Asthma and coagulation: A clinical and pathophysiological evaluation
Majoor, C.J.

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Chapter 4

Prothrombotic state in patients with severe and prednisolone-dependent asthma

Marlous M.S. Sneeboer, Christof J. Majoer, Anne de Kievit, Joost C.M. Meijers, Tom van der Poll, Pieter W. Kamphuisen, Elisabeth H. Bel

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Clinical Implications
Patients with asthma, in particular those with severe disease, have a prothrombotic state under stable conditions which may explain the increased risk of these patients to develop venous thromboembolism.

Capsule summary
Patients with asthma have a prothrombotic state as demonstrated by higher levels of ETP, PAPc, PAI-1 and vWF in peripheral blood compared to healthy controls. This prothrombotic state increases with asthma severity.

Abbreviations
ACQ = asthma control questionnaire  
BMI = body mass index  
CRP = C-reactive protein  
ETP = endogenous thrombin potential  
FEV1 = forced expiratory volume in 1 second  
PAI-1 = plasminogen activator inhibitor type 1  
PAPc = plasmin-α2-antiplasmin complex  
PE = pulmonary embolism  
TATc = thrombin-antithrombin complex  
vWF = von Willebrand factor
Abstract

Background
Epidemiological studies have shown that patients with asthma, in particular those with severe disease, have increased risk of pulmonary embolism. It is unknown whether these patients have a prothrombotic state under stable conditions.

Objective
To compare coagulation and fibrinolysis parameters between healthy subjects, and patients with mild, severe and prednisolone-dependent asthma under stable conditions and to investigate whether hemostatic markers correlate with airway inflammation.

Methods
In 126 adults (33 healthy controls, 31 mild asthma, 32 severe asthma, and 30 prednisolone-dependent asthma) parameters of inflammation (peripheral blood eosinophils and neutrophils), and markers of hemostasis (endogenous thrombin potential (ETP), thrombin-antithrombin complex (TATc), plasmin-α2-antiplasmin complex (PAPc), plasminogen activator inhibitor type 1 (PAI-1), D-dimer and von Willebrand factor (vWF)) were measured in plasma. One-way ANOVA with post-hoc Bonferroni test was used for group comparison, and linear regression analysis was used for correlations.

Results
We observed increased ETP levels (121 vs. 99%; overall p<0.01), increased PAPc levels (520 vs. 409 µg/L; overall p=0.04), increased levels of PAI-1 (10 vs. 7 ng/mL; overall p=0.02) and increased levels of vWF (142 vs. 87%; overall p<0.01) in asthma patients as compared to healthy controls. Levels of ETP, PAI-1 and vWF increased with increasing asthma severity. In addition, we found a correlation between ETP and vWF with neutrophils but not with eosinophils.

Conclusion
Patients with asthma have a prothrombotic state that increases with asthma severity. This may explain why patients with asthma, in particular those with severe disease, have an increased risk of venous thromboembolism.
Introduction

Patients with asthma, in particular those with severe disease and frequent exacerbations are at increased risk of venous thromboembolism. Epidemiologic studies have shown that the risk of pulmonary embolism (PE) in patients with severe asthma is increased up to 9 fold as compared to non-asthmatic individuals. The reason why patients with asthma are at increased risk of PE is unclear, but a pro-coagulant influence of the underlying inflammatory process has been suggested. This fits with the observation that also other chronic inflammatory diseases such as inflammatory bowel disease and rheumatoid arthritis are associated with increased risk of PE. Another contributing factor could be the use of corticosteroids, the mainstay of asthma treatment, which has also been associated with altered hemostasis and increased risk of PE. It is therefore conceivable that in asthma, in particular in severe asthma, hemostasis is activated since patients with severe asthma have severe airway inflammation and require high doses of corticosteroids for control of their disease.

In the present study, we hypothesized that patients with asthma have a prothrombotic state, which increases with asthma severity and is related to the number of inflammatory cells (eosinophils and neutrophils) in peripheral blood. The aim of our study was to compare coagulation and fibrinolysis parameters between healthy subjects, and patients with mild, severe and prednisolone-dependent asthma under stable conditions. For this, we measured markers of hemostasis (thrombin-antithrombin complex (TATc), endogenous thrombin potential (ETP), plasmin-α2-antiplasmin complex (PAPc), plasminogen activator inhibitor type 1 (PAI-1), D-dimer, and von Willebrand factor (vWF)) in peripheral blood.

