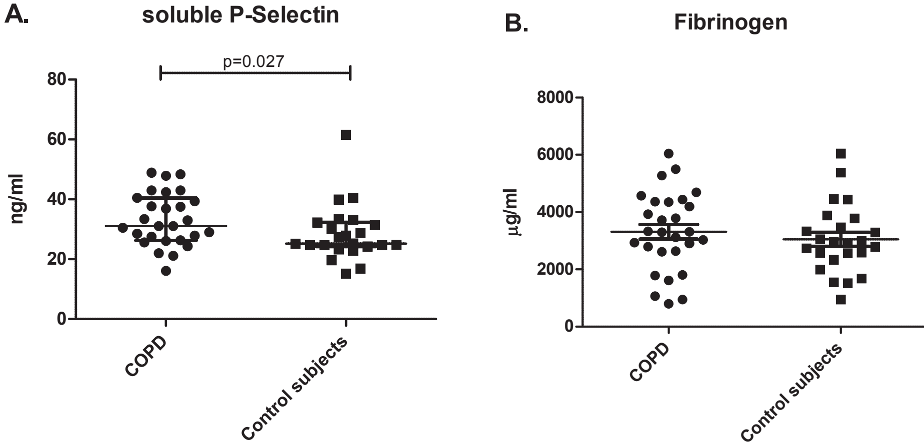

Figure 4. Soluble markers for platelet activation in COPD patients and controls.
Soluble markers for platelet activation were measured in platelet poor plasma. **A.** Soluble P-selectin was higher in COPD patients, median 31.07 ng/ml (IQR 26.3 – 40.5) versus 25.25 ng/ml (IQR 24.3 – 32.3) in controls, p=0.03. **B.** Fibrinogen was not statistically different, mean* 3314 µg/ml ± 259 versus 3051 µg/ml ± 245, p=0.53. Lines represent median with IQR (A) and mean with SEM (B). (Abbreviations: *COPD: Chronic Obstructive Pulmonary Disease; IQR: Interquartile Range; SEM: Standard error of the mean*). *normally distributed data is presented as mean ± SEM

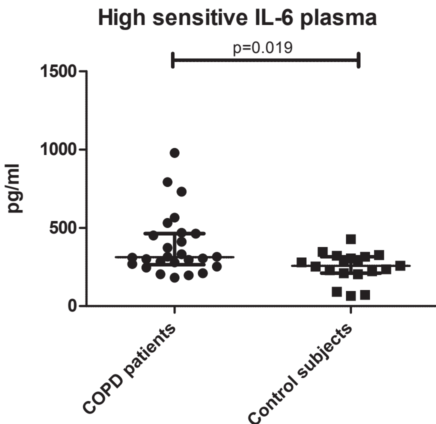

Figure 5. Systemic inflammation in stable COPD patients and controls.
HsIL-6 was higher in COPD patients, however only a subgroup shows increased systemic inflammation. Median 313.5 pg/ml (IQR 265 – 465) in stable COPD, versus 258.3 pg/ml (IQR 310 – 316) in controls, p=0.02. Lines represent medians with IQR. (*Abbreviations: COPD: Chronic Obstructive Pulmonary Disease, hsIL-6: high sensitive Interleukin-6, IQR: Interquartile range)*

To further investigate the underlying mechanisms we determined activation of plasmatic coagulation. D-dimer, a measure for fibrinolysis, was not different between stable COPD patients and controls (median 636.8 ng/ml (IQR 293 – 535) versus 294.3 ng/ml (232 – 455), p=0.16). An automated thrombin generation assay was used to investigate hypercoagulable phenotypes. Thrombin generation was measured in the presence and absence of TF. Clotting time (Lag time), total amount of thrombin generated (ETP) and maximum thrombin concentration (thrombin peak) was not different between stable COPD patients and controls (Table 3).

Table 3. Thrombin generating capacity of stable COPD patients and control subjects.

		Lag time (min)	**ETP** (nM.min)	**Peak** (nM)
	Stable COPD	5.24 ± 1.72	1110 ± 334	216.9 ± 99.8
0 pM TF	Control subjects	5.59 ± 3.37	1068 ± 423	1.92.2 ±100.2
		p = 0.79	*p=0.96*	*p=0.55*
	Stable COPD	13.39 ± 5.64	1327 ± 392	298.3 ± 75.8
1 pM TF	Control subjects	14.90 ± 6.77	1276 ± 402	285.3 ± 94.6
		p=0.33	*p=0.85*	*p=0.68*

No statistical significant differences were seen in terms of clotting time (Lag time), total amount of thrombin generated (ETP), and maximum thrombin concentration (Peak). Values are mean ± SD. *(Abbreviations: COPD: Chronic Obstructive Pulmonary Disease; ETP: Endogenous thrombin potential; SD: Standard deviation; TF: Tissue Factor)*

DISCUSSION

The present study shows that stable COPD patients have increased platelet-monocyte interaction in absence of increased platelet activation, platelet hyper-reactivity and activation of plasmatic coagulation. Increased expression of platelet marker CD61 was observed on monocytes in stable COPD patients compared to controls. The lack of platelet hyper-reactivity was reflected by platelet fibrinogen binding and platelet P-selectin expression in unstimulated samples and after stimulation with 3 different platelet agonists. Though a minority of COPD patients had a certain degree of systemic inflammation, this could not be related to platelet-monocyte interaction and platelet activation. Also, no differences in D-dimer and thrombin generation were detected between stable COPD patients and control subjects.

COPD patients are at increased risk for CVD and studies that improve insight in the underlying pathophysiological mechanisms are warranted.[3,30-32] We hypothesized that platelet-monocyte interaction would be increased in COPD, in concurrence with increased platelet activation and platelet hyper-reactivity. In contrast to our hypothesis, no alterations in platelet function were found. We confirm previous findings by Maclay

and colleagues showing that COPD patients have increased platelet-monocyte interaction,[21] however in absence of platelet hyper-reactivity. PMC formation is often used as a surrogate marker for platelet activation, as platelet and monocytes can interact via platelet P-selectin and PSGL-1 on monocytes and through platelet GPIIb/IIIa and monocyte-activating complex-1 (MAC-1) via binding of fibrinogen, interactions that are facilitated by platelet activation.[33] These interactions seem less relevant in COPD, as platelet activation and response to stimulation was not different.

Platelets and monocytes can interact in multiple ways[13,15,16] and monocyte-related factors, such as monocyte activation, may play an important role. Platelets constitutively express GPIbα, a receptor that directly interacts with MAC-1 on monocytes.[34] MAC-1 expression increases with monocyte activation, thereby promoting this interaction.[34,35] Our data suggests that not primarily platelet activation, but monocyte activation may be a key factor in their interaction. This is supported by the trend that was observed between platelet-monocyte interaction and plasma CRP levels. CRP is thought to play an important role in atherosclerosis[36] and studies have shown that CRP induces monocyte-endothelial cell adhesion, an early event in atherosclerosis.[37] Moreover, increased plasma CRP levels are linked in plaque instability, that may be explained by monocyte activation.[36,38,39]

These findings add to the current debate on whether COPD patients would benefit from anti-platelet therapy. At first sight, the benefits of anti-platelet therapy in COPD patients seem limited. Increased platelets-monocyte interaction, however, is thought to play a key role in cardiovascular disease pathogenesis as it augments development of atherosclerotic lesions and is involved in plaque instability.[13,40,41] P2Y12 receptor blockers, such as Clopidogrel, Prasugrel and Ticagrelor, are known to decrease platelet-monocyte interaction and systemic inflammation, partially via reduced monocyte activation,[42-45] whereas these effects are less clear for the COX-inhibitor Aspirin.[44,45] Our data suggests that P2Y12 receptors may have a beneficial effect in COPD and clinical confirmation is warranted.

Interestingly, a small increase in soluble plasma P-selectin levels was observed in COPD patients. P-selectin can be released by endothelial cells during endothelial inflammation, a mechanism that has been previously described for COPD.[46,47] This may explain our observation. In addition to platelet function, we assessed markers for plasmatic coagulation. Risk for venous thrombosis, especially pulmonary embolism, is increased in COPD.[2,3,6,7] Pulmonary embolism often results from thrombus dislocation from deep venous thrombosis, a mechanism dependent on blood stasis and plasmatic coagula-

tion.[48] We found that COPD patients did not show a hypercoagulable phenotype in stable disease, as reflected by D-dimer and thrombin generation.

An important, yet unresolved question to address is whether platelet function is affected during and after acute exacerbations. Harrison and colleagues showed that thrombocytosis during AE-COPD is associated with increased 1-year mortality (OR 1.53 (95% CI 1.03-2.29, p=0.030) and patients on anti-platelet therapy showed improved survival, although no explanation was given.[20] Data on platelet function and platelet-monocyte interaction during and after AE-COPD is crucial to assess future implications for specific anti-platelet therapy in COPD.

This study has several limitations; first limitation is the heterogeneity in COPD patients which makes our findings less robust and adds to the difficulty to extrapolate our findings. Tremendous research efforts focus on identification of different COPD 'phenotypes', however today we are not able to differentiate clinically relevant subgroups from an immunological perspective. A second limitation is the difference between COPD patients and controls in terms of tobacco smoke exposure and age. Tobacco smoke exposure and ageing may increase platelet hyper-reactivity. However, these factors would have resulted in platelet hyper-reactivity in the COPD group which was not shown by our data. Lastly, data on risk factors for CVD were not collected and therefore no stratification for cardiovascular risk factors could be performed. It was recently shown that comorbidities in COPD tend to cluster in different phenotypes[49] and therefore it would have been of great interest to investigate the effects of these factors on our findings from a mechanistical point-of-view.

In summary, we demonstrated that stable COPD patients have increased platelet-monocyte interaction in absence of increased platelet activation, platelet hyper-reactivity and activation of plasmatic coagulation. The mechanisms underlying cardiovascular risk in COPD may be more dependent on platelet-monocyte interaction than platelet function alterations. The benefits of common anti-platelet therapy may differ for the COX-inhibitor Aspirin, compared to P2Y12 receptor blockers, as the latter interferes with platelet-monocyte interaction. Further confirmation of the effects of anti-platelet therapy in COPD is warranted.

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Online supplement to

Increased platelet-monocyte interaction in stable COPD in absence of platelet hyper-reactivity

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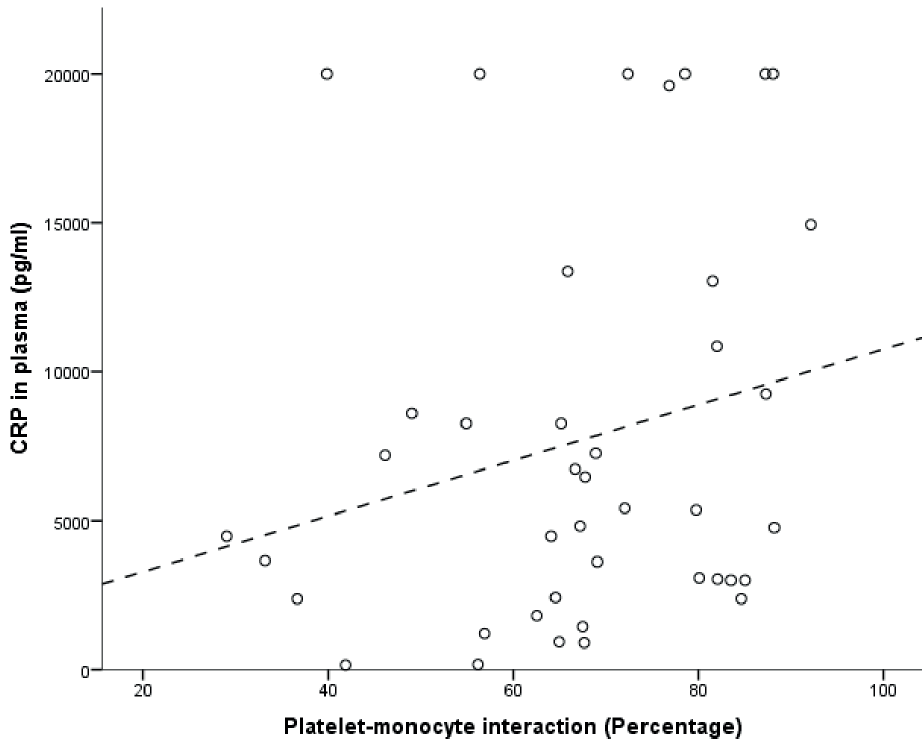

Supplementary figure. The relation between plasma CRP and platelet-monocyte interaction in the study cohort.
In the entire study cohort, a trend for a modest positive correlation was found between plasma CRP and platelet-monocyte interaction (Spearman's rho= 0.264, p=0.095). (*Abbreviations: CRP: C-reactive protein)*.

CHAPTER 4

Platelet-monocyte complexes and platelet function in Acute Exacerbations of COPD

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Submitted.

ABSTRACT

Cardiovascular risk is increased during acute exacerbations of COPD (AE-COPD). Increased platelet-monocyte complex (PMC) formation is suggested to contribute this this increased risk and may be associated with platelet function alterations. This study investigates platelet function in exacerbated COPD patients. PMCs and platelet reactivity were determined by flow cytometry in 22 exacerbated COPD patients during and post-exacerbation. PMCs decrease in AE-COPD, median 10.6% (IQR 8.5-13.6%) versus 11.9% (IQR 10.2-15.3%), p= 0.04, without platelet function alterations. Monocyte MAC-1 expression was increased during AE-COPD (p=0.005) and inversely correlated with PMCs. This study suggests that platelet function alterations may not explain cardiovascular risk during AE-COPD.

INTRODUCTION

COPD patients are at increased risk for cardiovascular diseases (CVD) and around 50% of hospital admissions in COPD patients can be attributed to CVD and 25% of deaths.[1] Myocardial infarction and stroke are the most commonly observed CVD in COPD.[2] Acute exacerbations of COPD (AE-COPD) are characterized by increased inflammation and further increase the cardiovascular risk.[2,3] Inflammation and hemostasis are closely linked biological systems and interact in many ways.[4] Blood platelets function at the cutting edge and play a pivotal role in development of cardiovascular disease.

A previous publication by Maclay and colleagues in Thorax suggested increased platelet activation in AE-COPD.[5] This implicates that COPD patients may benefit from anti-platelet therapy as preventive measure for CVD. We have shown that no clear platelet function alterations were present in stable COPD patients, but confirm increased platelet-monocyte complexes (PMCs). PMCs augment the development of atherosclerosis and plaque instability, thereby contributing to CVD.[6]

The presence of platelet function alterations during AE-COPD has not yet been studied. Moreover, the mechanisms by which platelet and monocytes interact in COPD are not known. Recent evidence suggested that macrophage receptor 1 (MAC-1) on monocytes may play a role in their interaction.[7] This study investigated platelet-monocyte interaction, platelet reactivity and MAC-1 expression on monocytes in AE-COPD and post-exacerbation.

METHODS

See the Online supplement for a detailed description of the methods.

In summary, COPD patients admitted for an exacerbation (as defined by the Anthonisen criteria) were included. The study was approved by the Ethical Committee of Radboud University Nijmegen, the Netherlands. Blood samples were taken during hospital admission and after 6 – 10 weeks post-exacerbation.

PMCs were determined by flow cytometry after incubation of samples with monoclonal antibodies to monocyte marker CD14 and platelet marker CD61. Erythrocytes were lysed and the reaction was stopped by dilution with distilled water.

The binding of fibrinogen to the activated fibrinogen receptor $\alpha IIb\beta 3$ and the expression of P-selectin (CD62P) on the platelet surface were used to determine platelet reactivity.

Platelet reactivity was defined as the expression of these markers after stimulation of whole blood with platelet agonists, as previously reported.[8] Samples were incubated with antibodies for CD61, anti-human fibrinogen and P-selectin and the platelet agonists. After stimulation, cells were fixated with 0.2% paraformaldehyde.

To measure MAC-1, whole blood samples were stained with monoclonal antibodies to CD14, CD45 and CD11b, after which the erythrocytes were lysed. After lysis, samples were measured within 10 minutes on the flow cytometer. The gating strategies and information on the statistical analysis can be found in the online supplement.

RESULTS

Twenty-two patients were included in this study Two patients died during hospital admission and one patient retracted consent. PMCs were decreased during AE-COPD compared to post-exacerbation, median PMCs 10.6% IQR 8.5-13.6% during AE-COPD versus 11.9% IQR 10.2-15.3% post-exacerbation, p = 0.04, (Figure 1A). In contrast, expression of MAC-1 by monocytes was higher during AE-COPD (MFI 26.01 IQR 20.1-34.8 versus 19.54 IQR 18.3-24.6 post-exacerbation versus, p=0.005)(Figure 1B). There was an inverse correlation between monocyte MAC-1 expression and platelet-monocyte interaction post-exacerbation, Pearon's r = -0.534, p=0.03, however this association was not found during AE-COPD, Pearson's r = -0.289, p=0.217 (Figure 1C).

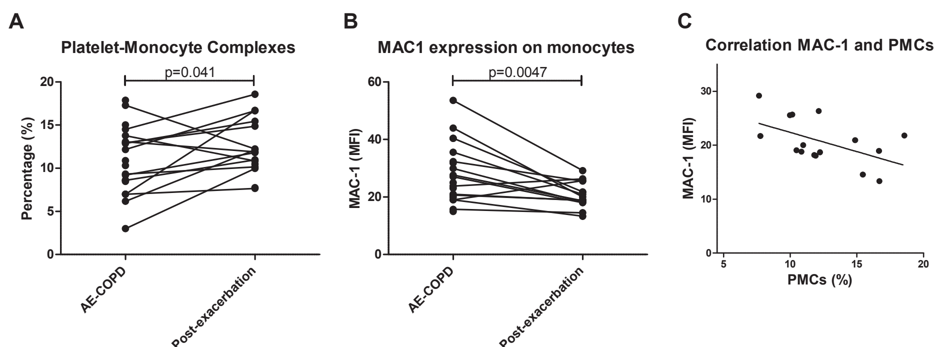

Figure 1. Platelet-monocyte complexes, MAC-1 and their relation.
A. Patients with AE-COPD had less PMCs compared to post-exacerbation, the median percentage PMCs of the total monocyte population was 10.6% IQR 8.5-13.6% during AE-COPD compared to 11.9% IQR 10.2-15.3% post-exacerbation, p=0.04. B. MAC-1 expression on monocytes was higher during AE-COPD compared to post-exacerbation, median MFI 26.01 IQR 20.1-34.8 during AE-COPD versus median MFI 19.54 IQR 18.3-24.6 post-exacerbation, p=0.005. C. The correlation between monocyte MAC-1 expression and platelet-monocyte interaction post-exacerbation (Pearon's r = -0.534, p=0.03). (*Abbreviations: AE-COPD: acute exacerbations of COPD; IQR: Inter-quartile range; MAC-1: macrophage-1 antigen; MFI: mean fluorescence index; PMC: Platelet-monocye complex)*

The binding of fibrinogen to platelets in unstimulated samples showed higher fibrinogen binding during AE-COPD, whereas P-selectin expression on platelets was not different. Upon stimulation of platelets with 3 different platelet agonists, ADP, CRP-XL and TRAP, in 2 concentration we did not observe any differences in platelet fibrinogen binding, nor did we observe changes in platelet P-selectin expression (Figure 2).

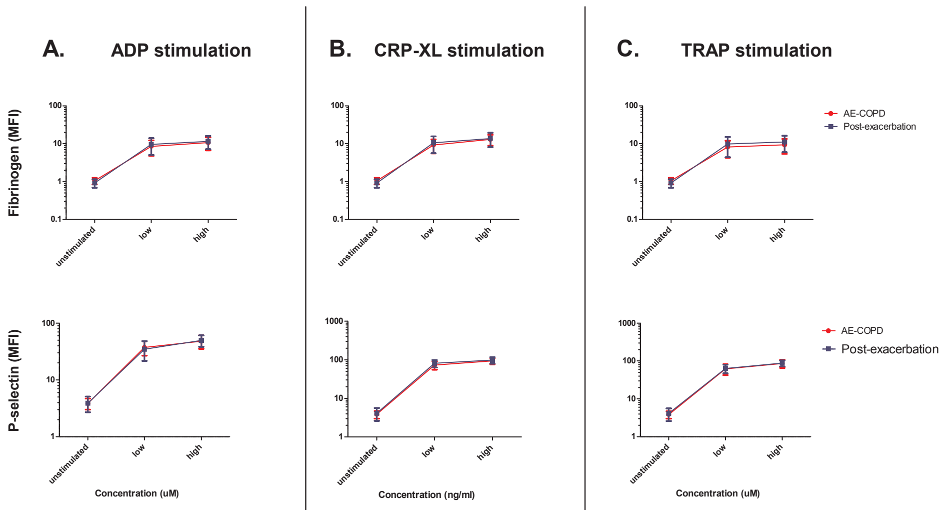

Figure 2. Platelet reactivity in response to stimulation with platelet agonists.
No differences in fibrinogen and P-selectin expression were found after stimulation with platelet agonists ADP (A), CRP-XL (B) and TRAP (C). (*Abbreviations: ADP: Adenosine di-phosphate; AE-COPD: Acute Exacerbations of COPD; CRP-XL: cross-linked collagen-related peptide; MFI: mean fluorescence index; TRAP: Thrombin receptor activating peptide)*

DISCUSSION

PMCs were lower in exacerbated COPD patients compared to post-exacerbation, whereas MAC-1 expression on monocytes was increased. Additionally, PMCs and MAC-1 were inversely correlated post-exacerbation. Platelet reactivity was similar during AE-COPD and post-exacerbation.

Clinical studies suggest that anti-platelet therapy may have a beneficial effect in COPD patients.[5,9] We, and others, reported increased PMCs in patients with COPD.[5] Platelet-monocyte interaction is often used as a surrogate marker for platelet activation, however, no clear platelet function alterations were detected in stable COPD patients.

It has been suggested that PMCs further increase during AE-COPD.[5] This has led to our hypothesis that platelet-monocyte interaction may be driven by monocyte activation, rather than platelet activation. Platelets and monocytes can interact in many ways and recent evidence suggests that MAC-1 is involved in PMC formation via binding to platelet GPIb, a mechanism dependent on monocyte activation.[7] This may be relevant as different platelet inhibitors can interfere with PMC formation.[10]

In contrast to the previous study,[5] we observed less PMCs during AE-COPD. An important difference is that we used a before-after model in which platelet-monocyte interaction was assessed during and after AE-COPD. Second, this cohort was slightly larger. Together this may explain the contradictory nature of the observations.

MAC-1 expression was higher during AE-COPD, however, had an inverse relationship with PMCs post-exacerbation. These data indicate that MAC-1 may not be crucially involved in platelet-monocyte interaction in COPD.

Similar to our previous study, no alterations in platelet function were found in exacerbated patients. This does not necessarily imply that anti-platelet therapy may not be beneficial in COPD. Some platelet-inhibitors, such as P2Y12 inhibitors, interfere with PMCs and may decrease systemic inflammation. Platelets also exhibit immunological functions, and these may influence COPD disease pathophysiology. Interestingly, COPD patients treated with platelet-inhibitors showed increased survival post-exacerbation, while this was not related to cardiovascular deaths.[9]

Our study has some limitations. The first limitation of our study is the small sample size. COPD is a heterogeneous disease and this makes our findings less robust. We assessed the effects of smoking, FEV1 and frequent versus infrequent exacerbator phenotype, and these had no influence. Second, we cannot state that the post-exacerbation measurement after 6 to 10 weeks resembles stable disease, as it may be period of convalescence.

In conclusion, this study reports increased platelet-monocyte interaction post-exacerbation of COPD, in absence of platelet hyper-reactivity. The increased cardiovascular risk during AE-COPD may therefore not be explained by platelet function alterations.

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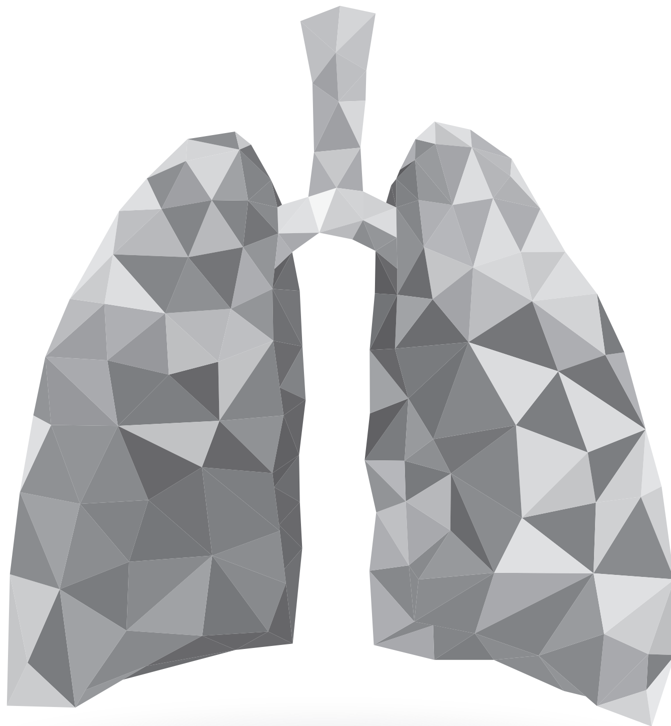
Online supplement to

Platelet-monocyte complexes and platelet function in Acute Exacerbations of COPD

Aleva FE, de Mast Q, de Groot PG, Heijdra YF, van der Ven AJAM



Submitted.

Study design.