Methods

Subjects and design

Hundred-twenty-six adult subjects (33 healthy controls, 31 patients with mild asthma, 32 patients with severe asthma and 30 patients with prednisolone-dependent asthma) were included in the study. Patients with asthma of different severities were recruited when visiting the outpatient pulmonary clinic of the Academic Medical Center Amsterdam, The Netherlands. Healthy subjects were recruited by advertisements in the local area outside the hospital. Assessment of asthma severity was based on the Global Initiative for Asthma (GINA) 2002 guideline for mild asthma and Innovative Medicine Initiative (IMI) criteria for severe asthma. All asthma patients had stable asthma and asthma was defined as a documented reversibility in forced expiratory volume in 1 second (FEV1)
of at least 12% after 400μg salbutamol, or airway hyperresponsiveness (concentration of methacholine <8mg/ml causing a 20% fall in FEV1 from baseline (PC_{20} <8mg/ml)) in the past 5 years. We divided patients into three categories based on the intensity of anti-inflammatory treatment; mild persistent asthma patients (using 250-500μg/day fluticasone or equivalent), severe asthma patients (using ≥1000μg/day fluticasone or equivalent and a second controller), and severe prednisolone-dependent asthma patients (using ≥1000μg/day fluticasone or equivalent and a second controller and >5mg/day prednisolone). Healthy subjects had no history of airway diseases, were non-atopic and had no airway hyperresponsiveness.

None of the subjects were current cigarette smokers and all had a history of a maximum of 10 pack years. Subjects were excluded if they had signs of a respiratory infection or a change in inhaled or oral corticosteroid dose within 4 weeks prior to screening and if they used omalizumab. Also patients using heparin, low-molecular-weight-heparin, aspirin, non-steroidal anti-inflammatory drugs (NSAIDs) or vitamin K antagonists were excluded. Patients who were pregnant or had a history of venous thromboembolism, and patients with concomitant disease or inherited coagulation disorders which could interfere with the study, were also excluded.

This study was part of a research program aimed at investigating risk factors of venous thromboembolism in patients with asthma. Study measurements included asthma control questionnaires (ACQ), spirometry, venous blood collection, and sputum induction and were conducted at the Academic Medical Center Amsterdam. All samples were collected on the same day. The study was approved by the Medical Ethics Committee of the Academic Medical Center Amsterdam and all subjects gave their written informed consent. The study was registered at the Dutch trial registry (www.trialregister.nl) number NTR3101.

Measurement of inflammation and hemostatic parameters in peripheral blood

Venous blood was obtained after 10 minutes rest. Complete white blood cell counting including automated differential cell counting was performed to calculate the number of neutrophils and eosinophils in peripheral blood. C-reactive protein (CRP) was measured by immunoturbidimetric determination. Citrated blood was used for measurement of hemostatic markers; vWF, TATc and D-dimer (in vivo coagulation), ETP (in vitro coagulation), and PAPc and PAI-1 (fibrinolysis). vWF was determined by ELISA with a polyclonal rabbit anti-human vWF antibody as catching antibody and horse radish peroxidase-labelled rabbit anti-human vWF antibody as detecting antibody (both DAKO, Glostrup, Denmark). TATc (Siemens Healthcare Diagnostics, Marburg, Germany), PAPc (DRG, Marburg, Germany), and PAI-1 (Hyphen BioMed, Andrésy, France) were determined by ELISA. D-dimer levels were measured
with a particle-enhanced immunoturbidimetric assay (Innovance D-dimer, Siemens Healthcare Diagnostics). *In vitro* thrombin generation was measured using the Calibrated Automated Thrombogram. This assay determines the generation of thrombin in clotting plasma using a microtiter plate reading fluorometer (Fluoroskan Ascent, ThermoLab systems, Helsinki, Finland), and Thrombinscope software (Thrombinscope BV, Maastricht, The Netherlands). The assay was carried out as described by Hemker et al.\textsuperscript{10} and the Thrombinscope manual. Coagulation was triggered with 5 pM recombinant human tissue factor (Innovin, Siemens Healthcare Diagnostics), 4 µM phospholipids, and 417 µM fluorogenic substrate Z-Gly-Gly-Arg-AMC (Bachem, Bubendorf, Switzerland). Fluorescence was monitored and the different parameters (lag time (time to initiate coagulation), peak thrombin, and area under the curve or endogenous thrombin potential (ETP)) were calculated using Thrombinscope software. Peak thrombin and ETP results were normalized to pooled normal plasma.