Patients admitted for an AE-COPD were included in the study. The inclusion criteria were; patients above 40 years of age, spirometry confirmed diagnosis of COPD (i.e. post-bronchodilator FEV1/FVC < lower limit of normal) and clinical diagnosis of COPD confirmed by a pulmonologist and > 10 pack years of smoking. General exclusion criteria were use of anti-coagulation or platelet function inhibitors, asthma, malignancies or chronic inflammatory diseases, such as rheumatoid arthritis, psoriasis and inflammatory bowel disease. An exacerbation is defined as sustained worsening of respiratory symptoms during 48 hours. Respiratory symptoms include at least one of the Anthonisen criteria, which are increased dyspnoea, sputum volume or sputum purulence, with or without minor symptoms such as cough, fever, common cold, wheezing or sore throat.[1]

Patients were recruited between January and April 2017 after admission at the department of Respiratory Medicine at Radboud university medical center, Nijmegen, The Netherlands. This study was approved by the Ethical Committee of Radboud University Nijmegen and was conducted according to the principles of the Declaration of Helsinki (version Oct 2008) and in accordance with the Dutch Medical Research involving Human Subjects Act. All participants gave written informed consent before blood was drawn. Blood samples were taken at two time points; first time point was within 96 hours after admission to the hospital and the second time point was after 6 to 10 weeks at a follow up visit at the outpatient clinic. Blood was collected in EDTA and 3.2% sodium citrate vacutainer tubes (Becton Dickinson, Plymouth, UK) and processed immediately. Blood counts were measured with a haematology analyzer (Sysmex XN-450, Kobe, Japan).

Platelet-monocyte interaction and platelet reactivity.

For measurement of platelet-monocyte interaction, samples were incubated with monoclonal antibodies to monocyte marker CD14 (phycoerythrin (PE)-conjugated) (Becton Dickinson) and platelet marker CD61 (PC7-conjugated) for 20 minutes. Optilyse B (Beckman Coulter) was used to lyse the erythrocytes and after 10 minutes the reaction was stopped by dilution with distilled water. Gating of monocytes was performed based on forward and sideward scatter and additionally for of CD14 positivity, followed by positivity for CD61.

The binding of fibrinogen to the activated fibrinogen receptor αIIbβ3 (GPIIb/IIIa complex) and the expression of P-selectin (CD62P) on the platelet surface were used to determine platelet activation, as these markers reflect platelet aggregation and degranulation, respectively. Platelet reactivity was defined as the expression of these markers after stimulation of whole blood with platelet agonists, as previously reported.[2,3] Whole blood samples were measured unstimulated and after stimulation with the agonists

adenosine 5' diphosphate (ADP) (Sigma-Aldrich), cross linked collagen-related peptide (CRP-XL) (kind gift from Prof. Dr. R. Farndale, Cambridge, UK) and thrombin receptor-activating peptide-6 (TRAP) (Sigma-Aldrich). Samples were incubated with antibodies for CD61 (PC7-conjugated) (Beckman Coulter), anti-human fibrinogen (fluorescein isothiocynate (FITC)-conjugated) (Dako) and P-selectin (CD62P, phycoerythrin (PE)-conjugated) (Biolegend) and two concentrations of the platelet agonists for 20 minutes. After stimulation cells were fixated with 0.2% paraformaldehyde. Expression of activation markers was measured by flow cytometry (FC500 flow cytometer, Beckman Coulter). Gating of platelets was performed based on forward and sideward scatter and additionally for of CD61 positivity.

MAC-1 expression on monocytes.

MAC-1 is a monocyte receptor that is upregulated during monocyte activation and is able to bind to platelets via platelet receptor GPIb and to platelet receptor GPIIb/IIIa via concurrent binding of fibrinogen.[4,5] To measure monocyte activation, whole blood samples were stained with monoclonal antibodies to CD14 (PE, Beckman coulter), CD45 (PC7) and CD11b (FITC) for 15 minutes, after which the erythrocytes were lysed with BD Pharm Lyse (BD Biosciences) for 10 minutes. After lysis, samples were measured within 10 minutes on the flow cytometer.

Soluble markers of platelet activation and inflammation.

High-sensitive Interleukin-6 (hsIL-6) was determined with human high sensitive IL-6 Quantikine kit (R&D systems, Minneapolis, USA). For platelet content whole blood was centrifugated for 15 minutes at 156 g without break to obtain platelet-rich-plasma (PRP). Platelet concentration was adjusted to 300×10^9/L by addition of autologous plasma. In order to lyse the platelets in PRP, samples were 1:1 diluted with triton X-100 2%, followed by ultra-centrifugation for 5 minutes at 10,000 RPM to spin down large particles and supernatants were taken for ELISA.

Data analyses

Statistical analysis was performed with Graphpad Prism 5.0 (San Diego, CA, USA) and IBM SPSS statistics 22.0 (New York, NY, USA). Data was tested for normality by use of the Shapiro-Wilkinson test and assessed in Q-Q plots. Normally distributed data were analyzed by paired T tests (two-sided) and results are presented as mean with the standard error of the mean (SEM). Non-normally distributed data were analyzed with the Wilcoxon-Rank sum test and data are presented as median with interquartile range (IQR). Correlations between inflammation and platelet markers were determined using Pearon's r when data were normally distributed and Spearman's rank correlation

coefficient when data were non-normally distributed. The differences were considered significant if the p-value < 0.05.

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4

CHAPTER 5

The effects of Signal Transducer and Activator of Transcription 3 mutations on human platelets.

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ABSTRACT

Involvement of STAT3 in inflammation is well-known. Recently, a role for STAT3 in platelet activation and platelet production has been suggested. Platelets exhibit important immune functions and engagement of STAT3 in platelet physiology may link inflammation and hemostasis. This study investigated the effects of STAT3 loss-of-function mutations and SNPs in STAT3 on Glycoprotein VI (GPVI)-mediated platelet activation and platelet numbers in humans. Two cohorts were studied. The first cohort concerned patients with STAT3 loss-of-function mutations. Platelet numbers were investigated in 8 patients and GPVI-mediated platelet activation was functionally tested in 4 patients. Additional experiments were performed to investigate underlying mechanisms. The second cohort concerned 334 healthy volunteers and investigated the consequences of SNPs in STAT3 on GPVI-mediated platelet activation and platelet numbers. Platelet activation was lower in STAT3 loss-of-function patients at baseline and after stimulation of the GPVI receptor, reflected by decreased P-selectin expression. This was independent of gene transcription. Blockade of the ADP pathway resulted in a further decrease of P-selectin expression, particularly in STAT3 loss-of-function patients. In contrast, the SNPs in STAT3 did not influence GPVI-mediated platelet activation. Also, platelet numbers were not affected by STAT3 loss-of-function mutations, nor was there an association with the SNPs. In conclusion, STAT3 signaling does not seem to play a major role in thrombopoiesis. We confirm that STAT3 is involved in GPVI-mediated platelet activation in humans, independent of gene transcription. GPVI-mediated platelet activation is highly dependent on secondary ADP release. Our findings suggest that STAT3 modulation may affect inflammation, hemostasis and their interaction.

INTRODUCTION

Signal Transducer and Activator of Transcription 3 (STAT3) is important for transduction of a variety of cell signals, including signals involved in inflammation.[1,2] In response to cytokines and growth factors, STAT3 is phosphorylated and translocated to the nucleus for transcription of specific genes.[1] Differentiation of T helper-17 (Th-17) cells is dependent on STAT3 and is essential for host defense against fungal infections.[3,4] Conversely, increased STAT3 activity results in hyperinflammation and disease, and contributes to the pathogenesis of certain types of cancer.[4-8] Therefore, a delicate balance of STAT3 activation is crucial to prevent disease.

In addition to regulation of inflammatory responses, recent evidence suggests that STAT3 is also involved in hemostasis. More specific, STAT3 was shown to play a role in platelet activation[9-11] and in thrombopoiesis.[12] Zhou and colleagues reported that platelet activation via surface receptor Glycoprotein VI (GPVI) is impaired in platelet STAT3-knockout mice.[9] Additionally, *ex vivo* STAT3 inhibition by pharmacological inhibitors resulted in decreased platelet activation and less thrombus formation after stimulation of GPVI with platelet agonist collagen-related peptide (CRP) in humans.[9] Grozovsky and colleagues showed involvement of STAT3 in platelet production.[12] Binding of desialylated platelets to the Ashwell-Morell receptor induced hepatic thrombopoietin (TPO) via a mechanism dependent on STAT3 in mice.[12] These studies suggest that STAT3 may be involved in primordial platelet physiology, however, most evidence is derived from mouse models and cell lines: further validation of its importance in humans is therefore warranted.

This study investigated involvement of STAT3 in platelet activation and platelet numbers in humans. Two independent cohorts were studied. The first cohort concerned patients with STAT3 loss-of-function mutations. STAT3 loss-of-function is rare but nonetheless observed in humans and results in the Hyper IgE syndrome, an immune deficiency disorder associated with recurrent infections, eczema, mucocutaneous candidiasis and extreme elevations of serum IgE.[13,14] Patients with STAT3 loss-of-function are deficient in STAT3-dependent cytokines, such as Interleukin (IL)-17. It is unknown whether thrombopoiesis and platelet function are affected in humans with mutations in STAT3. The second cohort concerned healthy human volunteers and investigated the effects of Single Nucleotide Polymorphisms (SNPs) in STAT3 on platelet activation and platelet numbers. These data are important to understand its function on a population level. We demonstrate that both mutations and SNPs in STAT3 do not influence platelet numbers in humans, whereas STAT3 loss-of-function mutations do affect GPVI-mediated platelet activation by CRP. Additionally, we observed that GPVI-mediated platelet activation was

largely dependent on secondary stimulation by adenosine di-phosphate (ADP), thereby partially restoring the defect observed in STAT3 loss-of-function patients.

METHODS

Patients selection.

Cohort 1. Subjects with STAT3 loss-of-function mutations.

Autosomal dominant cases of hyper IgE syndrome (AD-HIES) are caused by specific mutations that results in non-functional STAT3 activity.[15] Most mutations are found at positions that are well known for STAT3 function, such as the Src homology (SH2) domain that enables recruitment and binding of STAT3 to the activated receptor and the DNA-binding domain that enables binding of STAT3 to DNA target sites.[14] The vast majority of AD-HIES cases are due to these heterozygous mutations resulting in absent STAT3 activity, however, several other mutations have been described.[13,16] To examine the effects of STAT3 loss-of-function mutations on platelet numbers, medical records of 8 confirmed heterozygous AD-HIES patients were consulted. Additionally, we were able to functionally assess GPVI-mediated platelet activation in 4 of these patients and in 10 control subjects. Interleukin-17 production has been previously tested in peripheral blood mononuclear cells (PBMCs) of these patients and the interleukin-17 (IL-17) deficiency served as a further confirmation of the STAT3 loss-of-function.[17] Control subjects were healthy individuals that were age- and gender matched with AD-HIES patients. No genotyping for specific STAT3 mutations was performed in control subjects because the incidence is exceptionally low and severe medical complaints manifest early in life. The control subjects did not experience any medical complaints during recruitment. Subjects were recruited under a protocol approved by the Institutional Review Board of Radboud University Nijmegen Medical Center. AD-HIES patients are referred to as STAT3 loss-of-function patients.

Cohort 2. STAT3-related SNPs in healthy subjects.

Several SNPs in STAT3 that are associated with inflammatory diseases were identified from literature.[18-20] In contrast to the STAT3 loss-of-function mutations, the SNPs in STAT3 are associated with inflammatory diseases that are accompanied by increased IL-17 levels. Differentiation of Th-17 cells is dependent on STAT3, which suggests increased STAT3 activity.[20] We determined these SNPs using DNA from the 500 Functional Genomics (FG) cohort and correlated these with GPVI-mediated platelet activation and platelet numbers. This cohort consists of 534 healthy individuals of Caucasian origin and is part of the Human Functional Genomics Project (HFGP) aimed at characterizing variations in immune function.[21,22] More detailed information on the study design and

the characteristics of study participants can be found in previous publications.[22,23] Individuals were recruited under a protocol approved by the Institutional Review Board of Radboud University Nijmegen Medical Center.

Experiments cohort 1.

Platelet activation and responsiveness assay.

Blood from STAT3 loss-of-function patients during stable disease and healthy subjects was collected in 3.2% sodium citrate vacutainer tubes (Becton Dickinson, Franklin Lakes, New Jersey, USA). Whole blood was centrifugated for 15 minutes at 156 g without break to obtain platelet-rich-plasma (PRP). Platelet concentration was adjusted to 300x10^{9}/L by addition of autologous platelet-poor plasma (PPP). PPP was obtained after centrifugation of whole blood at 3800 RPM for 10 minutes. PRP was rested for 1 hour before platelet functions were assessed.

Platelet expression of activation marker P-selectin was measured in PRP at baseline and after incubation for 20 minutes with different concentrations of platelet agonist cross linked collagen-related peptide (CRP) (kind gift from Prof. Dr. R. Farndale, Cambridge, UK) to stimulate the GPVI surface receptor. Thrombin Receptor Activating Peptide-6 (TRAP) (Sigma-Aldrich, Saint Louis, Missouri, USA), an agonist for the thrombin receptor proteinase-activated receptor-1 (PAR-1), served as a positive control. Samples were incubated with 9 concentrations of CRP (range 1.3 – 320ng/ml) and two concentrations of TRAP (9.78μm and 156μm) together with antibodies for flow cytometry. The reaction was stopped by addition of 0.2% paraformaldehyde. P-selectin expression was measured with a Becton Dickinson flow cytometer. In order to identify platelets, platelets were gated based on forward and sideward scatter (FSC/SSC) and additional gating was performed based on expression of platelet surface marker CD61 (PC7 labeled anti-CD61, Beckman Coulter, Brea, California, USA). To determine platelet activation, the mean fluorescence intensity (MFI) exceeding that of the matched isotype of P-selectin (PE labeled anti-CD62P, Bio-legend, San Diego, California, USA) was determined.

Inhibition of ADP pathway with apyrase.

Activation via the GPVI surface receptor results in platelet degranulation of dense granules thereby releasing biologically active substances, most notably ADP. ADP can activate platelets via a secondary pathway. In order to avoid skewing of the responses by platelet activation via this positive feedback loop, additional experiments were performed after pre-incubation with apyrase, to block the ADP pathway. Apyrase was purchased from Sigma-Aldrich (Saint Louis, Missouri, USA). For experiments a final concentration 5 U/ml was used. After incubation with apyrase, PRP samples were stimulated with CRP at concentrations 320 ng/ml, 80 ng/ml, 20ng/ml in combination with the

antibodies for flow cytometry. The same protocol and gating strategies as described above were used.

Inhibition of transcription during platelet activation.

STAT3, as a transcription factor, regulates DNA transcription and exerts its effects after phosphorylation, dimerization and translocation to the nucleus.[24] Platelets do not have a nucleus and exclusively contain mitochondrial DNA. Experiments with transcription blocker actinomycin were performed to investigate the role of DNA transcription in this process. Actinomycin was purchased from Sigma-Aldrich (Saint Louis, Missouri, USA) and dissolved in DMSO, stock concentration 6270ug/ml. PRP was pre-incubated with actinomycin D in two concentrations (50ug and 5ug) and DMSO control, before samples were stimulated with CRP. The CRP concentration of 40ng/ml was chosen as it was estimated that platelet responses would be in the steep part of the platelet reactivity curve. The same protocol for stimulation and similar gating strategies as described above were used for measurement of P-selectin expression.

Platelet content and plasma markers.

Soluble plasma markers and platelet content of P-selectin, fibrinogen were determined by ELISA. For platelet content, PRP (concentration 300×10^9 platelets/ml) were freeze-thawed for 3 cycles to fragment the platelets. Samples were centrifugated at room temperature 5 for minutes at 5000 RPM in an ultracentrifuge to spin down large particles. Supernatants were used for ELISA. PPP was used for plasma markers. Soluble P-selectin and soluble fibrinogen were measured using a human P-selectin/CD62P duoset ELISA kit (R&D systems, Europe, Abingdon, UK) and a human Fibrinogen ELISA kit (Abcam, Europe, Cambridge, UK), respectively. Thrombopoietin (TPO) was measured with a human thrombopoietin Quantikine ELISA kit (R&D systems, Europe, Abingdon, UK).

Experiments cohort 2.

Single Nucleotide Polymorphisms (SNPs) in STAT3.

Genotyping of the SNPs was performed using a commercially available SNP chip, Illumina HumanOmniExpressExome-8 v.1.0, methods previously reported by Li et al.[23] In short, genotype calling was performed using Optical 0.7.0.[25] Call rates less than ≤0.99 were excluded from the dataset, as were samples with a Hardy-Weinburg equilibrium (HWE) ≤0.0001, call rate ≤0.99 and MAF ≤0.001. In total, 483 samples were included for further imputation, as described previously. Data was extracted for the following SNPs: rs744166, rs3816769 and rs4796793. Presence of the SNP was related to platelet activation in response to CRP and to platelet numbers.

Platelet activation and responsiveness assay.

For assessment of platelet activation and responsiveness, platelet P-selectin expression was measured in whole blood at baseline and after incubation for 20 minutes with 7 different concentrations of platelet agonists CRP, together with an antibody-mix for flow cytometry. The same protocol and gating strategies as described above were used for measurement of P-selectin expression. The area under the curve (AUC) of the P-selectin expression after stimulation (MFI) was correlated with the SNP data. In total, data was available for 334 healthy human volunteers.

Statistical analysis.

Cohort 1. Quantitative data are expressed as median with interquartile range (IQR) and were analyzed with GraphPad Prism 5. Repeated measures like platelet responsiveness upon stimulation was analyzed using a two-way ANOVA in order to correct for the multiple independent observations. To compare singular measurements between groups Mann-Whitney U tests were performed for non-normally distributed data.

Cohort 2. Platelet numbers were normally distributed, in contrast to P-selectin expression. To normalize P-selectin expression (AUC), data was log transformed. Formal correction for age- and gender effects in both outcome parameters was performed. SNP genotypes were coded 0, 1 and 2, before linear regression analyses was performed with SPSS with use of dummy variables.

P values < 0.05 were considered statistically significant.

RESULTS

Characteristics of study participants.

Main characteristics of the two study cohorts are presented in table 1. The first cohort concerned 8 STAT3 loss-of-function patients, five patients had a mutation in the DNA-binding domain, two in the SH2-domain and one patient had a mutation in the Linker-domain of STAT3. The second cohort involved 483 healthy human volunteers for which of 334 participants data on SNPs and GPVI-mediated platelet activation was available. A more detailed description of the specific mutations in the STAT3 loss-of-function patients can be found in table 2.

Table 1. Patient characteristics.

Cohorts	STAT3 loss-of-function Cohort		SNP Cohort
	AD-HIES	Control subjects	Healthy individuals
Number (n)	8	10	483
Age (mean, SD)	38 ± 10.5	32 ± 9.2	28.5 ± 13.7
Gender (% male)	37.5%	40%	44.3%
Mutation domain (n)		N/A	N/A
DNA binding domain	5		
SH2-domain	2		
Linker-domain	1		
SNP frequency*	N/A	N/A	
rs744166			31.5/48.4/20.1%
rs3816769			58.6/37.0/4.4%
rs4796793			37.7/48.9/13.4%
Medication	Anti-fungal therapy, prophylactic antibiotic treatment	No medication	No medication
Clinical complications	Recurrent RTIs, eczema, 'cold abscesses'	None	None

Characteristics of the two study cohorts. (*Abbreviations: AD-HIES: Autosomal-Dominant Hyper IgE Syndrome; N/A: Not Applicable; RTI: respiratory tract infection; SH2: Src Homology 2; SNP: Single Nucleotide Polymorphism)* *homozygous for common allele / heterozygous / homozygous for rare allele

Less platelet activation and GPVI-mediated platelet responsiveness in STAT3 loss-of-function patients.

To investigate the involvement of STAT3 in platelet activation, we determined baseline platelet activation in STAT3 loss-of-function patients. Platelet activation, determined by P-selectin expression, was significantly lower in STAT3 loss-of-function patients compared to healthy subjects (median MFI 3.03 (IQR 2.62 – 3.84) versus 4.82 (IQR 3.53 – 5.67), p=0.01) (Figure 1A). Next, we investigated whether collagen induced platelet activation by stimulation of GPVI surface receptor with CRP was affected. Stimulation of the GPVI receptor with different concentrations of CRP (range 1.3 – 320 ng/ml), resulted in significantly lower P-selectin expression in STAT3 loss-of-function patients compared to healthy subjects (p<0.001)(Figure 1B). To examine whether this observation was due to an intrinsic platelet defect or whether it resulted from the theoretical possibility of defective production of factors that prime platelets, platelets were stimulated with another agonist, TRAP, as a control. Although platelet responses were slightly lower with TRAP (Figure 1C), this effect was not statistically significant after correction for lower baseline P-selectin expression, suggesting a largely intrinsic defect in platelets.

Table 2. STAT3 loss-of-function mutations.

Protein domain	Site of mutation	DNA Sequence change	Predicted amino acid change	Number of patients
DNA-binding	Exon 13	1144C→T	R382W	2* (1)
	Exon 13	1145G→A	R382Q	1
	Exon 16	1387 deletion GTG	V463 deletion	1
	Intron 11	1110-2A→G	G380 deletion	1*
SH2	Exon 21	1909C→G	V637L	1
	Exon 21	1909G→A	V637M	1*
Linker domain	Exon 19	1679-1681 deletion	S560 deletion	1*

Specification of the STAT3 loss-of-function mutations. (*Abbreviations: DNA: Deoxyribonucleid acid; SH2: Src Homology 2).* Patients indicated with an asterisk (*) were functionally assessed.

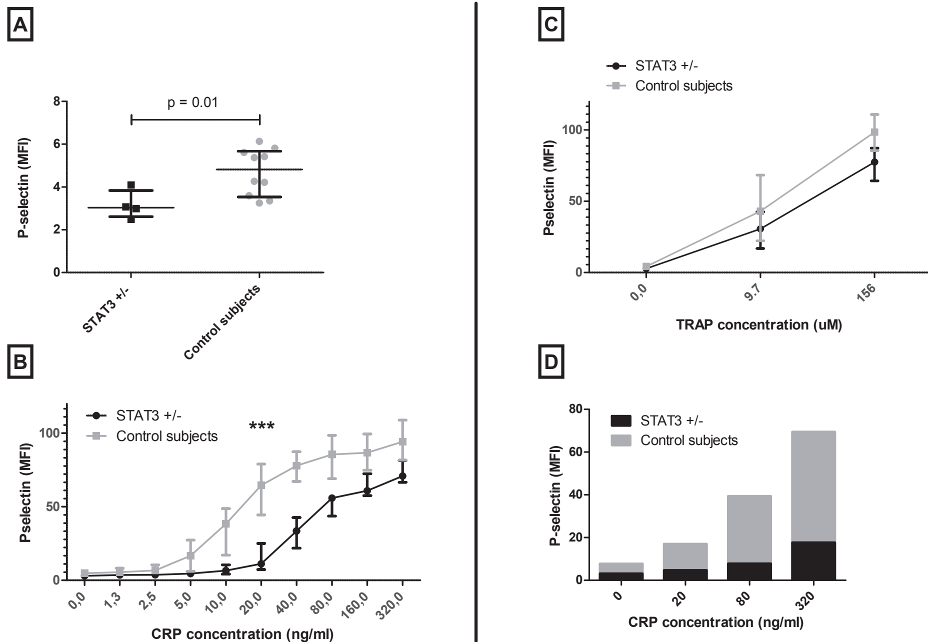


Figure 1. Functional assessment of GPVI-mediated platelet activation in STAT3 loss-of-function patients.
A. Platelet P-selectin expression in unstimulated samples (median MFI 3.03 (IQR 2.62 - 3.84) in STAT3 loss-of-function versus 4.82 (IQR 3.53 - 5.67) in control subjects, p=0.01). **B.** Platelet P-selectin expression in GPVI-mediated platelet activation by CRP stimulation ($P<0.001$). **C.** Platelet P-selectin expression in PAR-1 mediated platelet activation by TRAP (p=0.054). **D.** P-selectin expression in GPVI-mediated platelet activation by CRP after blockade of the ADP pathway by apyrase. Data are presented as median with IQR. (*Abbreviations: ADP: adenosine 5' diphosphate; CRP: Collagen-related Peptide; GPVI: Glycoprotein VI; MFI: Mean Fluorescence Intensity; TRAP: Thrombin Receptor Activating Peptide-6)*

Inhibition of the ADP pathway further attenuates platelet responsiveness in STAT3 loss-of-function patients.

GPVI-mediated platelet activation results in platelet degranulation, thereby releasing biologically active substances, in particular ADP from dense granules.[26] In order to investigate dependency of the GPVI-mediated platelet activation pathway on secondary ADP release, we incubated samples with apyrase to block this pathway. Stimulation of the GPVI receptor by different concentrations of CRP after incubation of samples with apyrase resulted in considerably lower P-selectin expression in both groups. Inhibition of the ADP pathway seems to have a more profound effect in STAT3 loss-of-function patients than in healthy subjects, as the relative increase from baseline to the highest CRP concentration was 5.7 fold in STAT3 loss-of-function patients compared to 11.4 fold in control subjects (Figure 1D).

STAT3 signaling in GPVI-mediated platelet activation is independent of gene transcription.

Platelets do not have a DNA containing nucleus, however platelets do possess functional mitochondrial DNA.[26] In order to exclude the theoretical possibility of involvement of novel DNA transcription in platelet activation in response to CRP stimulation, transcription was inhibited by general transcription blocker actinomycin D. After incubation of PRP samples with actinomycin D, samples were stimulated with CRP. No changes in expression of P-selectin was seen after inhibition of transcription in both patients and healthy subjects (data not shown).

No differences in platelet content and soluble markers were found in STAT3 loss-of-function patients.

Previous experiments suggest an intrinsic platelet defect in patients with STAT3 loss-of-function mutations. In addition, inhibition of the ADP pathway had a relatively higher impact on GPVI-mediated platelet activation in STAT3 loss-of-function patients. To examine whether these observations were a result of alterations in granule content, we measured P-selectin and fibrinogen in platelet lysates. No differences were seen in platelet content for P-selectin and fibrinogen (327.0 ng/ml (IQR 153.9 – 523.5) versus 261.1 ng/ml (IQR 204.3 – 325.5), p-value 0.84 and 2420 μg/ml (IQR 1786 – 3813) and 2092 μg/ml (IQR 1038 – 2938), p-value 0.52, respectively). This suggests that previous observations are more likely due to lower platelet granule release in response to stimulation than alterations in granule content.

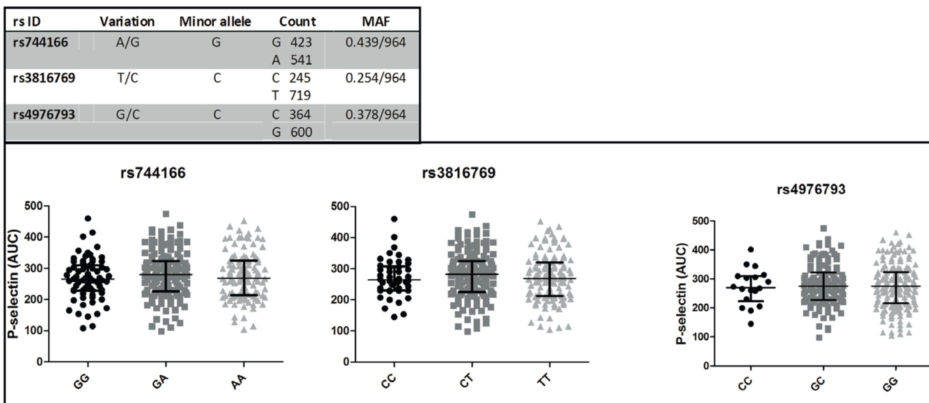
Lastly, P-selectin and fibrinogen in plasma was determined as a measure for platelet granules release. Interestingly, no significant differences were found in soluble plasma P-selectin and fibrinogen between patients and healthy subjects, (median 41.7 ng/ml

(IQR 27.0 – 47.3) versus 27.9 ng/ml (21.9 – 44.2), p-value 0.45 and mean 2513 μg/ml (IQR 1826 – 3016) versus 2640 μg/ml (IQR 2240 – 3114), p-value 0.74, respectively).

SNPs in STAT3 do not correlate with GPVI-mediated platelet activation in healthy volunteers.

To investigate the involvement of STAT3 on platelet activation on a population level, several SNPs in STAT3 that are associated with inflammatory diseases were investigated. The 3 SNPs; rs744166, rs3816769 and rs4796793, were determined in the second cohort, concerning healthy volunteers. These SNPs are known from literature to be functional and are likely to influence STAT3 activity in relation to several diseases, such as Crohn's disease, ulcerative colitis and autoimmune thyroid disease.[18-20] The minor allele frequencies (MAF) of rs744166, rs3816769, rs4796793 were C=0.439/964, C=0.254/964 and C=0.378/964, respectively. The MAFs were similar to the MAFs reported in the NCBI database (http://www.ncbi.nlm.nih.gov/SNP), except for rs3816769 with a MAF of C=382/1912 in the NCBI database.

Linear regression analysis was performed for SNP genotype and platelet activation in response to GPVI stimulation (AUC). No significant correlations could be detected for rs744166, rs3816769, and rs4796793 with GPVI-mediated platelet activation (p>0.05). The SNP characteristics and the relation between the SNPs and GPVI-mediated platelet activation is shown in Figure 2. In contrast to previous observations, SNPs in STAT3 do not seem to affect GPVI-mediated platelet activation.



rs ID	Variation	Minor allele	Count	MAF
rs744166	A/G	G	G 423 A 541	0.439/964
rs3816769	T/C	C	C 245 T 719	0.254/964
rs4976793	G/C	C	C 364 G 600	0.378/964

Figure 2. SNPs rs744166, rs3816769 and rs4796793 and P-selectin expression (AUC).
SNP characteristics are shown in the upper figure. P-selectin expression (AUC of MFI) after GPVI-mediated platelet activation by 7 concentrations of CRP is shown in the lower figure. Data are presented as median with IQR. *(Abbreviations: AUC: Area Under the Curve; CRP: Collagen-related Peptide, GPVI: Glycoprotein VI; IQR: Interquartile range; MAF: Minor Allele Frequency; MFI: Mean Fluorescence Intensity; SNP: Single Nucleotide Polymorphism)*

Platelet numbers are not influenced by STAT3 mutations and STAT3 SNPs.

Lastly, we studied platelet production. Platelet production is primarily regulated by TPO concentrations in blood and it has recently been suggested that hepatic TPO production is dependent on signaling via STAT3.[12] Platelet numbers retrieved from medical records of 8 confirmed STAT3 loss-of-function patients were mostly within the normal range (Table 3). Interestingly, thrombocytosis was observed in two patients during an acute infection. Thrombocytopenia was not observed in STAT3 loss-of-function patients. In addition, plasma TPO levels were determined in the 4 functionally assessed STAT3 loss-of-function patients and the 10 healthy subjects. Although most values were below the detection limit of 15.6 pg/ml, two STAT3 loss-of-function patients had detectable TPO levels (24.0 pg/ml and 79.7 pg/ml) (Figure 3A).

Table 3. Median platelet numbers (range).

STAT3 loss-of-function cohort	Highest (platelets x10^9/L)	Lowest (platelets x10^9/L)	
1. ♀ , 46 yrs.	264	229	
2. ♀, 46 yrs.*	247	160	
3. ♀, 23 yrs.*	505	231	
4. ♂, 47 yrs.*	317	168	
5. ♂, 26 yrs.	242	133	
6. ♀, 34 yrs.	314	205	
7. ♀, 32 yrs.*	431	175	
8. ♀, 50 yrs.	257	206	
SNP cohort	**Wild type**	**Heterozygous**	**Homozygous for SNP**
rs744166	274 (132-470)	257 (132-491)	268 (133-488)
rs3816769	276 (132-470)	263 (132-491)	266 (133-488)
rs4796793	280 (159-401)	275 (132-491)	261 (132-488)

Platelet numbers from STAT3 loss-of-function patients, platelets x 10^9/L, and platelet numbers from the SNP cohort, median platelet numbers x10^9/L (range). Patients indicated with an asterisk (*) were functionally assessed. *(Abbreviations: ♀: female; ♂: male; SNP: Single Nucleotide Polymorphism)*

Several studies suggest that IL-6 stimulates thrombopoiesis.[27,28] Therefore, we determined IL-6 levels in plasma. A trend for higher IL-6 was observed in STAT3 loss-of-function patients (Figure 3B).

Also the effects of the SNPs in STAT3 were examined. Consistently with the observation in the STAT3 loss-of-function patients, no significant influence of the SNPs on platelet numbers were observed in 334 healthy subjects (Table 3). Importantly, both thrombocytopenia and thrombocytosis were rarely observed.

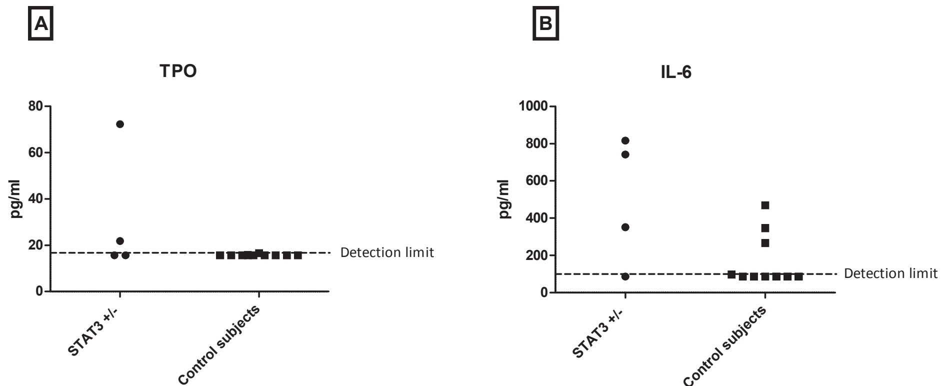


Figure 3. TPO and IL-6 levels in plasma.
A. Plasma TPO levels in STAT3 loss-of-function patients and control subjects, B. Plasma IL-6 expression in STAT3 loss-of-function patients and control subjects. *(Abbreviations: IL-6: Interleukin-6; TPO: Thrombopoietin)*

DISCUSSION

The present study investigated the involvement of STAT3 in GPVI-mediated platelet activation and platelet numbers in humans. Our findings indicate that GPVI-mediated platelet activation is affected by STAT3 loss-of-function mutations confirming its involvement in platelet function in humans. In contrast, neither clinically relevant STAT3 loss-of-function mutations, nor SNPs in STAT3 affected platelet numbers in humans.

Further experiments were performed to increase our understanding of its function. GPVI-mediated platelet activation was shown to be largely dependent on secondary stimulation by platelet ADP release, both in patients and in healthy subjects. Blockade of this mechanism strongly attenuated platelet responses, particularly in STAT3 loss-of-function patients. These observations were independent of gene transcription, as previously reported.[9] Although STAT3 is commonly known as a transcription factor, its involvement in platelet activation appears to be non-transcriptional and few non-transcriptional properties of STAT3 have been previously described.[29,30] SNPs in STAT3 did not directly affect GPVI-mediated platelet activation in a healthy population. In some inflammatory diseases the effects of SNPs in STAT3 on platelet activation may become apparent, however, in healthy individuals robust mechanisms seem to balance inflammation and hemostasis.

Interestingly, P-selectin levels in plasma were not different between STAT3 loss-of-function patients and control subjects, whereas platelet degranulation was decreased in patients. Another source for soluble P-selectin is the vascular endothelium.[31] Both

vascular abnormalities and endothelial dysfunction are common in Hyper IgE syndrome and may explain this observation.[32]

With the lack of an effect on platelet numbers we report an opposite effect compared to a previous study that suggests an important role for STAT3 in the hepatocytic production of TPO, the most important regulator of platelet formation by megakaryocytes.[12] In addition to a mouse model, it was shown that desialylated platelets are taken up by a human HepG2 cells and these cells subsequently produce TPO in a Janus Kinase-2/STAT3-dependent manner.[12] In contrast, we did not find a decrease in platelet numbers in patients with defective STAT3 signaling, moreover thrombocytosis was observed in STAT3 loss-of-function patients during acute infections. TPO levels were detectable in plasma in two patients, whereas these were low or undetectable in control subjects. A direct comparison between groups is limited by the fact that TPO was undetectable in most subjects and these results should be interpreted with caution. A trend was seen for increased IL-6 in patients and this may also stimulate thrombopoiesis.[27,28] Our findings suggest that *in vivo* other mechanisms also regulate platelet production in humans.

The observation that STAT3 is involved in GPVI-mediated platelet activation further validates that the mechanism reported by Zhou and colleagues may be functional and relevant in humans too.[9] Involvement of STAT3 in platelet activation may provide a relevant link between inflammation and hemostasis. Increased activation of the STAT3 signaling pathway has been described in cancer and many inflammatory diseases, including cardiovascular diseases.[4-8,33] Cardiovascular disease is the leading cause of death worldwide and interestingly, risk for cardiovascular diseases is strongly increased in many inflammatory diseasese.[34] To examine whether GPVI-mediated platelet activation was affected by SNPs in STAT3 we studied a cohort of healthy human volunteers, however, no relation was observed. The role of STAT3 activation in inflammatory diseases and its consequences for platelet activation needs further exploration. Especially since it is unknown if the SNPs in STAT3 affect STAT3 function in healthy subjects, or whether aberrant STAT3 function becomes apparent in inflammatory diseases.

Blockade of STAT3 is currently being explored as a new therapy for cancer.[35,36] Several STAT3-inhibitors have recently proceeded into clinical trials and also drugs that block Janus Kinase 2 (JAK2), such as Ruxolitinib.[35,37,38] It is important that involvement of STAT3 in platelet activation is recognized. Bleeding complications may occur, especially in patients that are concurrently treated with platelet inhibitors that target other pathways such as Ticagrelor and Clopidogrel, targets of the ADP receptor, P2Y12. GPVI-mediated platelet activation is highly dependent on secondary activation via ADP and suppression of both pathways may have important consequences in terms of bleedings.[39-41]

The relevance of the functional impairment in patients with AD-HIES seems to be limited due to the positive feedback loop by ADP, which partially restores platelet responses. Although severe bleeding complications are reported, they mostly coincide with opportunistic infections in AD-HIES patients that often have bronchiectasis, both features known to provoke pulmonary hemorrhages.[42,43] Interestingly, in one AD-HIES patient a spontaneous bleeding was recorded that could not be explained from a clinical perspective. The bleeding occurred in the iliopsoas muscle and was not preceded by any kind of trauma. Examination of the medical history of the other patients did not reveal any unexplained spontaneous bleedings.

Our study has a few limitations. The first limitation is that we were unable to relate platelet responses directly to STAT3 function and different STAT3 isoforms. STAT3 has several isoforms that mostly result from alternative mRNA splicing. These isoforms can have distinct functions, STAT3β, for example, lacks the Ser727 phosporylation site and is thought to be a negative regulator.[44] The exact mechanisms by which STAT3 mutations cause STAT3 loss-of-function are still largely unknown.[14] It would have been of great interest to directly relate STAT3 isoforms and STAT3 activity to GPVI-mediated platelet responses. Also the question whether there is an compensatory increase in other STATs needs further investigation, as STATs can both form homodimers and heterodimers with different functions.[44] The second limitation is the limited sample size of STAT3 loss-of-function patients (AD-HIES patients). This makes our findings less robust. AD-HIES is a very rare disease with an estimated prevalence of 1:100.000.[14,45] It has to be noted that the effects observed were consistent in patients and in different concentrations of CRP. Lastly, STAT3 loss-of-function patients are treated with prophylactic antimicrobial therapy and we were unable to correct for those differences in the healthy subjects. Therefore, we cannot completely exclude a potential effect of medication on our findings.

In conclusion, our findings indicate that STAT3 is involved in GPVI-mediated platelet activation by CRP in humans. Additionally, GPVI-mediated platelet activation was shown to be largely dependent on secondary stimulation by ADP. In contrast, neither clinically relevant STAT3 loss-of-function mutations, nor SNPs affecting STAT3 activity influence platelet numbers in humans. These data improve our understanding on the interaction between inflammation and hemostasis and suggest that STAT3 modulation may affect both inflammation and hemostasis and their interaction in humans.

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CHAPTER 6

Platelet integrin αIIbβ3 activation is associated with 25-hydroxyvitamin D concentrations in healthy volunteers

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ABSTRACT

Background

Cardiovascular events are often associated with low vitamin D concentrations, although the underlying mechanisms are poorly understood. This study investigated associations between 25-hydroxyvitamin D concentrations, single nucleotide polymorphisms (SNPs) in genes influencing vitamin D biology and platelet function in the 500 Functional Genomics (500FG) cohort.

Methods

The 500FG cohort consists of approximately 500 healthy participants of Western-European ancestry and is part of the Human Functional Genomics Project (HFGP). Among other immunological and metabolic parameters, this observational study measured platelet activation and function by binding of fibrinogen to the activated fibrinogen receptor integrin αIIbβ3 and platelet expression of P-selectin, markers of platelet aggregation- and degranulation, respectively, by flow cytometry. The platelet function parameters were correlated to serum 25-hydroxivitamin D and genotyping was performed to investigate SNPs in genes important for vitamin D biology.

Results

25-hydroxyvitamin D circulating concentrations were inversely correlated with baseline platelet binding of fibrinogen to integrin αIIbβ3 (Pearson's $r = -0.172$, $p=0.001$) and platelet responses to platelet agonist CRP-XL (Pearson's $r = -0.196$ $p=0.001$). No differences in platelet fibrinogen binding were observed between subjects with normal 25-hydroxyvitamin D concentrations (>75nmol/L) and a 25-hydroxyvitamin D insufficiency (50-75 nmol/L), while there was an effect in comparison to vitamin D deficient subjects (≤50nmol/L). There were no correlations between 25-hydroxyvitamin D concentrations and platelet P-selectin expression. Several SNPs in the GC region of the vitamin D binding protein (VDBP)-gene were associated with platelet responses to CRP-XL.

Conclusions

Low circulating vitamin D concentrations are associated with increased platelet fibrinogen binding to integrin αIIbβ3 in unstimulated samples and after stimulation with CRP-XL and ADP. These findings may partially explain the higher incidence of cardiovascular events in vitamin D deficient patients and its seasonal variation. Further studies are needed to investigate causality.

INTRODUCTION

Cardiovascular diseases, such as myocardial infarction, are the leading causes of global mortality and morbidity.[1] A seasonal variation of its incidence is observed, with a higher rate of events in the winter and a nadir in the summer.[2-5] Vitamin D concentrations show a similar seasonal pattern as vitamin D concentrations are influenced by skin exposure to sunlight.[6] Many studies have shown associations between low vitamin D concentrations and cardiovascular diseases[7-11] and the majority of patients with acute myocardial infarction are vitamin D deficient.[12,13] However, the underlying mechanisms remain poorly understood.

Vitamin D has many functions beyond its traditional role in bone health, including a regulatory role in inflammation and infection.[14-16] Inflammation and hemostasis are closely linked biological systems[17,18] and during episodes of increased systemic inflammation, thrombotic complications are more frequently observed.[19] Platelets play a pivotal role in this process and platelet activation, degranulation and aggregation are essential steps in arterial thrombus formation.[20] Interestingly, it has been reported that both platelets and their megakaryocyte precursors express the vitamin D receptor (VDR).[21] However, the direct association between vitamin D and platelet function in humans is poorly studied.

The vitamin D pathway is a complex metabolic pathway that has many steps before the substrate 25-hydroxyvitamin D is converted in its active metabolite 1,25-dihydroxyvitamin D.[6,14] As a result, there is a large variation of genetic inter-individual factors that influence vitamin D homeostasis.[22-25] Single nucleotide polymorphisms (SNPs) in genes of several key factors influencing vitamin D biology, such as vitamin D binding protein (VDBP), cytochrome P2R1 (CYP2R1) and VDR are reported to influence 25-hydroxyvitamin D concentrations, but may also influence 1,25-dihydroxivitamin D bio-availability and thereby its physiological effects.[22-24,26-28]

For this study, data from the 500 Human Functional Genomics Project (500FG) were used. The 500FG is part of the Human Functional Genomics Project (HFGP) that is aimed to characterize of variations of immune cell function and platelet function in healthy human volunteers[29] and thereby provided an unique opportunity to study the association between 25-hydroxyvitamin D and platelet function. The objective of this study was to investigate whether 25-hydroxyvitamin D concentrations and SNPs in genes encoding for proteins important for vitamin D biology influence platelet function in healthy human volunteers.

6

METHODS

Study design and population.

The 500FG cohort consists of 534 healthy volunteers and is part of the HFGP (http://www.humanfunctionalgenomics.org/site/) aimed at characterizing variations in immune function.[30] The study design and population have been previously described.[29,31] In summary, between August 2013 and December 2014 a total of 534 healthy human subjects of Caucasian origin were recruited in the Radboud university medical center, Nijmegen, the Netherlands. Participants were scheduled for a study visit between 8-10 am to donate blood. After their visit, participants received an online questionnaire on dietary habits, lifestyle and disease history.

Ethics.

This study was approved by the local Ethical Committee (NL42561.091.12, 2012/550) and was conducted according to the principles of the Declaration of Helsinki (version Oct 2008) and in accordance with the Dutch Medical Research involving Human Subjects Act. All participants gave written informed consent before blood was drawn.

Blood sampling and 25-Hydroxyvitamin D measurement.

Blood was drawn in sterile EDTA, serum and 3.2% sodium citrate vacutainer tubes (Becton Dickinson, Plymouth, UK). 25-Hydroxyvitamin D3 was measured with liquid chromatography tandem mass spectrometry (LC- MS/MS) after precipitation of the protein and solid-phase extraction as described in further detail by ter Horst et al.[29] In summary, an internal standard of ^{2}H3] 25OH-vitamin D3 was added before 50ul NaOH (2M) was added to release protein-bound 25-OH vitamin D3 and a combination of Acetonitrile/Methanol (9:1) was added for protein precipitation. H_2O was added followed by solid phase extraction (Oasis HLB 1cc, Waters). The eluate (300 µL methanol/isopropanol 95:5) was diluted with H_2O (3:1) and injected (10 µL) into an Agilent Technologies 1290 Infinity VL UHPLC-system (Agilent Technologies, Santa Clara, CA), equipped with a BEH C18 (1.7 µm 2.1 × 50mm) analytical column (Waters) at 45°C. An Agilent 6490 tandem mass spectrometer (Agilent Technologies) was operated in the electrospray positive ion mode, with a capillary voltage 3.5 kV, fragmentor voltage 380 V, sheath gas temperature 350°C and gas temperature 100°C with N_2 collision gas. Both 25OH-vitamin D3 and 25OH-vitamin D3 [-H2O] (in-source fragmentation) were used for quantification (results were averaged) with both two transitions (qualitative and quantitative) monitored. An 8-point calibration curve was used and absolute concentration of the calibrator (Sigma-Aldrich) was assessed by spectrophotometry (264nm). The method was linear assessed by CLSI EP6 protocol and recovery rates were within 90-109%.

Platelet activation and function assessment.

Platelet activation was defined by the binding of fibrinogen to the activated fibrinogen receptor integrin αIIbβ3 (GPIIb/IIIa complex) and the expression of P-selectin (CD62P) on the platelet surface, markers of platelet aggregation and degranulation, respectively. Platelet activation was measured in whole blood samples at baseline and after incubation with different platelet agonists, to assess its functional capacity. The agonists used were adenosine 5' diphosphate (ADP) (Sigma-Aldrich, Saint Louis, Missouri, USA) and cross-linked collagen-related peptide (CRP-XL) (kind gift from Prof. Dr. R. Farndale, Cambridge, UK). The blood samples were incubated for 20 minutes with 7 different concentrations of the agonists in combination with anti-bodies for flow cytometry at room temperature, followed by fixation with 0.2% paraformaldehyde. Staining of samples was performed with antibodies for CD61 (PC7-conjugated) (Beckman Coulter Brea, California, USA), anti-human fibrinogen (fluorescein isothiocynate (FITC)-conjugated) (Dako) and P-selectin (CD62P, phycoerythrin (PE)-conjugated) (Biolegend, San Diego, California, USA). Expression of these markers was measured by flow cytometry (FC500 flow cytometer, Beckman Coulter, Brea, California, USA). Gating of platelets was performed based on forward and sideward scatter and additionally for of CD61 positivity. The area under the curve (AUC) of fibrinogen binding and P-selectin expression after stimulation (MFI) was used for correlations with 25-hydroxyvitamin D concentrations and SNPs.

Genotyping.

The DNA samples of the participants were genotyped with a commercially available SNP chip, Illumina HumanOmniExpressExome-8 v.1.0, methods previously reported by Li et al.[31] In short, genotype calling was performed using Optical 0.7.0. Call rates less than ≤0.99 were excluded from the dataset, as were samples with a Hardy-Weinburg equilibrium (HWE) ≤0.0001, call rate ≤0.99 and MAF ≤0.001. A total of 483 samples were left for the genetic analysis, as described previously.[31] Of the 39 SNPs involved in the vitamin D pathway that were identified from literature,[25,32-35] thirty-one SNPs were available in our dataset, i.e.: rs10741657, rs10877012, rs2134095, rs2282679, rs3829251, rs10766197, rs218174, rs1155563, rs12785878, rs12794714, rs2762933, rs7041, rs6599638, rs10500804, rs7975232, rs4588, rs6055987, rs7116978, rs3755967, rs12800438, rs1562902, rs17467825, rs3794060, rs1993116, rs7968585, rs4945008, rs2060793, rs222020, rs4944957, rs2298849 and rs1801222.

Statistical analyses.

Statistical analysis was performed with IBM SPSS statistics 22.0 (New York, NY, USA) and Graphpad Prism 5.0 (San Diego, CA, USA). All data was tested for normality with the Shapiro-Wilkinson test and assessed in corresponding Q-Q plots. Non-normally

distributed data were log transformed before further analyses. Pearson's R correlation coefficients were calculated in R using the standard cor.test.routine. Multiple regression analyses were performed to assess the effects of covariates age and gender. The nominal p value < 0.05 was used as significance threshold. Correction for multiple comparisons was applied using False Discovery Rate (FDR).

RESULTS

The demographics of the study population can be found in table 1. Most participants were in their early adulthood and had a normal BMI. The Netherlands has a strong annual variation in terms of sunlight exposure and as participants were included throughout the year, we found an absolute vitamin D deficiency (≤ 50nmol/L) in 105 participants. The variation in vitamin D concentrations during recruitment was previously published by ter Horst and colleagues.[29]

Table 1. Demographics of study participants

Characteristics	N = 533
Gender (% male)	44,5%
Mean age, years (SD)	28,5 (13.9)
BMI (SD)	22.7 (2.9)
Oral contraceptive use (% of women)	53.0%
Current smoking (% of whole cohort)	13.3%
25-Hydroxyvitamin D concentrations, n (% of whole cohort)	
> 75nmol/L	206 (38.6%)
50 – 75 nmol/L	174 (32.6%)
≤ 50 nmol/L	105 (19.7%)
Missing value	48 (9.1%)

(Abbreviations: BMI: body mass index; SD: standard deviation)

Low 25-hydroxyvitamin D3 concentrations correlate to platelet binding of fibrinogen to integrin αIIbβ3.