**Statistical analysis**

Non-normally distributed data were log-transformed before analysis. Variables were summarized by descriptive statistics. Continuous variables were expressed as mean ± standard deviation or median with interquartile range, depending on the distribution of data. Categorical variables were presented as percentages. Overall comparison between the groups was done by one-way ANOVA which resulted in an overall p-value. In addition, post-hoc Bonferroni comparisons were performed between groups, which resulted in separate p-values. Multiple linear regression analysis, corrected for age and gender, was used to determine the association between coagulation and fibrinolysis parameters and inflammation parameters. A p-value of <0.05 was considered significant. SPSS Statistics (Version 20.0. Armonk, NY: IBM Corp) and Graphpad Prism (version 5.0. GraphPad Software, San Diego, California USA) was used for the data analysis.

**Results**

Characteristics of the patients with mild, severe and prednisolone-dependent asthma as well as healthy control subjects are shown in table 1. Patients with asthma were older as compared to healthy controls. Lung function (FEV1 prebronchodilator) decreased with asthma severity, whereas ACQ score increased with asthma severity (both overall p<0.01; table 1). Compared to healthy controls, patients with asthma had increased levels of neutrophils (overall p<0.01; table 1), eosinophils (overall p<0.01; table 1) and CRP (overall p<0.01; table 1) in peripheral blood.
Hemostatic parameters

Coagulation and fibrinolysis parameters of patients with mild, severe and prednisolone-dependent asthma and healthy control subjects are shown in Table 2. Compared to healthy control subjects, patients with asthma showed increased thrombin generation, as demonstrated by a significant higher level of ETP in asthma patients (overall p<0.01; figure 1). Post-hoc analysis showed a significant difference in ETP between healthy controls (99%) and severe asthma patients (114%; p<0.01), between healthy controls (99%) and prednisolone-dependent asthma patients (121%; p<0.01) and between mild asthma patients (105%) and prednisolone-dependent asthma patients (121%; p<0.01). A similar result was obtained with peak thrombin levels that were higher in asthma patients as compared to healthy controls (overall

<table>
<thead>
<tr>
<th>Table 1. Characteristics of healthy controls, mild asthma, severe asthma and prednisolone-dependent asthma patients</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Healthy controls</strong></td>
</tr>
<tr>
<td><strong>N = 33</strong></td>
</tr>
<tr>
<td>Age (years) *</td>
</tr>
<tr>
<td>Gender (% males)</td>
</tr>
<tr>
<td>BMI (kg/m²) *</td>
</tr>
<tr>
<td>Duration of asthma (years) ‡</td>
</tr>
<tr>
<td>Allergy (% yes)</td>
</tr>
<tr>
<td>Never smokers (% yes)</td>
</tr>
<tr>
<td>Smoking history (pack years) ‡</td>
</tr>
<tr>
<td>ICS dose (µg/day fluticasone or equivalent) ‡</td>
</tr>
<tr>
<td>OCS dose (mg/day prednisolone) *</td>
</tr>
<tr>
<td>ACQ score ‡</td>
</tr>
<tr>
<td>FEV₁ pre (%) *</td>
</tr>
<tr>
<td>Eosinophils (10⁹/L) ‡</td>
</tr>
<tr>
<td>Neutrophils (10⁹/L) *</td>
</tr>
<tr>
<td>CRP (mg/L) ‡</td>
</tr>
</tbody>
</table>

*mean (standard deviation) or ‡ median (interquartile range)

BMI = body mass index, NA = not applicable, ICS = inhaled corticosteroids, OCS = oral corticosteroids, ACQ = asthma control questionnaire, FEV₁ pre = forced expiratory volume in 1 second prebronchodilator, CRP = C-reactive protein

Eosinophils and neutrophils were measured in peripheral blood.
Patients had allergy if there ever was a positive skin prick test for twelve common Aeroallergens such as house dust mite, cockroach, grass or tree mix.
Asthma control was assessed by the Juniper asthma control questionnaire (ACQ)²⁷.
A trained lung function technician performed spirometry²⁸.
There was again a trend with severity of disease with peak thrombin levels in mild asthma of 109% (p>0.05), 118% in severe asthma (p<0.01) and 132% in prednisolone-dependent asthma (p<0.01) when compared to 108% in healthy controls. There was also a longer lag time (overall p<0.01) in asthma patients compared to healthy controls.