A small, but statistically significant inverse correlation was observed between 25-hydroxyvitamin D concentrations and platelet binding of fibrinogen to the activated fibrinogen receptor integrin αIIbβ3 in unstimulated samples, Pearson's $r = -0.172$, $p=0.002$, $n= 393$ (Figure 1). Platelet fibrinogen binding in response to platelet stimulation of GPVI receptor by CRP-XL also inversely correlated to vitamin D concentrations, Pearson's $r = -0.196$ $p=0.002$, $n= 299$, (Figure 1). These data show higher platelet activation and reactivity in participants with low vitamin D concentrations. No difference was

seen between platelet reactivity in response to stimulation with platelet agonist ADP (Pearson's r -0.097, p = 0.08, n= 393) (Figure 1). Interestingly, P-selectin expression, a marker of platelet degranulation, was not affected by vitamin D concentrations in unstimulated samples (Pearson's r = 0.037, p= 0.52, n= 467, nor was there a correlation in stimulated CRP-XL and ADP stimulated samples (Pearson's r= -0.12, p= 0.08, n= 299 and Pearson's r = 0.02, p= 0.66, n= 486, respectively) (Figure 2).

25-Hydroxyvitamin D deficiency (≤ 50 nmol/L), but not insufficiency, seems to be correlated to platelet integrin αIIbβ3 activation.

Next, study participants were grouped according to the commonly used cut-off values for 25-hydroxyvitamin D concentrations; vitamin D concentrations >75 nmol/L were considered sufficient, vitamin D concentrations from 50-75 nmol/L were considered insufficient, and the threshold for vitamin D deficiency was ≤ 50 nmol/L. A significant increase was found in platelet fibrinogen binding to integrin αIIbβ3 in unstimulated samples and in response to stimulation with CRP-XL in vitamin D deficient participants compared to sufficient and insufficient participants (Figure 3). Importantly, no significant differences were observed between vitamin D sufficient and vitamin D insufficient participants in terms of fibrinogen binding.

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When applying the commonly used conservative threshold of 50 nmol/L, fibrinogen binding in unstimulated samples was statistically different between groups (mean of participants with vitamin D > 50nmol/L 0.44 ± 0.005 versus 0.49 ± 0.009 in participants with vitamin D ≤ 50nmol/L, p<0.0001). This difference was also observed after stimulation with CRP-XL and after stimulation with ADP (mean fibrinogen binding of participants with vitamin D > 50nmol/L 1.997 ± 0.01 versus 2.088 ± 0.02 in participants with vitamin D ≤ 50nmol/L, p=0.0002, and mean of participants with vitamin D > 50nmol/L 1.696 ± 0.01 versus 1.744 ± 0.02 in participants with vitamin D ≤ 50nmol/L, p=0.021, respectively), supplementary figure 1. Together, these data suggest that the association between 25-hydroxyvitamin D and platelet fibrinogen binding to integrin αIIbβ3 is stronger in vitamin D deficient participants. Interestingly, a significant difference was seen in P-selectin expression after CRP-XL stimulation (mean of participants with vitamin D > 50nmol/L 269 ± 4 versus mean 301 ± 9 in participants with vitamin D ≤ 50nmol/L, p= 0.02), whereas this was not observed in unstimulated samples (mean of participants with vitamin D > 50nmol/L 0.71 ± 0.004 versus mean 0.70 ± 0.010 in participants with vitamin D ≤ 50nmol/L, p=0.32), nor after stimulation with ADP (mean of participants with vitamin D > 50nmol/L 1.82 ± 0.01 versus mean 1.83 ± 0.01 in participants with vitamin D ≤ 50nmol/L, p=0.58), supplementary figure 2.

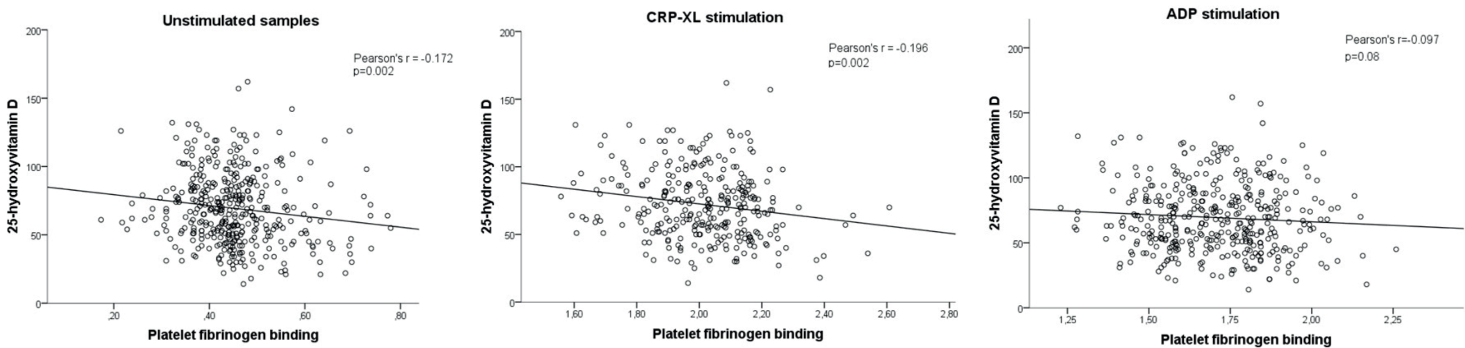

Figure 1. Correlation between vitamin D and platelet fibrinogen binding.

From left to right: the correlation between 25-hydroxyvitamin D and platelet fibrinogen binding at baseline (MFI), the correlation between 25-hydroxyvitamin D and platelet fibrinogen binding in response to stimulation with platelet agonist CRP-XL (AUC of fibrinogen MFI). and the correlation between 25-hydroxyvitamin D and platelet fibrinogen binding in response to ADP stimulation (AUC of fibrinogen MFI) *(Abbreviations:* ADP: Adenosine 5'diphospate; A*UC: area under the curve; CRP-XL: cross-linked collagen-related peptide; MFI: mean fluorescence intensity)*

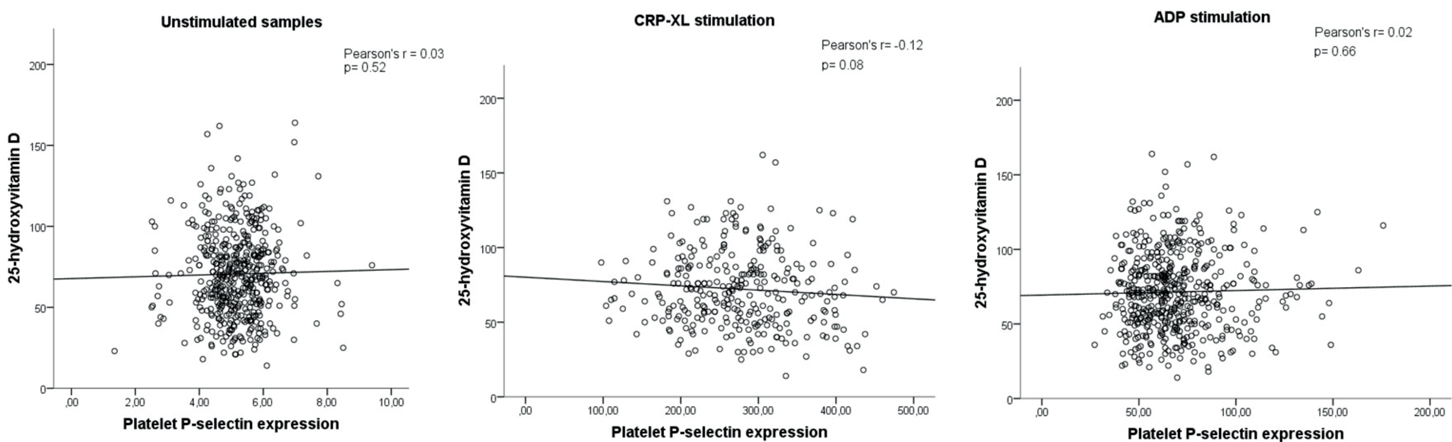

Figure 2. Correlation between vitamin D and platelet P-selectin expression.
From left to right: the correlation between 25-hydroxyvitamin D and platelet P-selectin expression in unstimulated samples (P-selectin MFI), the correlation between 25-hydroxyvitamin D and platelet P-selectin expression in response to stimulation with platelet agonist CRP-XL (AUC of P-selectin MFI). and the correlation between 25-hydroxyvitamin D and platelet P-selectin expression in response to stimulation with platelet agonist ADP (AUC of P-selectin MFI). *(Abbreviations: ADP: Adenosine 5'diphosphate; AUC: area under the curve; CRP-XL: cross-linked collagen-related peptide; MFI: mean fluorescence intensity)*

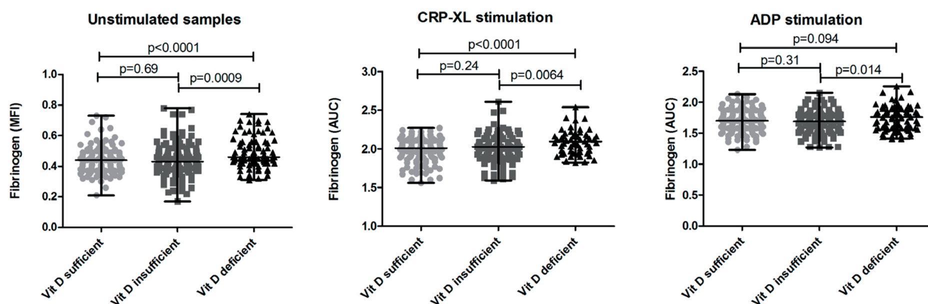

Figure 3. Vitamin D status and platelet fibrinogen binding.
Fibrinogen binding at baseline and after stimulation with CRP-XL and ADP in vitamin D sufficient (>75 nmol/L), insufficient (50-75 nmol/L) and deficient (≤ 50 nmol/L) participants. *(Abbreviations: ADP: adenosine 5'diphosphate; AUC: area under the curve; CRP-XL: cross-linked collagen-related peptide; MFI: mean fluorescence intensity; Vit D: 25-hydroxyvitamin D)*

The influence of SNPs in key players of the vitamin D pathway on platelet activation and function.

The vitamin D pathway is a complex metabolic pathway that is regulated on many levels. SNPs in key players, such as *VDBP*, *CYP2R1* and *VDR*, were determined and related to platelet activation and platelet function. Thirty-nine functional SNPs in the vitamin D pathway were identified through thorough literature search, of which 31 SNPs were available in the dataset. In total, 9 SNPs had a significant influence on fibrinogen binding to integrin αIIbβ3 in unstimulated samples and after stimulation with CRP-XL or ADP, table 2.

Six out of 9 associated SNPs were present in the GC region of *VDBP*, among 5 that were associated to platelet fibrinogen binding to integrin αIIbβ3 in response to stimulation with CRP-XL, indicating that there may be crosstalk in these signaling pathways. Next, we examined whether these SNPs exerted their effects on platelet activation and function through vitamin D concentrations or in an independent manner by performing causality test.[36,37] The analyses on each SNP-vitamin D- platelet triple result in "independent" model, suggesting that the genetic effect on both phenotypes are statistically independent, or there is a limited power to detect the actual causal relationship using the dataset available.

Table 2. Relation between fibrinogen expression and SNPs in the vitamin D pathway.

SNP	rs10877012	rs6599638	rs2282679	rs1155563	rs7041	rs4588	rs3755967	rs17467825	rs1801222
Gene	*CYP27B1*	*C10orf88**	*GC (VDBP)*	*GC (VDBP)*	*GC (VDBP)*	*GC (VDBP)*	*GC (VDBP)*	*GC (VDBP)*	*CUBN***
Alleles	G/T	G/A	T/G	T/C	A/C	G/T	C/T	A/G	G/A
Minor allele	T	A	G	C	A	T	T	G	A
Minor allele effect	Decreasing	Increasing	Decreasing	Decreasing	Increasing	Decreasing	Decreasing	Decreasing	Increasing
MAF	0.35	0.43	0.27	0.30	0.59	0.27	0.27	0.27	0.63
Unstimulated	0.036	ns	ns	ns	ns	ns	ns	ns	Ns
Reactivity – CRP-XL	ns	ns	0.05	0.03	ns	0.02	0.04	0.05	0.05
Reactivity – ADP	ns	0.004	ns	ns	ns	ns	ns	ns	ns

The p-values of the association between fibrinogen binding in unstimulated samples and samples stimulated with CRP-XL or ADP and the different SNPs in the vitamin D pathway are displayed. The raw p-values are listed in the table. * The region harboring the open-reading frame 88 (C10orf88) on chromosome 10q26.13 ** Cubilin is important for vitamin D uptake into cells by binding vitamin D to VDBP. *(Abbreviations: ADP: adenosine 5'diphosphate; CRP-XL: cross-linked collagen-related peptide; CUBN: cubilin; MAF: minor allele frequency; orf:* open-reading frame; *VDBP: vitamin D binding protein)*

DISCUSSION

This is the first study that investigated the correlation between 25-hydroxyvitamin D concentrations and platelet activation and reactivity in a large cohort of healthy human subjects. We found an inverse correlation between 25-hydroxyvitamin D concentrations and platelet fibrinogen binding to the activated fibrinogen receptor integrin αIIbβ3 in unstimulated samples and after stimulation with the platelet agonist CRP-XL, which activates platelets through the collagen pathway, showing increased platelet activation and reactivity in those with low vitamin D concentrations.

This association was stronger for 25-hydroxyvitamin D concentrations ≤ 50nmol/L, and, when this concentration was used as a cut-off value, platelet fibrinogen binding to integrin αIIbβ3 in response to agonist ADP was also significantly associated. No differences were observed between 25-hydroxyvitamin D insufficiency and normal 25-hydroxyvitamin D concentrations, further supporting the presence of a threshold. Several SNPs in key genes contributing to the vitamin D pathway were significantly associated with platelet fibrinogen binding to integrin αIIbβ3 at baseline and/or in response to stimulation. Five out of 9 SNPs in the GC region of VDBP, were associated with fibrinogen binding in response to CRP-XL. Several associations between the SNPs, 25-hydroxyvitamin D and platelet function parameters were found, however, causality could not be formally confirmed.

We report a modest, but significant effect of vitamin D concentrations on platelet fibrinogen binding to the activated fibrinogen receptor integrin αIIbβ3. However, on a population level, small effects may have considerable impact. Myocardial infarction and stroke are the leading causes of death worldwide with 8 million deaths a year.[38] Cardiovascular diseases are multifactorial in origin and a combination of factors, such as atherosclerosis, plaque instability and platelet reactivity contribute to its development.[39] Associations with vitamin D concentrations exist, although these are poorly understood.[11]

Previously, only few studies investigated direct associations between vitamin D concentrations and platelet parameters in different diseases.[40-42] An association between low vitamin D concentrations and high-residual platelet reactivity in patients with cardiovascular diseases receiving anti-platelet therapy has been reported, as well as antithrombotic activities of vitamin D supplemented to hemodialysis patients.[40,42] These findings, together with our observations, may point out specific pathways that are functionally involved.

The strongest association with 25-hydroxyvitamin D was found for platelet fibrinogen binding in response to CRP-XL stimulation. Interestingly, 5 of the investigated SNPs in the GC region, coding for VDBP, also showed an association with platelet responses to CRP-XL. The platelet agonist CRP-XL signals through the collagen pathway to activate platelets and is dependent on phosphorylation of Signal Transducer and Activator of Transcription 3 (STAT3).[43] Interactions between vitamin D and JAK-STAT signaling pathways have been reported.[44,45] Olson and colleagues showed that vitamin D decreases STAT3 phosphorylation and this may explain increased platelet fibrinogen binding in response to CRP-XL in vitamin D deficient participants.[45] On the other hand, VDBP may also play a key role as it has been suggested that VDBP is involved in aspirin-resistant platelet activation.[46]

In contrast to platelet fibrinogen binding, platelet P-selectin expression was only associated with 25-hydroxyvitamin D in response to stimulation with CRP-XL in the individuals in which vitamin concentrations was below 50nmol/L. Moreover, there was no association between P-selectin expression upon CRP-XL stimulation and any of the SNPs. The potential involvement of P-selectin seems far less than fibrinogen, and it is likely that the study is underpowered to detect such small effects. Others have suggested that platelet functional responses may be differentially regulated, which may be a plausible explanation for the different observations.[47]

A causal relationship between the SNPs studied here, vitamin D concentrations and platelet function parameters could not be confirmed, and caution is warranted as it may be suggested that vitamin D concentrations are merely a reflection of overall health status. People with a low health status stay may have a more sedentary lifestyle, may stay more indoors, and, in combination with a poor dietary variation, this results in low vitamin D concentrations. Therefore, vitamin D could be an epiphenomenon and may not be causally involved. This is further strengthened by supplementation studies that yield little to no effect on cardiovascular diseases to date.[48-50]

In order to increase our understanding of the involvement of vitamin D in hemostasis, future studies should focus on the underlying pathways. A combination of many factors ultimately determines disease susceptibility and progression. It may be worthwhile to investigate the effects of vitamin D supplementation on specific factors such as platelet reactivity. An example is the ongoing PRECOVID trial, which investigates the effects of vitamin D supplementation on immune responses and platelet function in vitamin D deficient COPD patients.[51] Furthermore, causal inference analyses should be pursued in large cohorts as it is pivotal to know whether vitamin D acts as a mediator or is just an epiphenomenon. Insight in the exact mechanisms is important to further understand

the clinical consequences of vitamin D deficiency as well as to identify effective therapeutic opportunities.

Our study has a few limitations. One important limitation is the observational design of the study and this study must therefore be seen as a hypothesis-generating study. This cohort was relatively large and we tried to perform formal causal inference analyses, however as the effect is relatively small, the study may be underpowered to detect these subtle differences and causality could not be shown.

In conclusion, this study shows increased platelet fibrinogen binding to integrin αIIbβ3 in healthy volunteers with low vitamin D concentrations, particularly below 50 nmol/L. This observation may partially explain the association with thrombotic diseases and its seasonal variation. Further studies are needed to investigate the underlying mechanisms and causality of vitamin D in platelet function.

ACKNOWLEDGEMENTS

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Online supplement to

Platelet integrin αIIbβ3 activation is associated with 25-hydroxyvitamin D concentrations in healthy volunteers

Aleva FE, Tunjungputri RN, Li Y, Heijdra YF, Oosting M, Smeekens SP, Jaeger M, Joosten LAB, de Groot PG, Netea MG, de Mast Q, van der Ven AJAM

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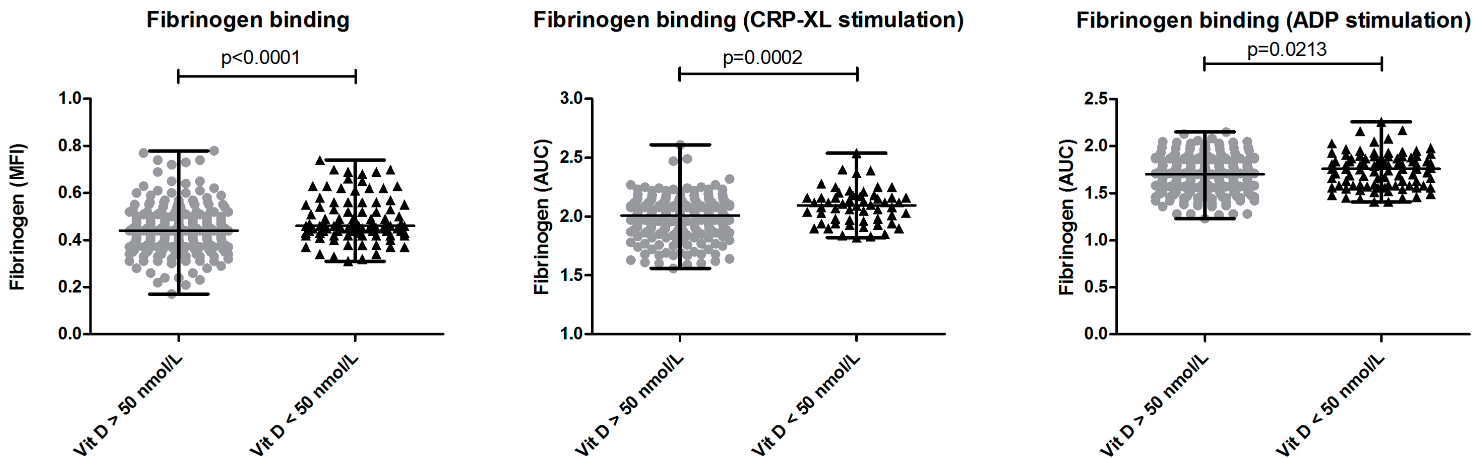
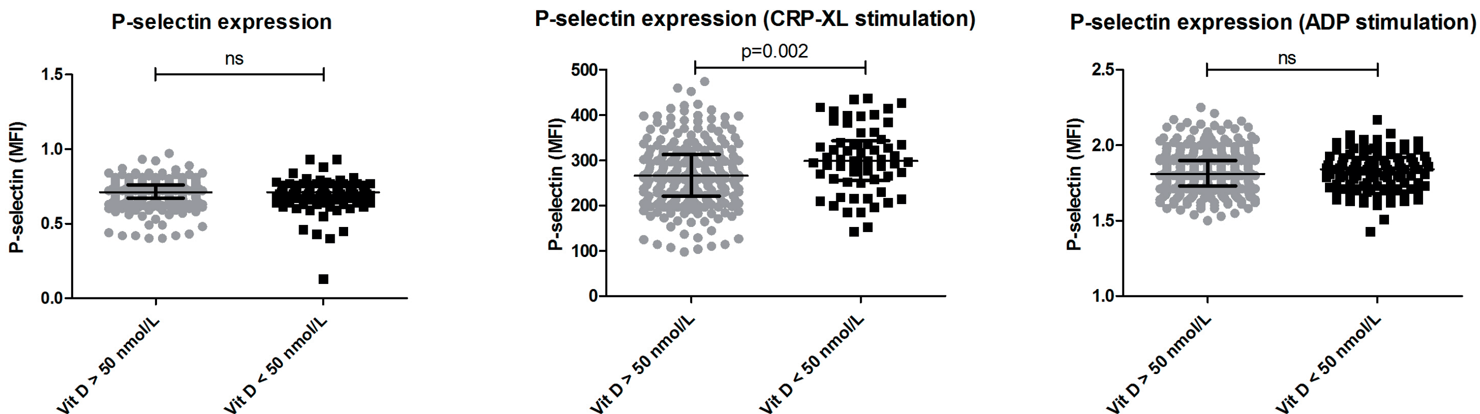


Supplementary Figure 1. Platelet fibrinogen binding in 25-hydroxyvitamin D deficient participants.
From left to right: Platelet fibrinogen binding to integrin αIIbβ3 in unstimulated samples and after stimulation with CRP-XL and ADP when using the threshold of 50nmol/L. *(Abbreviations: ADP: adenosine 5'diphosphate; AUC: area under the curve; CRP-XL: Cross-linked collagen-related peptide; MFI: mean fluorescence intensity)*



Supplementary Figure2. Platelet P-selectin expression in 25-hydroxyvitamin D deficient participants.
From left to right: P-selectin expression in unstimulated samples and after stimulation with CRP and ADP when using the threshold of 50nmol/L. *(Abbreviations: ADP: adenosine 5'diphosphate; AUC: area under the curve; CRP-XL:Cross-linked collagen-related peptide; MFI: mean fluorescence intensity)*

6

CHAPTER 7

Association between tobacco smoking and the number and function of monocytes and T cells in healthy humans

Aleva FE, Koenen HJPM, ter Horst R, Oosting M, Smeekens SP, Jaeger M, Joosten LAB, Netea MG, Heijdra YF, Joosten I, van der Ven AJAM

Submitted

ABSTRACT

Background

Tobacco smoking is still common and contributes to a variety of illnesses with significant mortality worldwide. A comprehensive analysis of the systemic effects of tobacco smoke on immune cell populations and function in humans is lacking.

Objectives

We investigated the association between smoking and the innate and adaptive immune cell populations in peripheral blood and its association with immune cell function and systemic inflammation in human subjects.

Methods

Cases (current- and former smokers) and controls (non-smokers) were identified among 500 healthy volunteers from the 500 Functional Genomics Cohort. Immune cell populations were determined in blood. *Ex vivo* cytokine production upon stimulation of whole blood and peripheral blood mononuclear cells and levels of circulating inflammatory mediators were compared between groups.

Results

Sixty-three current and 57 former smokers were matched to non-smokers. Current smokers showed increased numbers of circulating monocytes, mostly classical monocytes, and these correlated with pack years. Neutrophils, NK cells and B cells were not affected by smoking. Within the adaptive arm of the immune system, increased CD4 and CD8 positive central memory (CM) cells, CD4 positive effector memory (EM) cells and increased regulatory T cells were observed in smokers. Production of monocyte-derived cytokines after *ex vivo* lipopolysaccharide challenge was lower. Circulating concentrations of IL-6 and C-reactive protein positively correlated with pack years. No differences were observed in former smokers.