TATc showed a few outliers (values higher than the mean plus 5 times the standard deviation) that were excluded from the analysis. Thus, for the analysis of TATc, we had data from 32 healthy controls, 28 patients with mild asthma, 30 patients with severe asthma and 26 patients with prednisolone-dependent asthma.

$vWF = \text{von Willebrand factor}, \text{TATc} = \text{thrombin-antithrombin complex}, \text{PAPc} = \text{plasmin-\alpha2-antiplasmin complex}, \text{PAI-1} = \text{plasminogen activator inhibitor type 1}, \text{ETP} = \text{endogenous thrombin potential}$

<table>
<thead>
<tr>
<th></th>
<th>Healthy controls</th>
<th>Mild asthma</th>
<th>Severe asthma</th>
<th>Prednisolone-dependent asthma</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>33</td>
<td>31</td>
<td>32</td>
<td>30</td>
</tr>
<tr>
<td>vWF (%)*</td>
<td>87 (23)</td>
<td>107.8 (45)</td>
<td>107.6 (37)</td>
<td>142 (53)</td>
</tr>
<tr>
<td>TATc (µg/L)†</td>
<td>2.4 (2.0 – 2.8)</td>
<td>2.4 (2.1 – 2.8)</td>
<td>2.6 (2.3 – 2.9)</td>
<td>2.2 (2.0 – 2.5)</td>
</tr>
<tr>
<td>PAPc (µg/L)*</td>
<td>409 (149)</td>
<td>449 (141)</td>
<td>501 (183)</td>
<td>520 (183)</td>
</tr>
<tr>
<td>PAI-1 (ng/mL)‡</td>
<td>7.0 (4.0 – 12.5)</td>
<td>9.0 (5.0 – 14.0)</td>
<td>9.5 (6.0 – 13.0)</td>
<td>10.0 (8.0 – 22.5)</td>
</tr>
<tr>
<td>D-dimer (µg/L)‡</td>
<td>0.1 (0.1 – 0.3)</td>
<td>0.2 (0.1 – 0.4)</td>
<td>0.3 (0.2 – 0.4)</td>
<td>0.2 (0.1 – 0.3)</td>
</tr>
<tr>
<td>Lag time (min)*</td>
<td>2.8 (0.5)</td>
<td>3.2 (0.8)</td>
<td>3.4 (0.6)</td>
<td>3.6 (0.6)</td>
</tr>
<tr>
<td>Peak thrombin (%)*</td>
<td>108 (18)</td>
<td>109 (16)</td>
<td>118 (25)</td>
<td>132 (31)</td>
</tr>
<tr>
<td>ETP (%)†</td>
<td>99 (13)</td>
<td>105 (13)</td>
<td>114 (20)</td>
<td>121 (25)</td>
</tr>
</tbody>
</table>

*mean (standard deviation) or ‡ median (interquartile range)

† For analysis of TATc we had data of 32 healthy controls, 28 patients with mild asthma, 30 patients with severe asthma and 26 patients with prednisolone-dependent asthma.

**Figure 1.**
Endogenous thrombin potential (ETP) in plasma of healthy controls, mild asthma, severe asthma, and prednisolone-dependent asthma patients.

$p<0.01$. There was again a trend with severity of disease with peak thrombin levels in mild asthma of 109% (p>0.05), 118% in severe asthma (p<0.01) and 132% in prednisolone-dependent asthma (p<0.01) when compared to 108% in healthy controls. There was also a longer lag time (overall p<0.01) in asthma patients compared to healthy controls.

TATc showed a few outliers (values higher than the mean plus 5 times the standard deviation) that were excluded from the analysis. Thus, for the analysis of TATc, we had data from 32 healthy controls, 28 patients with mild asthma, 30 patients with
severe asthma and 26 patients with prednisolone-dependent asthma. There was no significant difference for TATc between the four groups of patients (healthy 2.4 vs. mild 2.4 vs. severe 2.6 vs. prednisolone-dependent 2.2 µg/L; overall p=0.73). Furthermore, D-dimer levels were increased in asthma patients as compared to healthy controls (healthy 0.1 vs. mild 0.2 vs. severe 0.3 vs. prednisolone-dependent 0.2 mg/L; overall p=0.01).

There was an overall significant difference for PAPc between asthma patients and healthy controls (overall p=0.04). Higher PAPc levels were observed in the severe asthma (501 µg/L) and prednisolone-dependent asthma patients (520 µg/L) compared to healthy controls (409 µg/L) but post-hoc testing for differences between separate groups did not reveal significant results. Furthermore, there were higher levels of PAI-1 (overall p=0.02) in asthma patients as compared to healthy controls (figure 2). We observed statistically higher levels in prednisolone-dependent asthma patients (10.0 ng/mL) than in healthy controls (7.0 ng/mL; p<0.01).

Lastly, we found higher levels of vWF (overall p<0.01) in asthma patients as compared to healthy controls (figure 3). Post-hoc analysis showed the highest level in prednisolone-dependent asthma patients (142%) when compared to healthy controls (87%; p<0.01). Also, patients with mild asthma (107.8%; p<0.01) had higher levels than healthy controls, while this was not different between severe asthma and healthy controls. Also, there was a difference in vWF between mild asthma patients (107.8%) and prednisolone-dependent patients (142%; p<0.01) and between severe asthma patients (107.6%) and prednisolone-dependent asthma patients (p<0.01).

**Figure 2.**
Plasminogen activator inhibitor type 1 (PAI-1) in plasma of healthy controls, mild asthma, severe asthma, and prednisolone-dependent asthma patients.
Correlation of hemostatic and inflammatory parameters

vWF and ETP correlated significantly with peripheral blood neutrophils (R=0.6; p<0.01 and R=0.3; p<0.01) (figure 4 a, b), whereas no correlation was found between hemostatic parameters with blood eosinophils or CRP.

Figure 3.
von Willebrand factor (vWF) in plasma of healthy controls, mild asthma, severe asthma, and prednisolone-dependent asthma patients.

Correlation of hemostatic and inflammatory parameters

Figure 4.
Correlation of a) von Willebrand factor (vWF) with neutrophils in plasma b) endogenous thrombin potential (ETP) with neutrophils in plasma
Discussion

This study shows that patients with asthma have a prothrombotic state, as demonstrated by higher levels of ETP, PAPc, PAI-1, and vWF in peripheral blood as compared to healthy controls. The results also show that the prothrombotic state increases with increasing asthma severity. In addition, vWF and ETP correlated with peripheral blood neutrophils but no associations were found between hemostatic parameters and eosinophils. The enhanced prothrombotic state may explain why patients with asthma, in particular patients with severe disease, are predisposed to develop venous thromboembolism.

This study is the first to systematically compare coagulation and fibrinolysis parameters in peripheral blood between healthy subjects and patients with mild, severe and prednisolone-dependent asthma. Previous studies have suggested increased hemostatic activity in asthma in general\textsuperscript{11-14}, and some studies have suggested a relationship between coagulation activity and asthma severity. For example, TATc in sputum correlated with the degree of bronchial responsiveness\textsuperscript{15} and increased PAI-1 levels in sputum were associated with poorer lung function\textsuperscript{16}. One study showed increased PAI-1 levels in the blood of patients with asthma during an asthma attack\textsuperscript{17}. In our study, we showed a prothrombotic state which was measured by multiple parameters, including increased \textit{in vitro} coagulation (ETP), and higher levels of PAPc, PAI-1, D-dimer and vWF. This imbalance of hemostasis might explain why patients with asthma have an increased risk of venous thromboembolism.

We observed a prothrombotic state, with the highest values in patients with severe asthma, and lower values in patients with mild asthma. In fact, the prothrombotic state in patients with mild asthma resembled that in healthy controls. Apparently, in patients with mild asthma the airway inflammation was well controlled with inhaled corticosteroids, resulting in very low levels or no activation of coagulation. In contrast, in patients with severe asthma the inhaled corticosteroids were not fully capable of dampening the airway inflammation, which may lead to more coagulation activation.