Conclusion

In healthy volunteers, tobacco smoking leads to a defective capacity of immune cells to respond upon microbial stimulation, yet it induces inappropriate systemic inflammation.

INTRODUCTION

Despite global initiatives for tobacco regulation, smoking is still common with an estimated 1 billion smokers worldwide.[1-3] Smoking impacts nearly every organ of the human body and contributes to numerous diseases with significant morbidity and mortality.[4-6] The most common diseases that are caused by smoking are chronic obstructive pulmonary disease (COPD), lung cancer, and cardiovascular diseases (CVD), most notably myocardial infarction.[5] Immune dysregulation by smoking is thought to play an important role in the development of these diseases. Moreover, strong associations between smoking and auto-immune diseases such as rheumatoid arthritis and Crohn's disease further emphasise the immune effects of smoking.[7-9]

Tobacco smoke consists of approximately 5,000 chemical components, some of which are very toxic, such as benzo-α-pyrenes and nitrogen oxides.[10-12] Many studies showed that tobacco smoke influences the human immune system, reviewed by,[13-15] affecting both innate and adaptive immunity.[16-20] Some tobacco smoke components have immunosuppressive effects, whereas other components induce inflammation.[7,21-23] Together, these factors determine the overall impact of tobacco smoke on the human immune system.

The aims of the various studies that investigated the effects of smoking vary widely, some aim to study specific tobacco smoke components,[23] some explore specific local and/or systemic immune cell lineages,[24-26] whereas others analyze effects in specific diseases in humans or in experimental models.[13,22] These studies often reported opposite effects of tobacco smoke, including up and down-regulation of natural killer cells,[18,27,28]regulatory T cells,[29,30] and release of pro- and anti-inflammatory cytokines.[17,19,31]

To the best of our knowledge, a comprehensive analysis of the systemic effects of tobacco smoking in healthy subjects, where smokers, former smokers and non-smokers are carefully matched, is lacking. These data are needed to identify and further study those pathways that are involved in the pathophysiology of smoking-related diseases at a systemic level.

The objectives of this study were: 1) to perform comprehensive analyses of the association between smoking and the variation of innate and adaptive immune cell populations in peripheral blood, and 2) to investigate its association with immune cell function and systemic inflammation in healthy human subjects. Pack years were used to investigate the effects of prolonged tobacco smoke exposure.

METHODS

Study design.

In this cross-sectional study, cases (current smokers and former smokers) and controls (non smokers) were identified among participants of the 500FG cohort.[32] This cohort is part of the Human Functional Genomics Project (HFGP) aimed at characterizing variations in human immune function.[33] The study design and population have been published previously.[32,34] In short, between August 2013 and December 2014 a total of 534 healthy human subjects of Caucasian origin were recruited at the Radboud university medical center, Nijmegen, the Netherlands. Forty-five participants were excluded, mainly because of the use of medication. Participants were scheduled for a study visit between 8-11 am to donate blood. After their visit, participants received an online questionnaire on dietary habits, lifestyle and disease history. Current smokers, former smokers and non-smokers were identified based on the answers to smoking-related questions in the questionnaire.

The study received ethical approval from the local Ethical Committee (NL42561.091.12, 2012/550) and was conducted according to the principles of the Declaration of Helsinki (version Oct 2008) and in accordance with the Dutch Medical Research Council involving Human Subjects Act. All participants gave written informed consent before blood was drawn.

Experimental procedures.

A detailed description of the experimental study procedures has been previously published.[32,34,35] In summary, myeloid and lymphoid immune cell populations were measured within 2-3 hours by 10-color flow cytometry (10-color Navios Flow cytometer, Beckman Coulter, Brea, California, USA). Cellular functional capacity in terms of cytokine production was measured in PBMCs and in whole blood. PBMCs were isolated by density centrifugation of diluted whole blood samples over Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden), followed by two washing steps in saline and final resuspension in RPMI 1640 medium supplemented with gentamicine, L-glutamine and pyruvate. Cells were stimulated for 24 hours or 7 days for measurement of cytokines.[34] For whole blood experiments, heparin blood was added to 48-wells plates and stimulated 1:5 with different stimuli for 48-hours. PBMCs were stimulated with LPS (Toll-like receptor (TLR) 4 ligand), Pam3Cys (TLR2 ligand), poly I:C (TLR3 ligand) for 24 hours or 7 days. Culture supernatants were stored in -20 Celsius degrees until ELISA measurements were performed. The concentrations of IL-1β, IL-6 and TNFα were determined in cell culture supernatants of the 24 hours PBMC stimulation assays. IL-17, IL-22 and IFNγ concentrations were determined in the 7 days PBMC culture supernatants. In addition,

the circulating concentrations of hsCRP, hsIL-6 and alpha-1-antitrypsin (AAT) were measured in EDTA plasma using ELISA.

Definition of cell subsets.

A comprehensive analysis was performed on different immunophenotypes of innate and adaptive immune cell populations. The cluster of differentiation (CD)-markers that were used for the identification of the different immune cell populations are described in Table E1 in the online data supplement.

Statistical analyses.

All statistical analyses were performed in the R programming language and on absolute cell numbers and not on ratios.

Paired analyses

Control samples were matched as closely as possibly to the smokers and ex-smokers based on age and gender. P-values were calculated using the two-sided Wilcoxon signed-rank test. This test was preferred over the parametric t-test since the cell counts were not always normally distributed.

Calculation of the effect of pack years

The rank-based regression method "Rfit",[36] implemented as a package in the R programming language, was used to evaluate the association between pack years and cell counts and cytokine production. The results were corrected for age, gender, body mass index (BMI), season and usage of oral contraceptives.

Multiple testing correction

P-values were corrected with multiple testing each time multiple p-values were evaluated simultaneously. This correction was achieved by calculating a False Discovery Rate (FDR) using the Benjamini-Hochberg FDR-correction.[37]

RESULTS

Within the 500FG cohort, 63 subjects were classified as current smokers and 57 subjects as former smokers. These subjects were individually matched for age and gender with non-smokers. Demographics of the study participants can be found in Table 1. Smokers, former smokers and non-smokers were similar in terms of age, gender, BMI and oral contraceptives use in women.

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Table 1. Demographics of study participants

Characteristics	Smokers N= 63	Controls N= 63	Former smokers N= 57	Controls N= 57
Gender, % male	42.9	42.9	56.1	56.1
Age, mean years (SD)	28 (12)	28 (12)	38 (17)	36 (15)
Pack years smoking, mean (SD)	5.2 (9)	0 (0)	not available	0 (0)
Oral contraceptive use, % of women	48.1	44.4	34.4	34.5
BMI, mean (SD)	22.7 (3.5)	23.2 (3.0)	22.8 (3.7)	23.2 (3.5)

Characteristics of study participants. (*Abbreviations; BMI: Body mass Index, IQR: Inter-quartile range, SD: Standard Deviation)*

Monocyte and lymphocyte populations are associated with current tobacco smoking.

Phenotypic analysis revealed that CD14 positive monocytes, including classical monocytes (CD14 high, CD16 negative), lymphocytes, including T cells (CD3 positive, CD56 negative), and double negative αβ cells (CD4 and CD8 negative) were increased in smokers, see Figure 1.

No differences between current smokers and non-smokers were observed in terms of neutrophils (median 3.99 x10^6 IQR 3.0x10^6 versus median 3.98 x10^6 IQR 2.6 x 10^6 p=0.37), natural killer (NK) cells (median 6700 IQR 7473 versus median 5730 IQR 4148, p=0.58), B cells (median 135224 IQR 127513 versus median 142069 IQR 100230 p=0.58) and several other immune cell populations (see Table E2 in the online data supplement). The increased monocyte population in current smokers seems to be primarily driven by the classical monocytes, since no significant differences were observed in the non-classical monocytes (CD14 high, CD16 positive) and the intermediate monocytes (CD14 and CD16 positive), see Figure 1. Regression analysis showed a positive correlation between CD14 positive monocytes and pack years, and this observation was similar for classical monocytes, suggesting a dose-effect relationship, see Figure 2.

Increased CD4 and CD8 positive Central Memory (CM) T cells and CD4 positive Effector Memory (EM) T cells in current smokers.

Further differentiation of specific subtypes of adaptive immune cells showed an important association with 'antigen experienced' T cells in current smokers. CM T cell numbers were higher in smokers in both CD4 and CD8 positive lineages (CD3+ CD4+ CD45RA- CD27+, and CD3+ CD8+ CD45RA- CD27+, respectively). Additionally, CD8 positive CM cells showed dose-effect relationships with pack years, while this was not seen in CD4 positive CM cells, see Figure 3. Central memory cells are known for their 'reactive' memory and are able to rapidly proliferate and differentiate into effector cells upon antigenic stimulation.[38] Also, an increase of CD4 positive EM (CD3+ CD4+

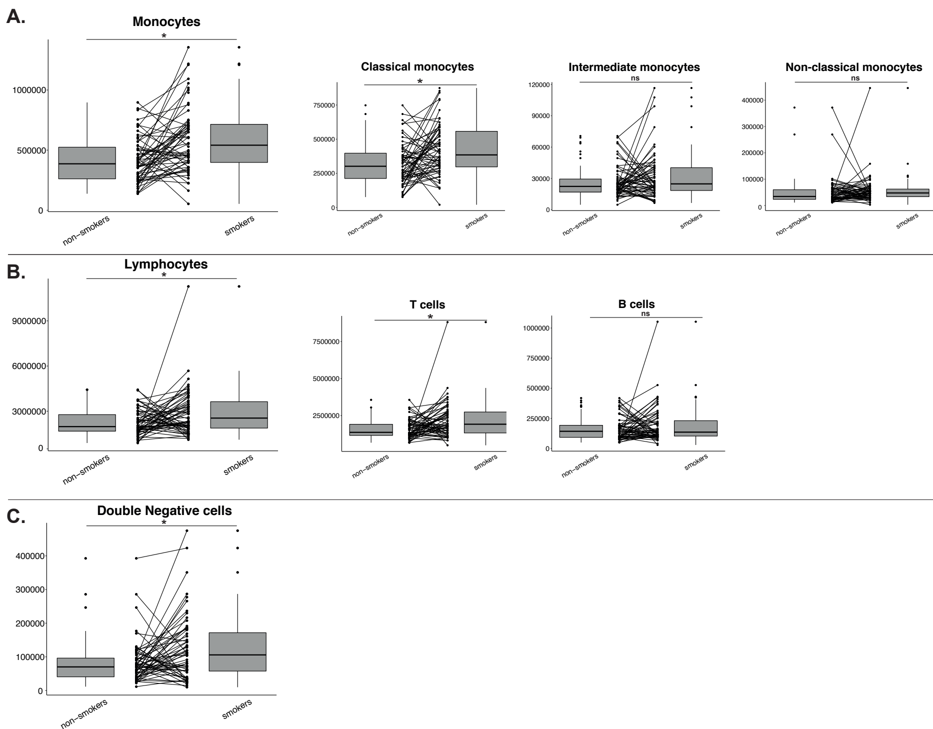

Figure 1. Several distinct immune cell populations are increased in smokers.
A. The increased numbers of monocytes in smokers (median 540897 IQR 316965 compared to non-smokers median 386200 IQR 263107, p=0.005) were mostly driven by increased classical monocytes (median 384952 IQR 260110 compared to median 301847 IQR 185030 in non-smokers, p=0.02), whereas no significant difference was observed between intermediate and non-classical monocytes (median 24782 IQR 21828 in smokers compared to non-smokers, median 22357 IQR 12477, p=0.37 and median 47683 IQR 28863 versus median 34301 and 36477, p=0.27, respectively) B. Smokers showed increased numbers of lymphocytes (median 2544103 IQR 1752675 compared to non-smokers median 1978454 IQR 1101036, p=0.036), primarily driven by T cells (median 1917035 IQR 1415245 compared to non-smokers median 1367745 IQR 751611, p=0.02). B-cells were not different between smokers and non-smokers (median 135224 IQR 127513 compared to non-smokers median 142069 IQR 100230, p=0.58). C. Increased alpha-beta double negative cells were increased in smokers (median 105769 IQR 113959 compared to non-smokers median 70087 IQR 55644, p=0.02)

CD45RA- CD27-) was observed in smokers, while this was not observed for CD8 positive EM cells (CD3+ CD8+ CD45RA- CD27-), see Figure 3. No correlation with pack years was observed for these T cell populations. Furthermore, an increase was seen in CD4 positive naive cells (CD3+ CD4+CD45RA+ CD27+) in smokers, data not shown.

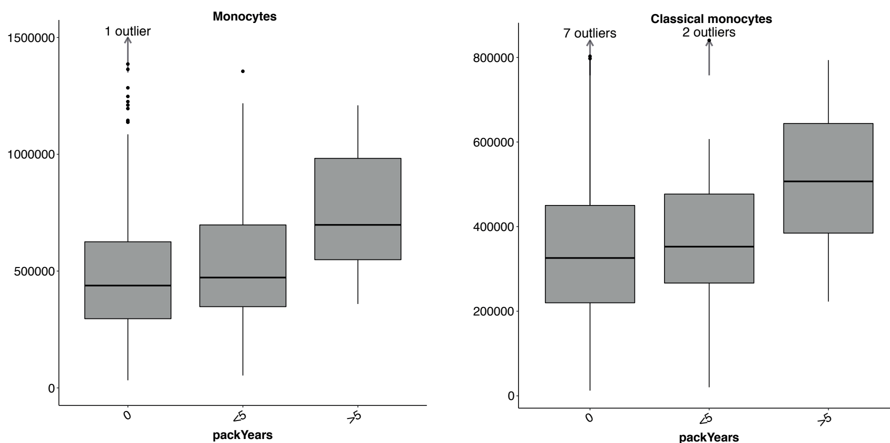

Figure 2. Monocyte populations according to pack years.
Both monocytes, and classical monocytes, were positively correlated with pack years suggesting a dose-effect relationship (regression, p=0.04 for both population). The data are categorized for display purposes only and were not categorized in the primary analyses).

Increased regulatory T-cells in current smokers compared to non-smokers.

Immune responses are delicately balanced in order to avoid infections on one hand and hyper-inflammation on the other. In addition to increased CM cells and increased CD4 positive EM cells in current smokers, an increase in regulatory T cells (CD3+ CD4+ CD25+ FOXp3+) was observed. Although controversial, regulatory T cells may be divided into thymic and peripherally differentiated regulatory T cells by expression of Helios. Both Helios positive and Helios negative regulatory T cells were increased in current smokers compared to controls, see Figure 4A.

Figure 3. CD4 and CD8 positive central memory (CM) T cells and CD4 positive effector memory (EM) T cells. *See page 131.*
A. Increased differentiation of CD4 positive CM and EM T cells in smokers compared to non-smokers (median 456462 IQR 421656 compared to non-smokers median 308106 IQR 200941, p=0.008 and median 54969 IQR 43178 versus 48221 IQR 27171, p=0.02, respectively). B. Increased differentiation of CD8 positive CM T cells (median 142537 IQR 121569 versus median 100849 IQR 72364, p=0.008), whereas no difference was observed in CD8 positive EM T cells (median 16410 IQR 14810 versus median 14227 IQR 13450, p=0.41) C. CM T cells showed a positive correlation with pack years, while this was not observed in EM T cells. The data on pack years are categorized for display purposes only and were not categorized in the primary analyses.

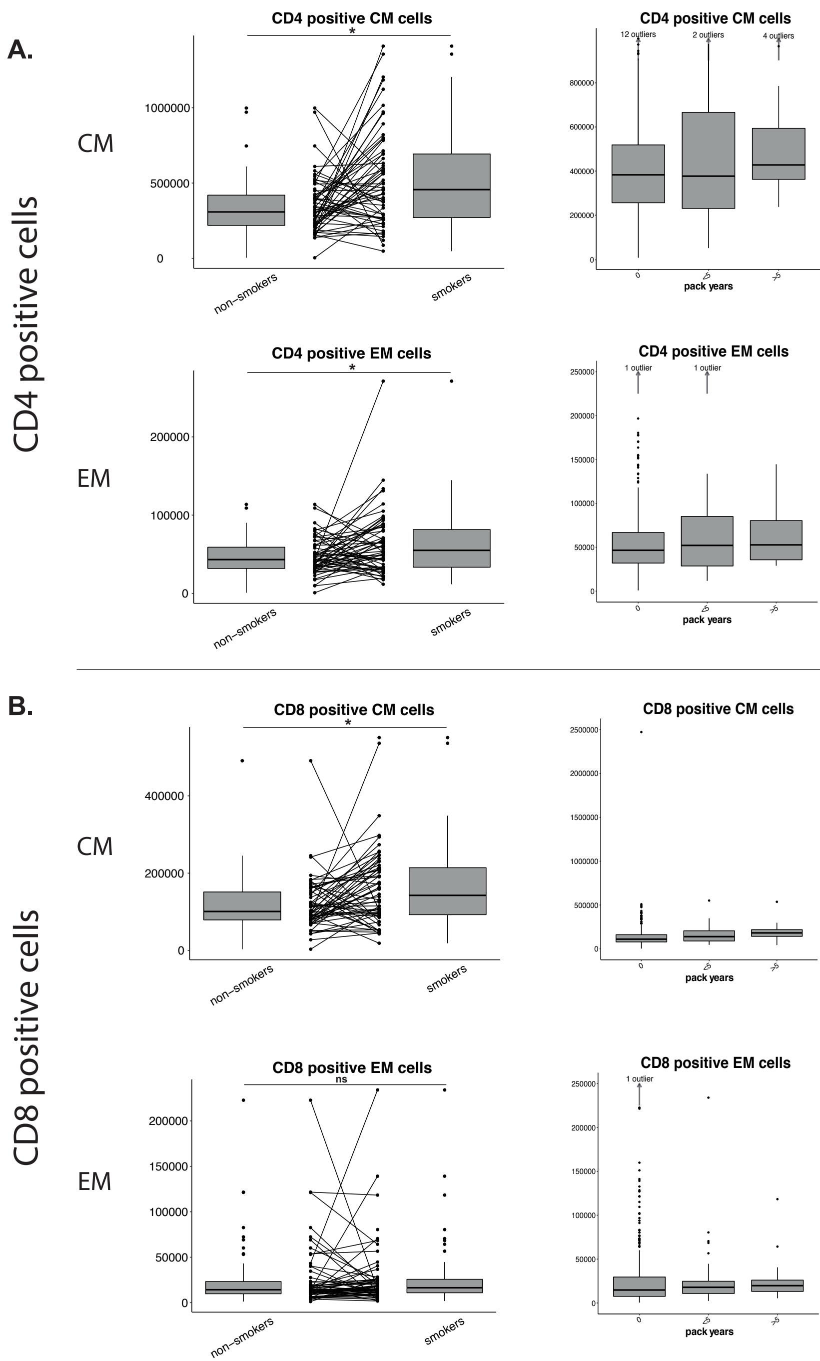

Figure 3. CD4 and CD8 positive central memory (CM) T cells and effector memory (EM) T cells.

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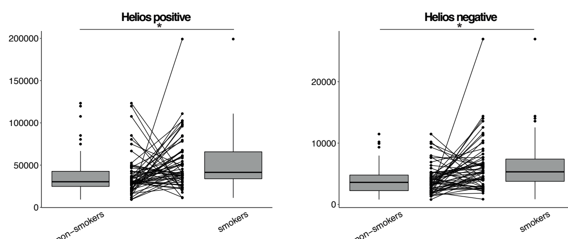
Further examination of the regulatory T cell compartment showed an increase in regulatory T cells positive for the activation marker Human Leukocyte Antigen - antigen D related (HLA-DR) in current smokers, and a positive correlation between this marker and pack years, see Figure 4B. Similarly, expression of proliferation/maturation marker Ki-67 on regulatory T cells was increased in current smokers and also showed a positive correlation with pack years, see Figure 4B. Together, these data show that in addition to increased CM and EM cells, also the regulatory T cell compartment is associated with current tobacco smoking.

A. Helios expression



B. Functional characteristics

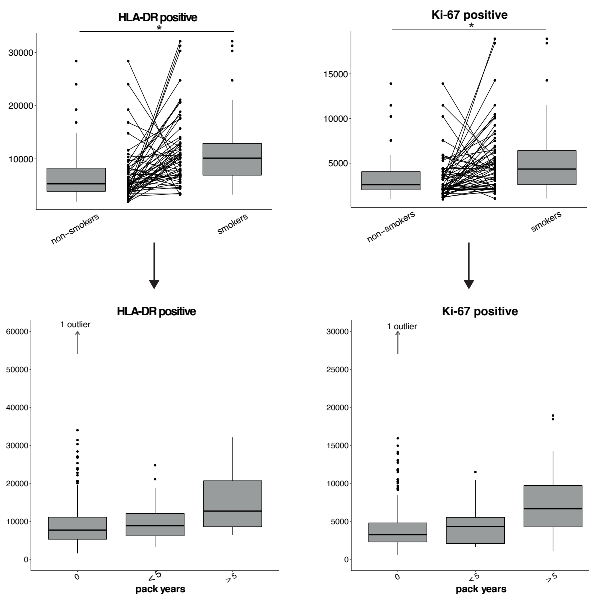


Figure 4. Increased expression of markers for activation and proliferation in regulatory T cells in smokers.

A. Increased expression of activation marker HLA-DR on regulatory T cells in smokers compared to non-smokers (median 10142 IQR 5971 versus 5309 IQR 4371, p=0.0003). B. Also, increased expression of proliferation marker Ki-67 was found in healthy smokers compared to healthy non-smokers (median 4342 IQR 3815 versus median 2581 IQR 2050, p=0.003) and both markers correlated with pack years (p=0.02 for HLA-DR positive regulatory T cells and p=0.009 for expression of Ki-67). The data are categorized for display purposes only and were not categorized in the primary analyses. (*Abbrevations: HLA-dr: Human Leukocyte Antigen - antigen D related; IQR inter-quartile range*)

B cells are not affected by smoking.

In contrast to monocytes and T cells, B cells were not associated with smoking in terms of absolute cell counts. The B cell compartment was divided in transitional B cells (CD19+ CD24++ CD38++), naive and memory B cells (CD19+ IgD+ IgM+ CD27- and CD19+ IgD+ IgM+ CD27+, respectively), and plasmablasts / plasma cells (CD19+ IgD- IgM- CD38++). Also several specific subsets of memory B cells were investigated, based on their differential expression of Ig-heavy chain isotypes, such as IgM and IgD.[39] No differences in these populations were observed between current smokers compared to non-smokers, see Figure 5. In contrast, regression analysis showed that class-switched memory B cells (IgD- IgM- CD38+ and CD27+) were positively correlated with pack years (p=0.01). The

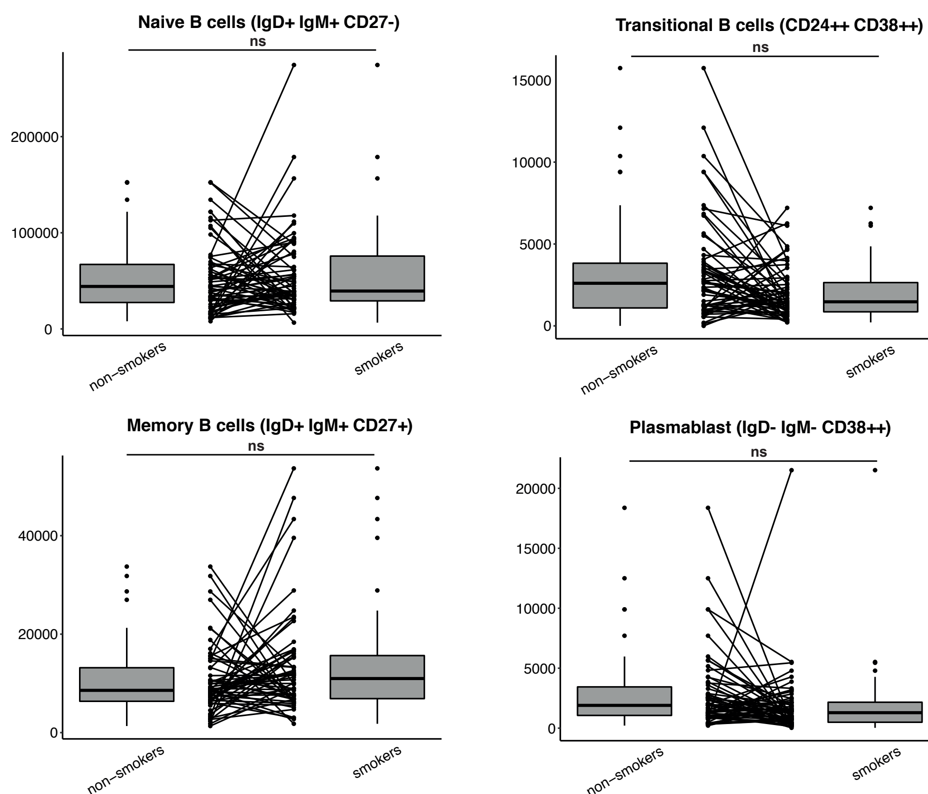


Figure 5. The effects of smoking on B cell populations.
No differences were observed with regards to naïve B cells (median 39390 IQR 46589 in smokers versus 44179 IQR 39594 in non-smokers, p=0.76), transitional B cells (median 1470 IQR 1786 in smokers versus median 2602 IQR 2731 in non-smokers, p=0.13), and memory B-cells (median 10983 IQR 8745 in smokers versus 8581 IQR 6816 in non-smokers, p=0.28) and plasma blasts/plasma cells (median 2296 IQR 2417 in smokers versus 2850 IQR 3048 in non-smokers, p=0.28). (*Abbreviations: CD: Cluster of differentiation; Ig: Immunoglobulin; IQR: inter-quartile range)*

lack of important effects is remarkable, as B cells have an important role in antigen specific adaptive responses.[40] Further definition and results on the different subpopulations that were investigated, including B cells, can be found in Table E3 in the online data supplement.

Tobacco smoking is associated with defective cytokine responses.

Several distinct innate and adaptive immune cell populations were upregulated in current smokers. In order to further investigate the association of smoking with immune cell function, we measured the cytokine production capacity upon stimulation and we measured the concentrations of several circulating mediators in cases and controls. Whole blood and PBMCs were stimulated with several TLR ligands and subsequently analysed for cytokine production. Whole blood samples and PBMCs were stimulated with LPS (TLR4 ligand) and Pam3Cys (TLR 2 ligand) for 48 hours (whole blood) and 24 hours and 7 days (PBMCs). In the whole blood LPS stimulation model, smokers had a trend towards lower TNFα production compared to non-smokers (2,92 pg/ml IQR 0,28 versus 3,00 pg/ml IQR 0,30, p=0.09 in the analyses corrected for multiple testing and p=0.010 in the uncorrected analysis). We also observed this trend in TNFα production in response to LPS stimulation of PBMCs from current smokers (TNFα 2,33 pg/ml IQR 0,44 versus 2,47 pg/ml IQR 0,40, p=0.09 in the corrected analyses and p= 0.015 in the uncorrected analyses).

The trend towards a decrease in the production of TNFα in current smokers was surprising since this is a monocyte-derived cytokine. Monocytes, including classical monocytes, were increased in current smokers, and these data may suggest that the decrease in cytokines results from a change in the function of monocytes.

We corrected cytokine levels for monocyte numbers and indeed lower production of the innate cytokine TNFα was observed in response to LPS stimulation in current smokers (p=0.0003 in whole blood and p=0.002 in PBMCs). Moreover, our analyses revealed that the production of IL-6, IFNγ and IL-1β was also decreased in whole blood from current smokers upon LPS challenge (p-values were all p=0.002), whereas in PBMCs this was only seen for Il-6 and IL-1β (p=0.01 and p=0.004, respectively). In response to stimulation with Pam3Cys, only TNFα was significantly lower in current smokers (p=0.01). These findings were further confirmed by the regression analysis of pack years and cytokines corrected for monocyte numbers that showed that both TNFα and IFNγ production capacity inversely correlate with pack years (both p=0.04), see Figure 6, while there was no significant impact on IL-6 and IL-1β production.

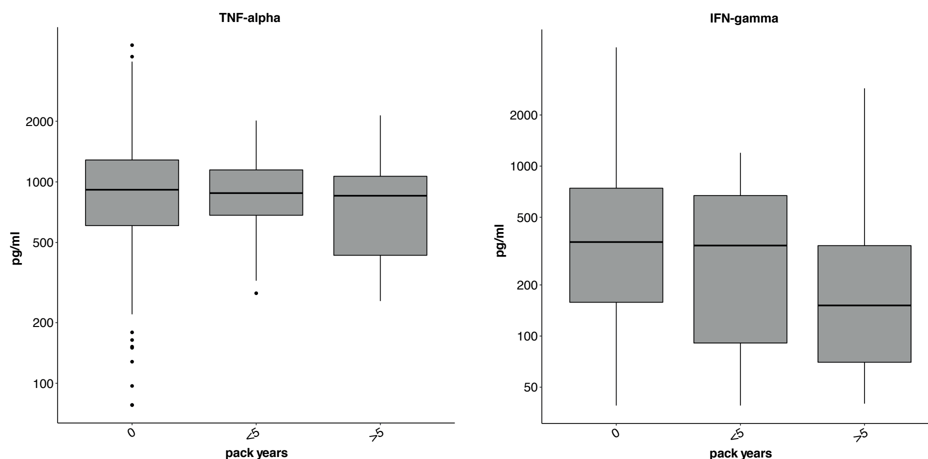


Figure 6. Relation between whole blood TNFα and IFNγ production in response to *ex vivo* LPS challenge and pack years.
A decrease in TNF-alpha and IFN-gamma was observed with increasing pack years. *(Abbreviations: IFN: Interferon; LPS: lipopolysaccharide; TNF: Tumor necrosis factor).*

Lastly, we determined plasma levels of circulating inflammatory mediators in cases and controls. No significant differences were detected for IL-6 (median 0,026 pg/ml IQR 0,54 versus 0,017 pg/ml IQR 0,25, p=0.82), hsCRP (median 2,76 pg/ml IQR 0,75 versus 2,86 pg/ml IQR 0,76, p=0.92), and AAT (median 9,10 pg/ml IQR 0,40 versus 9,17 IQR 0,18, p=0.41) in the paired analyses between smokers and non-smokers, see Figure 7A. Interestingly, a positive correlation between both IL-6 and hsCRP and pack years was observed in the regression analyses, p=0.03 and p=0.02, respectively, see Figure 7B. These data suggest that prolonged and/or intense smoking behaviour results in increased systemic inflammation.

Former smokers do not show the immune dysregulation that was observed in current smokers.

Interestingly, none of the observations above were found when former smokers were compared to non-smokers. Tables E4 – E6 in the online data supplement provides an overview of all the analyses that were performed and their findings. These findings may suggest that the immunodysregulation observed in current smokers normalizes after smoking cessation.

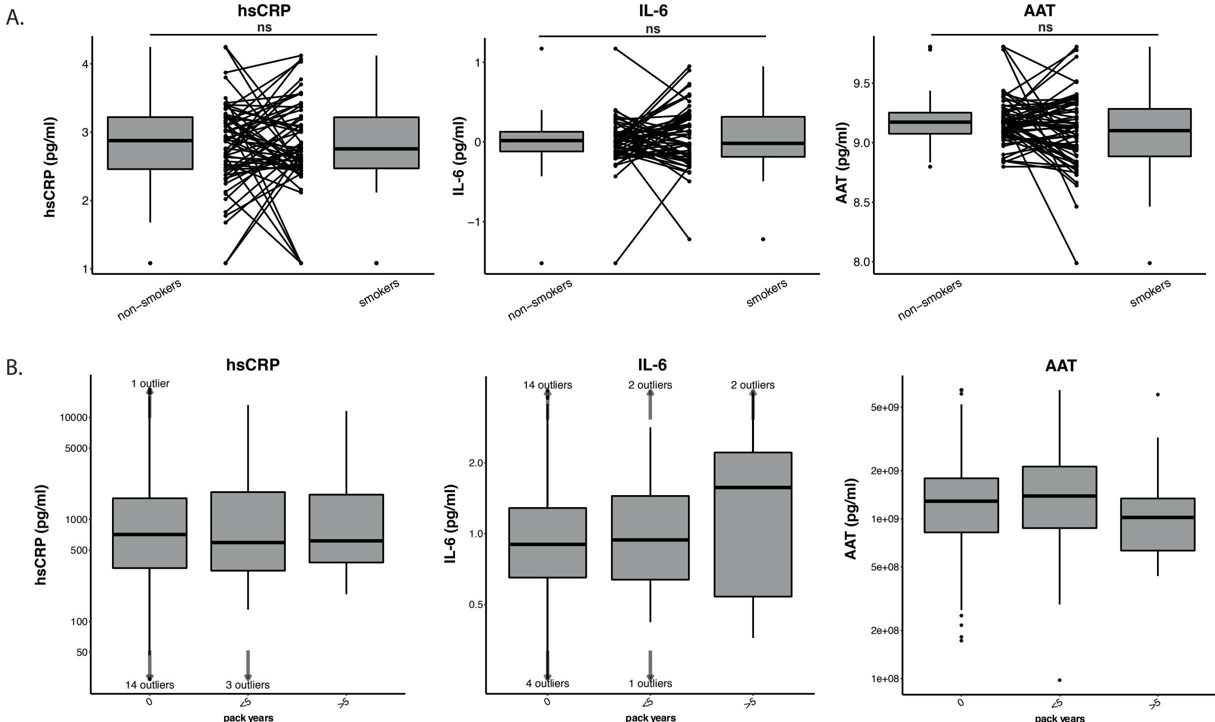


Figure 7. Circulating inflammatory mediators.
A. No differences were observed between smokers and non-smokers for inflammatory mediators hsCRP (median 2.76 pg/ml IQR 0.58 versus median 2.86 pg/ml IQR.0.25, p=0.92), IL-6 (median 0,026 pg/ml IQR 0,54 versus 0,017 pg/ml IQR 0,25, p=0.82) and AAT (median 9.10 pg/ml IQR 0.40 versus median 9.17 pg/ml IQR 0.18, p=0.41) B. Regression analyses showed a positive correlation between hsCRP and IL-6 and pack years, suggesting increased levels of these circulating pro-inflammatory mediators in smokers with increasing pack years. The data are categorized for display purposes only and were not categorized in the primary analyses. *(abbreviations:AAT: Alpha-1 antitrypsin; hsCRP: high sensitive C-reactive protein;IL-6: Interleukin-6; IQR: inter-quartile range)*

DISCUSSION

The present study provides a comprehensive analysis of the association between tobacco smoking and the variation of innate and adaptive immune cell populations, and its association with immune cell function in healthy human subjects. We report increased numbers of monocytes, mostly classical monocytes, in current smokers, whereas the production of the monocyte-derived cytokines after *ex vivo* LPS challenge was lower in this population. Furthermore, production capacity of TNFα and IFNγ were inversely correlated to pack years. Dysregulation of the adaptive arm of the immune system was reflected by increased CD4 and CD8 positive CM T cell populations, increased CD4 positive EM T cell populations and increased regulatory T cells in current smokers. Except for CD4 positive EM cells, these populations correlated with pack years. Interestingly, plasma IL-6 and hsCRP concentrations, markers for systemic inflammation, were not different between current smokers and non-smokers, but showed a positive correlation with pack years in the current smokers. B cells, on the contrary, showed no clear association with tobacco smoking. These findings indicate that tobacco smoking leads to a defective capacity of immune cells to respond upon microbial stimulation, yet it induces inappropriate systemic inflammation. No differences in immune cell populations were observed in former smokers, underlining the importance of smoking cessation.

Tobacco smoking is the second-leading risk factor for attributable mortality[3,5] and smoking-related diseases are not limited to the respiratory tract, but also develop at a distant level, like myocardial infarction.[4,6] In the present study, some intuitively conflicting observations co-occur that require further exploration. The previous publication on the 500FG cohort by our group[32] reported that smoking does not significantly affect host immune responses. In this previous study, however, only current smoking status (current-, former-, non-smokers) was taken into account, and subjects were not individually matched based on smoking. In addition, an in-depth analysis based on the number of pack-years has not been previously performed in the earlier study. In the present study, in which we carefully matched cases and controls, we report important systemic effects of smoking on the immune system.

In current smokers, increased numbers of monocytes, mostly classical monocytes, were observed that produce less pro-inflammatory cytokines, suggesting a defective capacity of monocytes to produce cytokines upon challenge with microbial ligands. This supports a previous publication by Noakes and colleagues, who compared immune response from cord blood of neonates between of maternal smokers and non-smokers during pregnancy, and report decreased TNFα, IL-6 and IL-10 in cord blood of neonates from maternal smokers.[41] Others investigated the effects of the tobacco component

nicotine and reported decreased cytokine production by monocytes via a mechanism dependent on nuclear factor-kappa B transcriptional activity.[42,43]

In addition, increased populations of double negative αβ cells and regulatory T cells were found in smokers. Aβ cells are mostly known from transplantation medicine where they act as suppressors of T cell proliferation and inducers of apoptosis.[44] Regulatory T cells, another suppressive cell type, showed increased activation and maturation in current smokers. Opposite effects regarding regulatory T cell numbers and function have been reported in the context of smoking, including up- and down-regulation of regulatory T cells.[13,45-47] These findings seem dependent on the tissue investigated (peripheral blood, bronchial alveolar lavage fluid) and the studied population, such as healthy (non) smokers or COPD patients.

T cell differentiation follows along a continuum from naive T cells to antigen experienced CM and EM T cells.[38,48,49] In smokers, CM T cells were increased both in CD4 and CD8 positive cell lineages and confirm previous studies that report an increase in these specific T cell populations in humans,[26,50,51] whereas this was not found in mice.[52] The correlation with pack years suggests that prolonged exposure contributes to their development. Upon antigenic stimulation, CM T cells can rapidly proliferate into effector T cells that migrate to the tissues to exert their pro-inflammatory effects.[38] The CD4 positive EM T cell population was higher in current smokers, but was not correlated to pack years. Increased EM T cell populations may result from antigenic stimulation of CM T cells in current smokers and seem less dependent on prolonged tobacco exposure.

Previous studies suggest that T cell differentiation is driven by antigen load and persistence in infectious diseases.[53-55] Indeed, several studies found alterations in airway colonization caused by tobacco smoke.[56,57] An important, yet unanswered, question is whether the defective capacity of immune cells in current smokers that was observed in this study contributes to these alterations and this requires further exploration.

In contrast, positive correlations between pack years and circulating concentrations of both IL-6 and hsCRP suggest that during prolonged and/or intense tobacco smoke exposure, progression into inappropriate systemic inflammation occurs.

These data are important as they contribute not only to our understanding of the systemic immunological consequences of smoking, but may help to identify pathophysiological mechanisms that result in smoking-related diseases. It is well known that not all subjects that are exposed to tobacco smoke will develop smoking-related diseases. Around 30-40% of smokers develop COPD,[58] suggesting that a combination of environ-

mental, genetic and intrinsic individual factors determine disease susceptibility and the clinical course of disease.[59-61]

Many of our observations show important similarities with studies in smoking-related diseases. In COPD circulating CD4 and CD8 positive cell populations have shown to be increased[30,62,63] and correlated with GOLD stage and disease exacerbations. Increased CD4 positive EM T cell populations have also been reported to correlate with markers of atherosclerosis, such as intima-media thickness and low-density lipoproteins, and these EM cells were higher in patients with stable angina and acute myocardial infarction.[64] Comprehensive analysis of immune cells in these diseases is pivotal to identify which mechanisms prevail in the development of smoking-related diseases and may identify those at risk.

Our study has also limitations. First, the design of the study is cross-sectional and must be seen as a hypothesis-generating study. Second, many participants are relatively young and have a low number of pack years. This may result in missing some of the true effects of tobacco smoke exposure on immune responses. Lastly, we do not know what substances our participants exactly smoke, especially since the Netherlands has a tolerance policy on cannabis. Despite these limitations, important conclusions can be drawn from this comprehensive analysis and these require further exploration.

In conclusion, smoking affects monocyte and T-cell populations and monocyte-derived cytokine production in healthy human smokers. The upregulation of pro-inflammatory cells and systemic inflammation is, however, associated by a defective response to bacterial stimulation, which suggest a deleterious combination of low-grade chronic inflammation and immunodeficiency. Comprehensive analyses of immune cell populations in smoking-related diseases may identify those pathways involved in pathophysiology of smoking-related diseases at a systemic level.

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Online supplement to

Association between tobacco smoking and the number and function of monocytes and T cells in healthy humans

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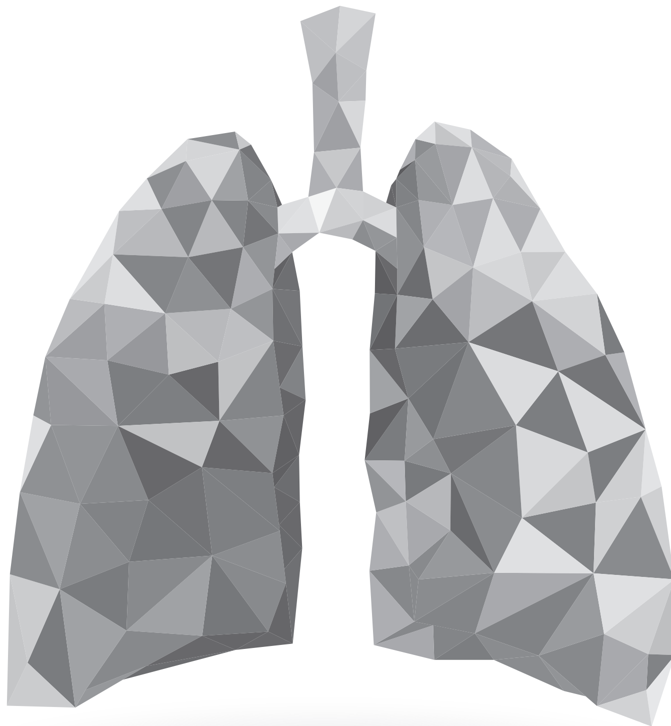

Submitted

Supplementary table 1. Definition of peripheral immune cell populations.

Immune cell phenotype	CD markers
Monocytes	CD3+ CD14+
Classical monocytes	CD3+ CD14++ CD16 -
Intermediate monocytes	CD3+ CD14++ CD16+
Non-classical monocytes	CD3+ CD14+ CD16++
T cells	CD3+ CD56-
Double negative αβ cells	CD3+ CD4- CD8-
Neutrophils	Based on FSC and SSC
NK cells	CD3- CD56+ CD16-
NKT cells	CD3+ CD56+
B cells	CD3+ CD19+
CD4+ central memory (CM) T cells	CD3+ CD4+ CD45RA- CD27+
CD8+ central memory (CM) T cells	CD3+ CD8+ CD45RA- CD27+
CD4+ effector memory (EM) T cells	CD3+ CD4+ CD45RA- CD27-
CD8+ effector memory (EM) T cells	CD3+ CD8+ CD45RA- CD27-
CD4+ naive T cells	CD3+ CD4+CD45RA+ CD27+
CD8+ naive T cells	CD3+ CD8+CD45RA+ CD27+
Regulatory T cells Helios+	CD3+ CD4+ CD25+ FOXp3+ Helios +
Regulatory T cells Helios-	CD3+ CD4+ CD25+ FOXp3+ Helios -
Activated regulatory T cells	CD3+ CD4+ CD25+ FOXp3+ HLA-DR+
Proliferating regulatory T cells	CD3+ CD4+ CD25+FOXp3+ Ki-67+
Transitional B cells	CD19+ CD24++ CD38++
Naive B cells	CD19+ IgD+ IgM+ CD27-
Memory B cells	CD19+ IgD+ IgM+ CD27+
Plasmablasts	CD19+ IgD- IgM- CD38++

The different CD-markers that were used to phenotype the peripheral immune cells are summarised. (*Abbreviations: CD; Cluster of differentiation, FOXp3: Forkhead box P3, FSC: Forward-scattered light, HLA-DR: Human Leukocyte Antigen – antigen D Related, Ig: Immunoglobulin, NK: Natural killer; NKT: Natural killer T; SSC: Side-scattered light)*

Supplementary table 2. Immune cell populations.

	smokers		non-smokers		
Population	median	IQR	median	IQR	P-value
Monocytes	540897	316965	386200	263107	0,005
CD4 Positive T cells	1302550,5	957422	849018	596037	0,020
T cells (CD3+ CD56-)	1917035	1415245	1367745	751611	0,020
Classical monocytes	384951,5	260111	301847	185030	0,020
Double negative αβ cells	105768,5	113959	70087	55644	0,021
Regulatory T cells	37812	34141	22947	22124	0,021
Lymphocytes	2544103	1752675	1978454	1101036	0,036
CD8 Positive T cells	520268	350565	448882	249713	0,092
NK bright (CD56++ CD16-)	8098	5892	8778	6847	0,166
Non-classical monocytes	47682,5	28863	34301	36477	0,270
Intermediate monocytes	24781,5	21828	22357	12477	0,373
Neutrophils	3985093	3029764	3984660	2618607	0,373
NKT cells	60769,5	97716	82038	80770	0,540
B cells	135224	127513	142069	100230	0,583
NK (CD56+ CD16-)	6699,5	7473	5730	4148	0,583
Double Positive cells (CD4+ CD8+)	9681	9582	9789	6710	0,981
NK diminished (CD56+ CD16+)	235317,5	189931	212292	158187	0,981
NK cells (CD3- CD56+)	249162	199997	239360	167915	0,981

The different immune cell populations in the general immune cell panel. (*Abbreviations: CD; Cluster of differentiation, IQR: inter-quartile range; NK: Natural killer; NKT: Natural killer T)*

Supplementary table 3. The different B-cell populations investigated in this analysis of peripheral blood immune cell phenotypes.

Population	smokers		non-smokers		P-value
	median	IQR	median	IQR	
IgM+ CD38++ CD27+	414	349	788	835	0,10
Transitional B cells (CD24++ CD38++)	1470	1786	2602	2731	0,13
Plasmablast (IgD- IgM- CD38++)	1290	1670	1899	2379	0,13
Transitional B cell (CD27- IgM+ CD24+ CD38high)	2391	2696	3420	3778	0,13
Class switched memory (IgD- IgM- CD38+ CD27+)	15178	14635	10320	13242	0,14
IgD+ CD5++	4964	5824	6939	8013	0,23
CD19+ CD20- Plasma blasts/cells	2296	2417	2850	3048	0,28
Natural effector (CD24+ CD38+ IgD+ IgM+)	10257	8403	8003	6407	0,28
Memory B cells (IgD+ IgM+ CD27+)	10983	8745	8581	6816	0,28
Class non switched memory (IgM+ CD38+ CD27+)	13200	9996	11097	9486	0,28
IgD- IgM- CD27-	3776	4495	3336	2853	0,28
IgD- IgM-	20970	21347	18257	15853	0,29
IgD- CD5+	23982	17375	20261	17641	0,29
CD19+ CD20+ B cells	84530	70335	69757	58504	0,44
IgD- IgM+ CD27-	1006	1140	711	862	0,44
CD24+ CD38+	75449	61197	61820	51338	0,46
IgD+ CD5+	55164	51446	49090	43257	0,46
IgM-	17275	16640	15025	17675	0,46
IgD+ IgM-	3398	5202	4733	5457	0,46
CD24+ CD38+ CD27+ IgM+	2604	2409	2197	1881	0,53
IgD+ IgM+	53628	49239	48701	46564	0,54
IgM+ CD27-	40884	46810	44334	39413	0,75
IgD- CD5++	799	1058	855	641	0,76
Naive B cells (IgD+ IgM+ CD27-)	39390	46589	44179	39594	0,76
Mature naive CD24+ CD38+	31530	43359	34519	31107	0,77
IgD- IgM+	4270	4625	3373	3902	0,80
IgM only memory (IgD- IgM+ CD27)	3018	3057	2640	2748	0,87

The different immune cell populations in the general immune cell panel. (*Abbreviations: CD: Cluster of differentiation; Ig: Immunoglobulin; IQR: inter-quartile range)*

Supplementary table 4. The different cell populations investigated in peripheral blood from former smokers.

	former smokers		non-smokers		
Population	**median**	**IQR**	**median**	**IQR**	**p-value**
B cells (CD19 +)	130634	88886	141481	120926	0.90
CD19 + CD20 - Plasma blasts/cells	1645	1659	1628	2937	0.84
CD19 + CD20 + B cells	69943	54755	70713	75796	0.84
CD24 + CD38 +	63031	47385	65458	74677	0.84
CD24 + CD38 + CD27 + IgM +	2002	1778	2069	2481	0.84
CD4 + CD25high Regulatory T cells	36057	19650	27773	30618	0.81
CD4 + CM CD45RA - CD27 +	429826	236528	388827	313478	0.32
CD4 + Effector CD45RA + CD27 -	2157	5395	822	4331	0.48
CD4 + EM CD45RA - CD27 -	57894	40525	49066	33294	0.26
CD4 + Naive CD45RA + CD27 +	500186	541467	414912	439557	0.28
CD4 + T cells	1046763	682681	1036096	704738	0.81
CD8 + CM CD45RA - CD27 +	124367	88474	110615	103452	0.52
CD8 + Effector CD45RA + CD27 -	23960	41265	13399	23746	0.26
CD8 + EM CD45RA - CD27 -	24092	29187	15911	22293	0.46
CD8 + Naive CD45RA + CD27 +	227739	248943	241166	190799	0.46
CD8 + T cells	455006	265260	419251	210053	0.90
Class non switched memory (IgM + CD38 + CD27 +)	10127	7051	10902	10077	0.84
Class switched memory (IgD - IgM - CD38 + CD27 +)	13311	9000	8500	14388	0.84
Classical monocytes (CD14 + +CD16 -)	359651	290790	269060	351510	0.90
Double Negative (CD4 - CD8 -)	60466	71999	65237	53607	0.90
Double Positive (CD4 + CD8 +)	11404	6173	8925	9047	0.90
IgD - CD5 +	21222	13307	17322	21142	0.84
IgD - CD5 + +	671	525	807	990	0.84
IgD - IgM -	19029	12761	14516	19208	0.84
IgD - IgM - CD27 -	3448	2089	2615	3336	0.84
IgD - IgM +	3372	1802	3226	3903	0.87
IgD - IgM + CD27 -	903	989	803	1298	0.84
IgD + CD5 +	45417	33608	49439	51794	0.84
IgD + CD5 + +	3625	4844	4469	7324	0.84
IgD + IgM -	4344	4648	3798	4736	0.84
IgD + IgM +	43549	38880	50164	55577	0.84
IgM -	17727	10998	14105	15412	0.84
IgM + CD27 -	36104	31556	40790	48444	0.84
IgM + CD38 + + CD27 +	416	443	478	740	0.84
IgM only memory (IgD - IgM + CD27)	2482	1615	2413	2725	0.84
Intermediate monocytes (CD14 +CD16 +)	25489	18681	26359	19204	0.90

Supplementary table 4. The different cell populations investigated in peripheral blood from former smokers. (continued)

Population	former smokers		non-smokers		p-value
	median	IQR	median	IQR	
Lymphocytes	2177388	1013214	2150268	1320234	0.90
Mature naive CD24 + CD38 +	28319	24272	28796	41820	0.84
Memory B cells (IgD + IgM + CD27 +)	7902	6225	8352	7226	0.87
Monocytes (CD14 +)	439566	297493	402252	432023	0.90
Naive B cells (IgD + IgM + CD27 -)	35235	32190	40455	39012	0.84
Natural effector (CD24 + CD38 + IgD + IgM +)	7319	6421	7605	6907	0.84
Neutrophils	4641964	2362811	4446735	2683722	0.90
NK (CD56 + CD16 -)	6396	6477	5102	5706	0.81
NK bright (CD56 + + CD16 -)	10243	7118	10315	8834	0.90
NK cells (CD3 - CD56 +)	247562	163109	242405	175158	0.90
NK dim (CD56 + CD16 +)	228522	159153	211024	166369	0.90
NKT cells (CD3 + CD56 +)	75971	88490	82165	120920	0.92
Non-classical monocytes (CD14 + +CD16 +)	41958	38604	42701	36467	0.90
Plasmablast (IgD - IgM - CD38 + +)	1009	1443	1255	1964	0.84
Ki-67 + CD4 + Tconv	16489	12279	13617	16984	0.31
Ki-67 + CD4 + Regulatory T cells	3857	3833	3353	2122	0.31
Ki-67 + CD8	7388	5350	6778	9017	0.77
Ki-67 + DN(CD4 -CD8 -)	40089	54792	42096	56767	0.77
Ki-67 + DP(CD4 +CD8 +)	681	949	410	916	0.26
T cells (CD3 + CD56 -)	1745891	926820	1591205	867307	0.81
Transitional B cell CD27 - IgM + CD24 + CD38high	2351	2592	2488	3581	0.84
Transitional B cells (CD24 + + CD38 + +)	1724	2011	1723	2683	0.84
Regulatory T cells CD25 + CD127low	44191	32339	33788	24706	0.32
Regulatory T cells CD45RA -	26503	13579	21242	15924	0.26
Regulatory T cells CD45RA +	21115	23414	18148	17189	0.51
Regulatory T cells FOXP3 + Helios -	5150	4304	4047	3605	0.26
Regulatory T cells FOXP3 + Helios +	41536	22001	36566	20793	0.31
Regulatory T cells HLA -DR +	10274	7196	7523	5935	0.26

The different immune cell populations in former smokers. (*Abbreviations: CD: Cluster of differentiation; Ig: Immunoglobulin; IQR: inter-quartile range; NK: Natural killer; NKT: Natural killer T)*

Supplementary table 5. The circulating mediators in peripheral blood from former smokers.