Our study has several strengths. First, it was specifically designed to test multiple coagulation and fibrinolysis parameters in patients with asthma of different severities. Asthma severity was well defined and only patients with stable disease were included to minimize the influence of acute inflammatory events. In addition, we excluded patients with factors that could potentially influence hemostasis. For example, we excluded women who used oral contraceptives since it has been described that this influences hemostatic markers\textsuperscript{18}. We also excluded patients with a history of arteriosclerotic disease, as previous studies suggest that asthma is a risk
factor for acute coronary syndrome and stroke which could have an influence on the thrombotic state.

Unfortunately, there were outliers of TATc which we had to exclude from analysis. The most likely explanation for these outliers is the sensitivity of TATc to blood withdrawal technique and workup that could cause artificial TATc generation. The thrombin generation assay which was also used is more robust and has been shown to have an inter-individual variability of 17.5% suggesting that within an individual the ETP is relatively constant over time. Therefore, based on the results of the Calibrated Automated Thrombogram, we think that our results on coagulation status in patients with asthma are valid.

There are several mechanisms by which airway inflammation could influence coagulation. Firstly, previous studies have shown that eosinophils can express prothrombotic factors such as tissue factor. Also, other cells that are present in the asthmatic airways, such as bronchial epithelial cells and mast cells have the ability to produce PAI-1. Therefore, it is likely that during chronic airway inflammation, there is continuous activation of coagulation, disturbing the balance towards a prothrombotic state. In our study we observed a correlation between the prothrombotic state and neutrophils but not with eosinophils. Apparently, for coagulation, eosinophilic inflammation is not the key factor. Neutrophils, on the other hand, might be more important in causing an imbalance of hemostasis. Asthma patients with neutrophilic or mixed neutrophilic/eosinophilic airway inflammation have more severe disease. However, the neutrophilia could also be a consequence of the use of oral corticosteroids. Nevertheless, the neutrophilic component of airway inflammation might be the reason why patients with severe asthma are at increased risk of venous thromboembolism.

A second mechanism of the prothrombotic state could be related to hypoxia. Patients with airway diseases such as asthma have unequal ventilation which may lead to hypoxia in particular parts of the lung. Hypoxia has been shown to induce systemic inflammation and activation of coagulation. A two hour hypoxic challenge in patients with chronic obstructive pulmonary disease (COPD) led to increased levels of TATc and a trend for vWF and D-dimer. Thus, in patients with asthma airway narrowing could locally lead to hypoxic areas in the lung where coagulation becomes activated.

A third mechanism of the prothrombotic state might be related to corticosteroid use. In patients with asthma, corticosteroids are the mainstay of treatment. A recent systematic review showed that use of oral corticosteroids had a significant effect on hemostasis by increasing coagulant and reducing fibrinolytic factors. Moreover, a population based case-control study in Denmark showed that current use of oral corticosteroids significantly increased the risk of venous thromboembolism.
(adjusted IRR 2.31; 95% CI 2.18 – 2.45). In our study, patients with mild, severe and prednisolone-dependent asthma were using different doses of corticosteroids; mild persistent asthma patients using the lowest doses, and prednisolone-dependent patients the highest doses. Different corticosteroid doses could therefore have contributed to the differences in hemostatic abnormalities as observed in the different patient groups in our study. Thus, corticosteroid use may add to the imbalance of the hemostatic system that is already present in chronic airway inflammation. Therefore, clinicians should be aware of the potential hemostatic effects when prescribing corticosteroids to patients, especially in patients who already have other risk factors of venous thromboembolism such as older age, high body mass index or hormone replacement therapy.

Overall, the results of our study show that in peripheral blood of patients with asthma, in particular those with severe disease, a prothrombotic state is present. This prothrombotic state is most likely caused by chronic airway inflammation combined with the effect of high dose corticosteroids, and may explain the increased risk of patients with severe asthma to develop venous thromboembolism.

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Each authors contribution
conception and design: C.J. Majoor, P.W. Kamphuisen, T. vd Poll, E.H. Bel;
acquisition and analysis of data: M.M.S. Sneeboer, C.J. Majoor, A. d Kievit; analysis and interpretation: M.M.S. Sneeboer, C.J. Majoor, J.C.M. Meijers, T. vd Poll, P.W. Kamphuisen, E.H. Bel; drafting the article: M.M.S. Sneeboer; read and approved final manuscript: all authors.

All authors declare no conflicts of interest
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