Circulating mediators	former smokers		non-smokers		p-value
	median	IQR	median	IQR	
Adiponectin	6.73	0.24	6.63	0.30	0.44
Leptin	3.91	0.63	3.73	0.84	0.44
IL-18	1.69	0.96	1.48	0.96	0.51
IL-1Ra	2.15	0.25	2.20	0.17	0.61
hsCRP	2.76	0.59	2.71	0.53	0.87
AAT	9.04	0.42	9.11	0.35	0.89
IL-18 binding protein	4.41	0.17	4.38	0.16	0.89
IL-18	2.18	0.24	2.17	0.12	0.92
IL-1β	0.92	0.50	0.71	0.50	0.92
VEGF-A	1.48	0.25	1.46	0.26	0.92
IL-6	0.01	0.29	0.02	0.31	0.92
Resistin	4.13	0.21	4.14	0.23	0.92

The circulating mediators in peripheral blood from former smokers. (*Abbreviations: AAT: Alpha-1 Antitrypsin; hsCRP: high-sensitive C-reactive protein; IL: Interleukin; IQR: Inter-quartile range; Ra: receptor antagonist; VEGF: Vascular Endothelial Growth Factor)*

Supplementary table 6. *Ex vivo* cytokine production upon stimulation with TLR-ligands in former smokers.

Sample	TLR-ligand	Cytokine (pg/ml)	formersmokers		non-smokers		p-value (uncorrected)
			median	IQR	median	IQR	
PBMC	LPS	IL-1β	3.30	0.38	3.39	0.35	0.31
PBMC	LPS	IL-6	3.81	0.25	3.88	0.26	0.04
PBMC	LPS	TNFα	2.35	0.58	2.39	0.37	0.49
PBMC	LPS	IL-1β	2.34	0.83	2.27	1.12	0.24
PBMC	LPS	IL-6	3.43	0.88	3.43	1.19	1.00
PBMC	LPS	TNFα	1.89	0.13	1.89	0.09	0.31
Whole blood	LPS	IFNγ	2.55	0.54	2.57	0.62	0.45
Whole blood	LPS	IL-1β	3.31	0.22	3.31	0.21	0.91
Whole blood	LPS	IL-6	3.93	0.18	3.97	0.20	0.27
Whole blood	LPS	TNFα	2.96	0.39	3.02	0.26	0.25
PBMC	Pam3Cys	IL-6	3.92	0.26	4.02	0.27	0.07
PBMC	Pam3Cys	TNFα	2.60	0.62	2.61	0.41	0.58
PBMC	Poly I:C	IL-1β	1.73	0.29	1.76	0.50	0.14
PBMC	Poly I:C	IL-6	2.55	0.61	2.66	0.56	0.13
PBMC	Poly I:C	TNFα	1.89	0.12	1.89	0.16	0.86

Ex vivo cytokine production. (*Abbreviations: IL: Interleukin; IQR: Inter-quartile range; LPS: Lipopoly-saccharide; PBMC: Peripheral Blood Mononuclear Cells; TLR: Toll-like receptor; TNFα: Tumor Necrosis Factor-α)*

CHAPTER 8

Summary and Discussion

SUMMARY AND DISCUSSION

Comorbidities play a significant role in COPD and especially cardiovascular comorbidities have important health consequences.[1-4] About half of the admissions of patients with COPD are accounted for by CVD.[3,5] COPD is a chronic inflammatory condition and although the crosstalk between inflammation and coagulation is well known, this crosstalk has been poorly studied in COPD. Therefore, the main aim of this doctoral thesis was to investigate the interaction between inflammation and coagulation in patients with COPD and to investigate the potential to modulate their interaction.

Although the presence of pulmonary embolism in patients with an acute exacerbation of COPD (AE-COPD) have been repeatedly reported, the exact prevalence still needed to be determined. A systematic reviewed about prevalence for pulmonary embolism in patients with an AE-COPD was therefore performed (**Chapter 2**). This is followed by studies that investigated pathophysiological mechanisms in which inflammation and coagulation interact in COPD (**Chapters 3-4**). Platelet function was studied both in stable disease and in AE-COPD, since platelets play a pivotal role in the development of cardiovascular diseases. **Chapter 5** investigates the role of STAT3 in platelet function. Recent work suggested that STAT3 is involved in the crosstalk between inflammation and coagulation, via activation of platelets through the collagen pathway, however, further confirmation of its relevance in humans was warranted.

It is increasingly appreciated that vitamin D is able to modulate immune responses. For this reason, vitamin D may be used as an additional treatment in COPD patients. Currently, the effects of vitamin D supplementation on exacerbation frequency in COPD is investigated.[6] The association between vitamin D and platelet function is less investigated. The 500FG, a large cohort study that is part of the Human Functional Genomics Project (HFGP, http://www.humanfunctionalgenomics.org/site/) that is aimed at characterizing variations in immune function and platelet function,[7,8] offered the opportunity to investigate the association between vitamin D and platelet function. Platelet function assays were performed in 500 healthy volunteers and its association with vitamin D is reported in **Chapter 6**. Lastly, the primary risk factor for COPD is tobacco smoking. Long-term tobacco smoke exposure causes not only an inflammatory response in the lungs, but also at a systemic level. The latter may contribute to the non-pulmonary complications of COPD such as CVD. Immune dysregulation because of smoking has been studied extensively, however, a comprehensive analyses of the systemic effects of tobacco smoke on peripheral immune cell populations in humans was lacking. Using the data from the 500FG cohort, current smokers and former smokers were carefully

matched to non-smokers and differences in immune cells were investigated (**Chapter 7)**.

Pulmonary embolism is common in patients with unexplained AE-COPD.

The risk for myocardial infarction and stroke in COPD patients is extensively studied and many studies report a significantly increased risk in this population.[9-11] The risk for these thrombotic complications increases even further during episodes of increased inflammation, such as seen in acute exacerbations of COPD,[9-12] suggesting that inflammation contributes to their development. It has also been suggested that venous thromboembolism is increased in COPD,[13,14] however, the prevalence of VTE during acute exacerbations is less well studied. To address this caveat, we have systematically reviewed the current literature in order to estimate the overall prevalence of PE during AE-COPD.

In **Chapter 2**, we report that PE is common in AE-COPD and our meta-analysis showed an overall pooled prevalence of PE in 16.1% (95%-Confidence Interval 8.3-25.8%) of patients with unexplained AE-COPD. Moreover, two-thirds of these emboli were found to be located more proximal than subsegmental, indicating that the majority of these embolisms have important clinical consequences. These embolisms require anticoagulant treatment and should therefore be recognized.[15] Of the 7 included studies, 6 additionally reported on the prevalence of deep venous thrombosis and our meta-analysis showed a pooled prevalence of 10.5% (95%-Confidence interval 4.3-19.0%) in AE-COPD. Our study confirms findings from a previous review by Rizkallah and colleagues who reported a prevalence of 19.9%.[16] Since publication of this previous review 5 relevant studies have been published and these were included in our meta-analyses.

The risk for venous thromboembolism in AE-COPD is substantial, especially in comparison to other inpatient populations that show a prevalence of 5.7 – 6.0%.[17,18] These findings merit clinical attention as PE has a 28-day case fatality rate of 21.2% in patients with COPD.[19] One-third of cases of PE in AE-COPD was limited to isolated subsegmental PE. Unfortunately, we were unable to relate outcomes to embolus localization. The clinical relevance of these small emboli is debated as studies report opposite effects.[15,20,21]

Future studies are needed to investigate the clinical relevance of these small emboli in COPD. In order to improve early identification of PE in AE-COPD, we identified several clinical signs that are suggestive of the presence of PE in AE-COPD, in particular pleuritic chest pain and signs of cardiac failure. With this study, we aim to increase awareness on the venous thrombotic complications of COPD and to underline the clinical relevance of the interaction between inflammation and coagulation in COPD.

Platelet function and plasmatic coagulation in COPD patients.

Inflammation and coagulation are highly integrated biological systems and to further explore the relevance of this crosstalk in COPD, several observational studies were performed to investigate the pro-coagulative mechanisms. As previously stated, the risk for myocardial infarction and stroke are strongly increased in COPD.[9-11] Platelets play a pivotal role in development of arterial thrombotic events, but are also increasingly recognized as immune cells.[22,23] Platelet function was studied both in stable COPD patients and in exacerbated COPD patients (**Chapters 3-4**). In **Chapter 3**, we investigated platelet-monocyte interaction and platelet function in 30 stable COPD patients and 25 control subjects. We showed increased platelet-monocyte interaction in stable COPD patients, in absence of increased platelet activation, platelet hyper-reactivity and activation of plasmatic coagulation. This study confirms a previous study by Maclay and colleagues that also found increased platelet-monocyte interaction,[24] however we did not find platelet hyper-reactivity. These findings are important as platelet-monocyte complexes contribute to development of CVD by augmented development of atherosclerosis and involvement in plague instability.[22,25,26]

In addition, these findings add to the current debate on the potential benefits of anti-platelet therapy in patients with COPD.[27,28] Since we could not show increased platelet activation and hyper-reactivity the benefits of anti-platelet therapy may seem limited from a pathophysiological point-of-view. However, P2Y12 receptor blockers, such as clopidogrel and ticagrelor, decrease platelet-monocyte interaction and systemic inflammation.[29,30] On the contrary, for the COX-inhibitor aspirin these effects are not clearly demonstrated.[31-33] This suggests that beneficial effects of the platelet-inhibitors in COPD may be different, depending on their mechanism of action.

Platelet-monocyte complex formation is often used as a surrogate marker for platelet activation, however, platelets and monocytes can interact in various ways.[22,23,34] The best understood interaction occurs via binding of P-selectin on platelets to PSGL1 on monocytes and concurrent binding of fibrinogen to GPIIb/IIIa on platelets and MAC-1 on monocytes, and these interactions are facilitated by platelet activation.[35] In stable COPD we did not observe increased expression of P-selectin on platelets, nor did we observe increased fibrinogen binding. In absence of these platelet function alterations, we explored the role of monocyte activation for this interaction in AE-COPD (Chapter 4). Platelets can interact with activated monocytes via their receptor GPIbα that directly interacts with MAC-1 on monocytes.[36] MAC-1 increases with increased monocyte activation and some suggest that during inflammation this interaction is crucial for platelet-monocyte interaction.[36,37] We hypothesized that MAC-1 plays an important

role in platelet-monocyte interaction in COPD patients and this hypothesis is further explored in **Chapter 4.**

Apart from platelet function, several markers for plasmatic coagulation were also assessed in stable COPD patients. In contrast to previous findings in asthma patients,[38] we did not find a hyper-coagulable state in stable COPD patients. In comparison to control subjects, no differences in clotting time, total amount of thrombin generated, maximum thrombin generation and plasma d-dimer were found. Majoor and colleagues found an important link between hypercoagulability in asthma, and use of glucocorticoids and asthma severity were identified as independent risk factors.[39] Interestingly, glucocorticoids are also regularly used by COPD patients, however, mostly in the context of acute exacerbations of COPD. This factor may play an important role in the risk of thrombotic events in COPD patients and requires further investigation.

In **Chapter 4,** we investigated platelet-monocyte interaction and platelet function in exacerbated COPD patients and we explored whether MAC-1 expression on monocytes correlated with platelet-monocyte interaction. Twenty-two patients were included, of which two died during their hospital admission and one patient retracted informed consent. In contrast to our hypothesis, this study showed lower platelet-monocyte interaction in COPD patients during AE-COPD compared to 6 to 10 weeks post-exacerbation. This finding challenges a previous study that suggested increased platelet-monocyte interaction in AE-COPD.[24] Maclay and colleagues investigated platelet-monocyte interaction in 12 patients with AE-COPD and compared them to 18 stable COPD patients.[24] In contrast to their study, we assessed platelet-monocyte interaction during and after AE-COPD in order to limit differences between the patients groups, and this may explain the contradictory nature of the observations. MAC-1 expression, on the contrary, was increased during AE-COPD and inversely correlated with platelet monocyte interaction post-exacerbation. These findings suggest that MAC-1 does not play a major role in platelet-monocyte interaction in COPD.

Similar to our previous study in stable COPD patients, no clear alterations in platelet function were observed. Some clinical studies showed increased survival of COPD patients that are treated with platelet-inhibitors.[27,28] Platelets also exhibit important immunological functions[23] and these may influence disease pathophysiology in COPD. Furthermore, Harrison and colleagues reported increased survival in COPD patients treated with platelet-inhibitors, while this was not related to cardiovascular deaths.[27]

An important limitation of this study is the small sample size. COPD is a very heterogeneous disease and many studies aim to differentiate between different COPD pheno-

types.[4,40] Vanfleteren and colleagues have recently shown that COPD patients can be grouped in five different clusters based on comorbidities, thereby distinguishing COPD patients that are at risk for CVD from other clusters of comorbidities.[40] To date, no clear distinction can be made from a immunological perspective and this may have affected our observations. Several confounding factors were assessed, such as frequent versus infrequent exacerbator phenotypes and the effects of smoking, however, these factors did not alter our observations. Together, these findings do not explain the increased risk for CVD during AE-COPD and further research on the exact mechanisms is warranted.

Signal Transducer and Activator of Transcription 3 (STAT3) is involved in glycoprotein VI (GPVI)-mediated platelet activation.

In **Chapter 5**, we investigated the involvement of STAT3 in platelet activation. STAT3 is important for transduction of a variety of cell signals, including signals involved in inflammation.[41,42] In response to cytokines and growth factors, STAT3 is phosphorylated and translocated to the nucleus for transcription of specific genes. Differentiation of T helper-17 (Th-17) cells is dependent on STAT3 and is essential for host defense against fungal infections.[43,44] Additionally, STAT3 function is associated with several in inflammatory diseases, including COPD.[43,45,46]

Recent evidence suggested that STAT3 function in platelets may provide a potential new link between inflammation and coagulation.[47,48] Zhou Zhou and colleagues showed that signal STAT3 is involved in platelet activation in response to collagen via platelet receptor GPVI.[47] The functional relevance of this crosstalk in humans is less well studied and understood. To investigate its importance in humans, we studied patients with dysfunctional STAT3 and healthy human subjects. Patients with autosomal dominant Hyper IgE syndrome have dominant-negative mutations in STAT3, resulting in impaired function of STAT3. We showed that GPVI-mediated platelet activation is affected by STAT3 loss-of-function mutations, thereby confirming functionality of this crosstalk in humans.

Involvement of STAT3 in platelet activation may provide a relevant link between inflammation and hemostasis. Increased activation of the STAT3 signaling pathway has been described in cancer and many inflammatory diseases, including cardiovascular diseases and COPD.[43,45,46,49-51] In contrast, platelet numbers were not affected by mutations in STAT3 loss-of-function patients nor was there an association with the SNPs in STAT3 in healthy human subjects. With the lack of an effect on platelet numbers, we report an opposite effect compared to a previous study that suggests an important role for STAT3 in the hepatocytic production of TPO, the most important regulator of platelet formation by megakaryocytes. In addition to a mouse model, it was shown that desialylated plate-

lets are taken up by a human HepG2 cells and these cells subsequently produce TPO in a Janus Kinase-2/STAT3-dependent manner.[52] In contrast, we did not find a decrease in platelet numbers in patients with defective STAT3 signaling, moreover thrombocytosis was observed in STAT3 loss-of-function patients during acute infections. A trend was seen for increased IL-6 in patients and this may be another factor that may stimulate thrombopoiesis.[53,54] Together, our findings suggest that *in vivo* STAT3 does not play a pivotal role in the production of platelets in humans.

Further experiments showed that GPVI-mediated platelet activation was largely dependent on secondary stimulation by platelet ADP release, both in STAT3 loss-of-function patients and in healthy subjects. However, the blockade of this mechanism with apyrase strongly attenuated platelet responses, particularly in STAT3 loss-of-function patients, indicating that STAT3 may be involved in the secondary ADPpathway as a result of platelet activation. The relevance of SNPs in STAT3 was further studied in a cohort of healthy volunteers and GPVI-mediated platelet activation was not affected by the SNPs studied.

Our study is limited by the fact that it is not known whether the studied SNPs in STAT3 affect STAT3 functionality in healthy subjects, or whether aberrant STAT3 function only becomes apparent when inflammatory diseases manifest. The role of STAT3 activation in inflammatory diseases and its consequences for platelet activation needs further exploration. Furthermore, blockade of Janus Kinase (JAK) - STAT3 pathway is currently being explored as a new therapy for cancer.[55-58] It is important that involvement of STAT3 in platelet activation is recognized as bleeding complications may occur, especially in patients that are concurrently treated with platelet inhibitors that target the ADP pathway such as Ticagrelor and Clopidogrel.

Involvement of 25-hydroxyvitamin D in platelet activation and reactivity.

Vitamin D has many functions beyond its traditional role in bone health, including a regulatory role in inflammation and infection.[59-61] Vitamin D deficiency is highly prevalent in COPD patients and is estimated to occur in 60% - 77% of patients with COPD GOLD stage 3 and 4, respectively.[62] Vitamin D supplementation is suggested to have a protective effect to respiratory infections, including viral infections,[63-65] a common trigger for AE-COPD.

In contrast to the immunomodulatory effects of vitamin D, associations between vitamin D and platelet function are currently scarcely studied. Several studies have shown strong associations between low vitamin D levels and cardiovascular diseases[66-69] and a seasonal variation of CVD incidence.[70-73] Moreover, the majority of patients with acute myocardial infarction is vitamin D deficient.[74,75]

In **Chapter 6,** we assessed the association between serum 25-hydroxyvitamin D levels and platelet activation and reactivity in a cohort of 500 healthy human subjects. For this observational study, data from the 500 Human Functional Genomics Project (500FG) were used.[8] The 500FG is part of the Human Functional Genomics Project (HFGP) that is aimed a characterisation of variations of immune cell function and platelet function in healthy human subjects[7,8] and thereby provided an unique opportunity to study the association between 25-hydroxyvitamin D and platelet function.

The vitamin D pathway has several important functional SNPs that influence bio-availibility of 25-hydroxyvitamin D and its active metabolite 1,25-dihydroxyvitamin D.[76-78] Thirty-nine SNPs were identified from literature,[78-82] out of which 31 of these SNPs were available in the 500FG dataset. This study is the first to show an inverse correlation between 25-hydroxyvitamin D levels and platelet fibrinogen binding in unstimulated samples and after stimulation with CRP and ADP in a large cohort of healthy subjects, whereas no relation with P-selectin expression was found. This association was stronger for 25-hydroxyvitamin D levels ≤ 50nmol/L, a commonly used cut-off value for vitamin D deficiency. No differences were observed between 25-hydroxyvitamin D insufficiency and normal 25-hydroxyvitamin D levels, further supporting the presence of a threshold.

Few studies investigated direct associations between vitamin D levels and platelet parameters in different diseases.[83-85] An association between low vitamin D levels and high-residual platelet reactivity in patients with CVD receiving anti-platelet therapy has been reported, as well as antithrombotic activities of vitamin D supplemented to haemodialysis patients.[83,85]

The strongest association with 25-hydroxyvitamin D was found for platelet fibrinogen binding in response to CRP-XL stimulation. Interestingly, 6 of the 9 vitamin D pathway related SNPs that were associated with platelet function also showed an association with platelet responses to CRP-XL. We among other have shown that the platelet agonist CRP-XL signals through the collagen pathway to activate platelets.[47] This mechanism is dependent on phosphorylation of Signal Transducer and Activator of Transcription 3 (STAT3).[47] Interactions between vitamin D and JAK-STAT signaling pathways have been reported.[86,87]

Olson and colleagues showed that vitamin D decreases STAT3 phosphorylation and this may explain increased platelet fibrinogen binding in response to CRP-XL in vitamin D deficient participants.[87] On the other hand, VDBP may also play a key role as 5 out of 9 SNPs were present in the GC region, encoding VDBP, and involvement of VDBP in platelet activation has been suggested.[88]

A causal relationship between the SNPs, vitamin D levels and platelet function parameters could not be confirmed. Critics suggest that vitamin D may be just a bystander and may reflect a combination of physical inactivity, poor health status and poor variation in diet that results in vitamin D deficiency. Vitamin D could be an epiphenomenon and may not be causally involved. This hypothesis is further strengthened by supplementation studies that yield little to no effect on cardiovascular diseases to date.[35,89,90] Furthermore, no association between vitamin D levels and immune cell function was found in the 500 human functional genomics projects after correction of seasonality effects.[8] On the other hand, studies exploring the effect of vitamin D supplementation on platelet reactivity are lacking. The PRECOVID trial[6] investigates the effects of vitamin D supplementation on COPD exacerbations as well as its effects on immune responses and platelet function in vitamin D deficient COPD patients. These kind of trials are crucial to further understand the mechanisms of action of vitamin D as well as to identify effective therapeutic opportunities.

The impact of cigarette smoking on the human immune system.

Despite global initiatives for tobacco regulation, smoking is still common with an estimated 1 billion smokers worldwide.[91-93] Smoking impacts nearly every part of the human body and contributes to numerous diseases with significant morbidity and mortality.[94-96] Smoking-related diseases do not only develop locally, such as in COPD and lung cancer, but also on a distant level, such as in myocardial infarction.[94,95] Immune dysregulation by smoking is thought to play an important role in the development of smoking-related diseases.[97]

Many studies have investigated the immunological effects of smoking, however, their study designs vary widely. Some studies aim to explore the effects of specific tobacco smoke components,[98] some analyse specific local and/or systemic immune cell lineages,[99-101] whereas others investigate tobacco effects in specific diseases, while humans or non-human species are used.[102,103] To the best of our knowledge, a comprehensive analyses of the systemic effects of tobacco smoking in healthy human subjects is lacking. These data are needed to identify and further study those pathways that are involved in the pathophysiology of smoking-related diseases at a systemic level. Therefore, the last study in this doctoral thesis, **Chapter 7**, describes a comprehensive analysis on the variation of peripheral blood immune cell population in carefully matched smokers, non-smokers and former smokers to investigate the systemic effects of smoking.

In this cross-sectional study, cases (current smokers and former smokers) and controls (non-smokers) were identified among 500 healthy volunteers participating in the Human Functional Genomics Project (HFGP).[8] Immune cell populations were determined in

peripheral blood and compared among the different study subjects. Furthermore, levels of circulating inflammatory mediators and *ex vivo* cytokine production upon stimulation of whole blood and peripheral blood mononuclear cells (PBMCs) were compared.

In current smokers, we observed an increased number of monocytes, mostly classical monocytes and these cells correlated with pack years. The production of the monocyte-derived cytokines TNFα, IL-6, IFNγ and IL-1β to *ex vivo* LPS challenge, in contrast, was lower in current smokers. Also dysregulation of the adaptive arm of the immune system was observed in current smokers, with increased numbers of CD4 and CD8 positive CM cells and increased numbers of CD4 positive EM cells. These observations were accompanied by increased numbers of regulatory T cells that showed a more activated and mature phenotype and this also correlated with pack years. IL-6 and hsCRP, markers for systemic inflammation, were not different between smokers and non-smokers, however these markers were positively correlated with pack years in current smokers. Together, these findings indicate that tobacco smoking leads to a defective capacity to produce cytokines upon microbial stimulation, yet it induces inappropriate systemic inflammation.

Our study reports important effects of tobacco smoking on human immune phenotype and immune function and confirms separate observations of previous studies, whereas it challenges others. Increased numbers of classical monocytes were observed that produce less pro-inflammatory cytokines. This supports a previous publication by Noakes and colleagues, who compared immune responses from cord blood of neonates between maternal smokers and non-smokers, and report decreased TNFα, IL-6 and IL-10 in cord blood of neonates from maternal smokers.[104] Others investigated the effects of the tobacco component nicotine and reported decreased cytokine production by monocytes via a mechanism dependent on nuclear factor-kappaB transcriptional activity.[105,106]

Furthermore, increased numbers of regulatory T cells were found in smokers. These natural suppressor cells of the immune system showed increased activation and maturation in current smokers. Opposite effects regarding regulatory T cell numbers and function have been reported in the context of smoking, including up- and down-regulation of regulatory T cells.[102,107-109] These findings seem dependent on the tissue investigated (peripheral blood, bronchial alveolar lavage fluid) and the population, such as healthy (non-) smokers or COPD patients.

These observations co-occur with increased numbers of antigen experienced pro-inflammatory T cells. T cell differentiation follows along a continuum from naive T cells

to antigen experienced CM and EM T cells.[110-112] In smokers, CM T cells were increased in CD4 and CD8 positive cell lineages and confirm previous studies that investigated specific T cell populations in humans,[100,113,114] but not in mice.[115] Upon antigenic stimulation, CM T cells are able to rapidly proliferate into effector T cells that migrate to the tissues to exert their pro-inflammatory effects.[112] It has been suggested that T cell differentiation is determined by antigen load and persistence in infectious diseases.[116-118] Indeed, several studies found alterations in airway colonization caused by cigarette smoke,[119,120] and the presence of pathogens may be an alternative source for antigenic stimulation.

We found that systemic inflammation was not increased in healthy smokers, whereas there was an association between pack years and plasma IL-6 and hsCRP. During prolonged and/or intense tobacco smoke exposure the balance may shift to a more pro-inflammatory phenotype. Indeed, several studies in COPD have shown increased inflammation, together with a decrease in regulatory T cells,[107,121,122] while others observed merely a dysfunction of the regulatory T cell compartment.[123,124] Similar observations are reported in cardiovascular diseases; decreased numbers of regulatory T cells and regulatory T cell dysfunction were found in patients with coronary artery disease and acute myocardial infarction.[125-127]

These data are important as they contribute not only to our understanding of the immunological consequences of smoking, but may help to identify pathophysiological mechanisms that result in the development of smoking-related diseases. Further investigation and comprehensive analysis of immune cells in these diseases is pivotal to identify which mechanisms prevail in the development of smoking-related diseases and may help to identify those at increased risk.

CONCLUSIONS

In this doctoral thesis the interaction between inflammation and coagulation in patients with COPD and the potential to modulate their interaction was investigated. We found that pulmonary embolism is common in patients with unexplained exacerbations of COPD and that the majority of these emboli have important clinical consequences. Further investigation of the mechanisms responsible for thrombotic complications in COPD revealed no differences in thrombin generation and d-dimer, markers for plasmatic coagulation, in stable COPD patients. Platelet-monocyte interaction, however, was increased in stable COPD patients and may augment atherosclerosis and plaque instability, thereby contributing to the development of cardiovascular diseases.

In healthy subjects, we found an increased number of circulating immune cells, including monocytes, in smokers compared to non-smokers, which further support the role of monocytes in COPD and atherosclerosis. Interestingly, during acute exacerbations platelet-monocyte interaction was lower and their interaction may not explain the increased risk for CVD during AE-COPD.

Several factors involved in inflammation may also exert effects on platelet function. Signal Transducer and Activator of Transcription 3, for example, is involved in glycoprotein VI-mediated platelet activation. Similarly, 25-hydroxyvitamin D is associated with platelet fibrinogen binding, in particular with glycoprotein VI-mediated platelet activation, among other platelet activation pathways. Although awareness for the thrombotic complications in COPD is increasing, the ability to modulate the interaction between inflammation and coagulation needs to be closely studied in COPD in order to prevent thrombotic complications in the future.

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CHAPTER 9

Nederlandse samenvatting

NEDERLANDSE SAMENVATTING

COPD is een ernstige ziekte die in de meeste gevallen wordt veroorzaakt door roken. Daarnaast spelen nevenaandoeningen, ook wel comorbiditeiten genoemd, een belangrijke rol in COPD. Vooral de cardiovasculaire comorbiditeiten hebben een belangrijke impact op de gezondheid van COPD-patiënten. Naar schatting wordt ongeveer de helft van de ziekenhuisopnames van COPD-patiënten veroorzaakt door cardiovasculaire comorbiditeiten en een kwart van de sterfgevallen.

COPD is een inflammatoire aandoening en het is bekend dat er intensieve interactie tussen ontsteking en stolling bestaat. Zowel arteriële als veneuze trombose komt vaak voor bij COPD en heeft een negatieve impact op de gezondheidstoestand. Deze interactie is in COPD nog niet uitgebreid bestudeerd. Het doel van dit academische proefschrift was daarom om de interactie tussen ontsteking en stolling te onderzoeken bij COPD-patiënten, alsmede om te onderzoeken of deze interactie kan worden beïnvloed.

In **Hoofdstuk 2** van dit proefschrift kijken we naar de aanwezigheid van longembolieën bij patiënten met een acute exacerbatie van COPD. In de literatuur is het voorkomen van deze longembolieën ten tijde van COPD-exacerbaties verscheidene malen gerapporteerd, echter bestaat er nog geen consensus over hoe vaak dit gemiddeld voorkomt. In deze studie hebben we een systematische review met meta-analyse uitgevoerd van de reeds gepubliceerde studies en gevonden dat de prevalentie van longembolieën bij patiënten met een onverklaarde exacerbatie van COPD rond de 16% procent ligt. Bovendien is tweederde van deze longembolieën groter dan subsegmenteel en daarmee klinisch relevant. Volgens de huidige richtlijn moeten deze longembolieën behandeld worden. Daarnaast hebben we verschillende studies uitgevoerd die het mechanisme verantwoordelijk voor het verhoogde risico op cardiovasculaire aandoeningen onderzochten, deze zullen in de volgende hoofdstukken besproken worden.

In **Hoofdstuk 3** zijn de functie van bloedplaatjes, bloedplaatjes-monocyt interactie en verschillende markers voor stolling vergeleken tussen patiënten met een stabiel COPD en gezonde proefpersonen. We vonden meer interactie tussen bloedplaatjes en monocyten in COPD-patiënten, in afwezigheid van plaatjeshyperreactiviteit. Deze bevinding was opmerkelijk, omdat deze fenomenen normaliter vaak samengaan. Plaatjeshyperreactiviteit speelt een belangrijke rol bij arteriële trombose. Bloedplaatjesremmers vormen een belangrijke therapie om onder andere hartaanvallen en beroertes te voorkomen. Interactie tussen plaatjes en monocyten speelt eveneens een rol, omdat deze bijdraagt aan het ontstaan van atherosclerose en plaque instabiliteit. De mate van stolling, gemeten met behulp van thrombine productie en D-dimeer, was niet verschil-

lend tussen COPD-patiënten en gezonde proefpersonen. In afwezigheid van functionele veranderingen in bloedplaatjes stelden we dat de interactie met monocyten mogelijk veroorzaakt wordt door monocyt-activatie. In bovengenoemde studie is er gekeken naar stabiel COPD, we wilden hierna graag verder kijken naar hoe bloedplaatjes en monocyten precies met elkaar interacteren en hoe bloedplaatjes functioneren gedurende acute exacerbaties van COPD.

In de hierop volgende studie, beschreven in **Hoofdstuk 4**, hebben we bovenstaande vragen onderzocht. De hypothese luidde dat onder invloed van ontsteking, meer bloedplaatjes-monocyt interactie zou bestaan tijdens exacerbaties van COPD in vergelijking met de herstelperiode daarna. Patiënten met een exacerbatie van COPD werden tijdens een opname onderzocht en tijdens een poliklinische controle, 6 tot 10 weken later. In tegenstelling tot onze hypothese vonden we minder bloedplaatjes-monocyt interactie tijdens acute exacerbaties van COPD. Er was tevens sprake van een inverse correlatie met monocyt-activatie marker MAC-1, wat het minder waarschijnlijk maakt dat activatie van de monocyt via deze receptor een cruciale rol speelt in de bloedplaatjes-monocyt interactie. Het beperkte aantal patiënten dat in deze studie geïncludeerd kon worden was een belangrijke beperking en daarom moeten we voorzichtig zijn met de interpretatie van de bevindingen van deze studie.

In **Hoofdstuk 5** is gekeken naar de rol van Signal Transducer and Activator of Transcription 3 (STAT3) mutaties in de functie van bloedplaatjes en in de productie van bloedplaatjes. Een recente studie liet in een muismodel zien dat STAT3 een cruciale rol speelde in bloedplaatjesactivatie via de collageen pathway. In gezonde proefpersonen werd dit ook getoond met behulp van een STAT3-remmer. Deze bevinding is interessant, omdat STAT3 vaak geassocieerd wordt met inflammatoire aandoeningen, waaronder COPD en cardiovasculaire ziekten. Om de relevantie van deze bevindingen verder te valideren bij mensen hebben we deze pathway onderzocht in patiënten met een defect in STAT3 functie en bij gezonde proefpersonen. De patiënten met een defect in STAT3 functie lijden aan het autosomaal-dominant Hyper IgE syndroom, een immuunstoornis die leidt tot recidiverende infecties met onder andere stafylokokken en schimmels.

We vonden in de patiënten met een defect in STAT3 functie inderdaad minder bloedplaatjesactivatie en een lagere respons van bloedplaatjes bij het stimuleren van de collageen pathway. Verdere experimenten naar de onderliggende mechanismen liet zien dat een andere pathway, de ADP pathway, dit defect grotendeels maskeerde. Na *ex vivo* remming van de ADP pathway met apyrase bleek dat bij de patiënten bloedplaatjesactivatie nauwelijks toenam na het stimuleren van de collageen pathway, terwijl dit in gezonde proefpersonen veel minder invloed had.

In een tweede cohort van gezonde proefpersonen hebben we verschillende SNPs in STAT3 onderzocht om te kijken of bovenstaande effecten zich ook in de algemene populatie voordoen. De SNPs in STAT3 beïnvloedden de mate van bloedplaatjesactivatie echter niet. Verder onderzoek moet uitwijzen of STAT3 leidt tot meer bloedplaatjesactivatie in inflammatoire aandoeningen, waarvan gesuggereerd wordt dat STAT3 activiteit is toegenomen. Indien dit fenomeen optreedt zou het bij kunnen dragen aan de ontwikkeling van cardiovasculaire aandoeningen bij toegenomen inflammatie.

Onze data zijn tevens relevant in de context van de ontwikkeling van nieuwe geneesmiddelen die STAT3 of zijn voorloper JAK2 remmen. Deze medicijnen worden momenteel in studieverband geëvalueerd voor hun effecten in oncologische aandoeningen. Deze middelen zouden potentieel kunnen leiden tot een verhoogd bloedingrisico, zeker bij patiënten die gelijktijdig behandeld worden met bloedplaatjesremmers die aangrijpen op de ADP pathway, zoals Ticagrelor en Clopidogrel.

Een andere factor die invloed zou kunnen hebben op bloedplaatjesactivatie en reactiviteit is vitamine D. Cardiovasculaire aandoeningen gaan vaak samen met een vitamine D-tekort en bovendien is er een seizoensgebonden patroon van het voorkomen van hartaanvallen die overeenkomt met vitamine D-spiegels in het bloed. In **Hoofdstuk 6** wordt de associatie tussen de bloedspiegel van 25-hydroxyvitamine D en bloedplaatjesfunctie onderzocht. Ondanks dat er een duidelijke relatie is tussen 25-hydroxyvitamine D en cardiovasculaire ziekten zijn de directe effecten op bloedplaatjes nog niet eerder bestudeerd.

We zijn de eerste die een inverse correlatie tussen 25-hydroxyvitamine D en bloedplaatjesactivatie van integrin αIIbβ3 laten zien in een groot cohort van gezonde proefpersonen, te weten het 500FG cohort. Zowel in ongestimuleerde monsters, als in monsters gestimuleerd met CRP-XL werd een inverse correlatie gevonden met de binding van fibrinogeen aan integrin αIIbβ3 op bloedplaatjes. Deze associatie was vooral sterk aanwezig bij proefpersonen met een 25-hydroxyvitamine D < 50nmol/L. We vonden geen verschil tussen een normaal vitamine D (>75nmol/L) en een relatieve vitamine D insufficiëntie (50nmol/L-75nmol/L), wat suggereert dat er sprake is van een zekere afkapwaarde. Daarnaast hebben we naar verschillende SNPs gerelateerd aan de vitamine D pathway gekeken en 9 van de 31 SNPs die aanwezig waren in de dataset hadden een associatie met vitamine D concentraties in het bloed.

De bovengenoemde associatie tussen vitamine D en bloedplaatjesfunctie hoeft niet te betekenen dat zij in direct verband met elkaar staan. Causal inference analysis liet geen causaal verband zien, echter dit kan te maken hebben met het kleine effect en

de omvang van het cohort. Critici stellen dat lage vitamine D concentraties mogelijk niet direct betrokken zijn bij het ontstaan van cardiovasculaire aandoeningen, maar dat het meer als epifenomeen optreedt. Mensen met een slechte gezondheidsstatus komen mogelijk minder buiten, hebben weinig beweging en mogelijk een minder gevarieerd dieet; factoren die allen bijdragen aan een vitamine D-tekort. Het risico op cardiovasculaire aandoeningen zou dan niet zozeer door het lage vitamine D bepaald worden, maar door de bovengenoemde factoren. Daarnaast laten verschillende studies die patiënten met vitamine D behandelen om cardiovasculaire ziekten te voorkomen niet een onomstreden voordeel zien van behandeling met vitamine D-supplementen.

De laatste wetenschappelijke studie in dit proefschrift beschrijft de effecten van roken op het immuunsysteem van gezonde vrijwilligers en is beschreven in **Hoofdstuk 7**. Ondanks dat verschillende mondiale initiatieven proberen om de tabaksconsumptie terug te dringen is het roken van tabak nog steeds veelvoorkomend en er zijn momenteel nog ongeveer 1 miljard rokers wereldwijd. Roken beïnvloedt elk deel van het menselijk lichaam en ligt ten grondslag aan vele aandoeningen. Sommige aandoeningen ontwikkelen zich lokaal, zoals COPD en longkanker, terwijl andere aandoeningen zich buiten het bereik van de long ontwikkelen.

Men denkt dat disregulatie van het immuunsysteem ten grondslag ligt aan het ontstaan van veel rokengerelateerde ziekten. De effecten van tabaksrook op het immuunsysteem zijn uitvoerig onderzocht, echter, studiedesigns verschillen enorm. Sommige studies richten zich bijvoorbeeld op specifieke componenten van tabak, zoals nicotine, terwijl andere studies naar specifieke cellijnen in de long of in bloed kijken. Daarnaast wordt onderzoek zowel in mensen als in dieren verricht. Een uitgebreide studie naar de effecten van roken op immuuncellen in bloed van gezonde mensen ontbrak nog en deze is door ons verricht in het 500FG cohort, waarin rokers, ex-rokers en niet rokers nauwkeurig aan elkaar gematched werden.

We vonden dat bij rokers, in vergelijking met niet rokers, verschillende immuun populaties waren toegenomen. Monocyten, met name klassieke monocyten, waren toegenomen in populatie en er bestond een positieve correlatie met het aantal gerookte pack years. De productie van monocyt afkomstige ontstekingscytokines TNFα, IL-6, IFNγ en IL-1β na *ex vivo* LPS stimulatie, daarentegen, was gedaald. Ook in het adaptieve immuunsysteem waren verscheidene populaties verandert, zoals het aantal CD4 en CD8 positieve central memory (CM) cellen en CD4 positieve effector memory (EM) cellen.

Tegelijkertijd observeerden we een toename van regulatoire T-cellen, de natuurlijke rem op het immuunsysteem. Regulatoire T-cellen waren bovendien meer geactiveerd

en vertoonden een verder gedifferentieerd fenotype dan bij niet rokers. IL-6 en hsCRP, markers voor systemische inflammatie, waren niet verschillend tussen rokers en niet rokers, terwijl er wel een correlatie was met pack years. Deze bevindingen suggereren dat er enerzijds sprake is van een zekere mate van immuundeficientie en dat er anderzijds sprake is van laag-gradige systemische inflammatie. Tussen ex-rokers en niet rokers werden geen signifiante verschillen gezien in immuuncellen, wat het belang van het stoppen met roken opnieuw benadrukt.

Samengenomen laat deze studie uitgebreide effecten zien van het roken op het immuunsysteem van gezonde proefpersonen en heeft het overlap met eerdere bevindingen in rokengerelateerde aandoeningen. Deze data dragen niet alleen bij aan de kennis over de consequenties van roken, maar kunnen mogelijk ook inzicht geven in de pathofysiologie van rokengerelateerde aandoeningen. Zo zijn in COPD ook toegenomen populaties van CD4 en CD8 positieve cellen gevonden en dit correleerde met GOLD stage en exacerbaties van COPD. In cardiovasculaire ziekten zouden CD4 positieve EM cellen correleren met markers voor atherosclerose. Verder onderzoek naar immuuncellen in deze aandoeningen is belangrijk om in beeld te krijgen welke mechanismen de overhand krijgen en resulteren in rokengerelateerde ziekten.

CONCLUSIE

9

In dit academische proefschrift werd de interactie tussen ontsteking en stolling bij patiënten met COPD onderzocht en werd onderzocht of deze interactie kan worden beïnvloed. We vonden dat longembolieën voorkomen bij 16% van de patiënten met onverklaarde exacerbaties van COPD en dat de meerderheid van deze longembolieën belangrijke klinische consequenties heeft. Verder onderzoek naar de mechanismen die verantwoordelijk zijn voor trombose lieten geen verschillen zien in thrombine productie en D-dimeer, markers van plasmatische stolling, in stabiel COPD. Bloedplaatjes-monocyt interactie, daarentegen, was toegenomen in stabiel COPD. Deze interactie versterkt de ontwikkeling van artherosclerose en plaque instabiliteit, en draagt daarmee bij aan de ontwikkeling van cardiovasculaire ziekten. Daarnaast vonden we bij gezonde mensen een toename van verschillende typen immuuncellen, zoals monocyten, in rokers in vergelijking met niet-rokers. In onze studie naar bloedplaatjes-monocyt interactie tijdens exacerbaties van COPD vonden we dat deze interactie juist minder sterk was en dit vormde geen verklaring voor het toegenomen cardiovasculaire risico tijdens exacerbaties van COPD.

Er zijn verschillende factoren die een rol spelen bij ontsteking en tevens de functie van bloedplaatjes kunnen beïnvloeden. Zo vonden we dat STAT3 een rol speelt bij glycoproteine VI-gemedieerde bloedplaatjesactivatie in mensen. 25-hydroxyvitamin D lijkt eveneens een rol te spelen, doordat het een inverse correlatie toonde met fibrinogeen binding met integrin αIIbβ3 op bloedplaatjes. Dit effect was het sterkst bij glycoproteine VI-gemedieerde bloedplaatjesactivatie. Ondanks dat we ons in toenemende mate bewust worden van de trombotische complicaties bij COPD, moet verder onderzoek leiden tot methoden om de interactie tussen stolling en ontsteking te beïnvloeden om zo cardiovasculaire ziekten bij COPD patienten in de toekomst te voorkomen.

CHAPTER 10

Epiloque

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Met dit proefschrift besluit ik een boeiende en uitdagende reis. Mijn dank gaat allereerst uit naar mijn promotoren en copromotor, voor de kans en het vertrouwen die jullie me gaven om deze uitdaging aan te gaan.

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CURRICULUM VITAE

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Floor Aleva werd geboren op 24 november 1988 in Zwolle, als oudste kind van Roel en Dionne. Enkele jaren later volgde Martijn, inmiddels in Groningen. Zij verhuisden in 1994 naar Eindhoven, waar Floor haar Atheneum volgde aan het Pleincollege Eckart en tevens deelnam aan het Pre-University Program aan de TU/e. Ze behaalde haar diploma in 2007, waarna zij in Nijmegen begon met de studie Geneeskunde. Gedurende haar studie werd het enthousiasme voor wetenschap al vroeg gewekt en werkte zij aan verschillende studies voor de afdelingen Pathologie en IQ Healthcare in het Radboudumc. In 2012 werd Floor geselecteerd voor het Honours Master Program 'Beyond the Frontiers' en werd ze in de gelegenheid gesteld om enkele maanden onderzoek doen aan het prestigieuze Imperial College in Londen. Dit leidde tot haar eerste wetenschappelijke publicatie.

Bij terugkomst werd de interesse voor de longziekten en de acute geneeskunde gewekt tijdens de coassistentschappen. In 2013 volgde zij haar senior co-schap bij prof. dr. Yvonne Heijdra op de afdeling Longziekten van het Radboudumc en werd zij aangenomen voor de specialisatie tot longarts. Met veel plezier heeft Floor hierna een keuze-coschap Intensive Care geneeskunde gevolgd in het Jeroen Bosch Ziekenhuis te 's-Hertogenbosch, waarna zij afstudeerde in 2014. De wetenschap lonkte nog steeds en toen Floor van Prof. dr. Yvonne Heijdra de mogelijkheid kreeg eerst om te promoveren alvorens te beginnen met de specialisatie, greep zij die kans direct aan. In samenwerking met de afdeling infectieziekten en mede onder supervisie van prof. dr. André van der Ven heeft zij promotieonderzoek gedaan wat heeft geresulteerd in dit proefschrift.

Floor volgt op dit moment de vooropleiding interne geneeskunde in het Jeroen Bosch Ziekenhuis te 's-Hertogenbosch. Zij zal na afronding van de vooropleiding haar opleiding tot longarts voortzetten in het Radboudumc.

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PUBLICATIONS

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ABBREVIATIONS LIST

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AAT	Alpha-1 Antitrypsin
AD-HIES	Autosomal-Dominant Hyper IgE Syndrome
ADP	Adenosine 5'diphosphate
AE-COPD	Acute Exacerbations of Chronic Obstructive Pulmonary Disease
AF	Atrial Fibrillation
ATS	American Thoracic Society
AUC	Area Under the Curve
BMI	Body Mass Index
CD	Cluster of Differentiation
CI	Confidence Interval
CM	Central Memory
COPD	Chronic Obstructive Pulmonary Disease
COX	Cyclooxygenase
CRP	C-Reactive Protein
CRP-XL	Cross linked Collagen-Related Peptide
CTPA	Computed Tomography Pulmonary Angiography
CVD	Cardiovascular diseases
CYP2R1	Cytochrome P2R1
DVT	Deep Venous Thrombosis
ED	Emergency department
EM	Effector Memory
ERS	European Respiratory Society
ETP	Endogenous Thrombin Potential
FDR	False Discovery Rate
FEV1	Forced Expiratory Volume per second
FG	Functional Genomics
FSC	Forward Scatter
FVC	Forced Vital Capacity
GP	Glycoprotein
GPVI	Glycoprotein VI
GOLD	Global initiative for Obstructive Lung Diseases
HFGP	Human Functional Genomics Project
HLA-DR	Human Leukocyte Antigen – antigen D Related
hsIL-6	High sensitive Interleukin-6
HWE	Hardy-Weinberg Equilibrium
Ig	Immunoglobulin
IFN	Interferon

IL	Interleukin
IQR	Inter-quartile range
JAK	Janus Kinase
LC- MS/MS	Liquid Chromatography tandem Mass Spectrometry
LPS	Lipopolysaccharide
MAC-1	Macrophage receptor 1
MAF	Minor Allele Frequency
MFI	Mean Fluorescence Intensity
MPV	Mean Platelet Volume
NK	Natural Killer
NT-proBNP	N-terminal prohormone of Brain Natriuretic Peptide
PAR-1	Proteinase-Activating Receptor-1
PBMCs	Peripheral Blood Mononuclear Cells
PE	Pulmonary Embolism
PMC	Platelet-Monocyte Complexes
PPP	Platelet-poor Plasma
PRP	Platelet-rich Plasma
PSGL-1	P-selectin Glycoprotein Ligand-1
RTI	Respiratory Tract Infection
SCC	Sideward Scatter
SD	Standard Deviation
SEM	Standard Error of the Mean
SH2	Src Homology 2
SNP	Single Nucleotide Polymorphism
STAT3	Signal Transducer and Activator of Transcription 3
STROBE	Strengthening The Reporting of Observational studies in Epidemiology
TF	Tissue Factor
Th-17	T Helper-17
TLR	Toll-like receptor
TNF-α	Tumor Necrosis Factor-Alpha
TPO	Thrombopoietin
TRAP	Thrombin Receptor Activating Peptide-6
VDBP	Vitamin D binding protein
VDR	Vitamin D receptor
VTE	Venous thromboembolism