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# Thrombin generation correlates with disease duration in multiple sclerosis (MS): Novel insights into the MS-associated prothrombotic state

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## Abstract

**Background:** Thrombin is well recognised for its role in the coagulation cascade but it also plays a role in inflammation, with enhanced thrombin generation observed in several inflammatory disorders. Although patients with multiple sclerosis (MS) have a higher incidence of thrombotic disease, thrombin generation has not been studied to date.

**Objectives:** The aim of this study was to characterise calibrated automated thrombography parameters in patients with relapsing–remitting MS (RRMS) and primary progressive MS (PPMS) in comparison to healthy controls (HCs).

**Methods:** Calibrated automated thrombography was performed on platelet poor plasma from 15 patients with RRMS, 15 with PPMS and 19 HCs.

**Results:** We found that patients with RRMS generate thrombin at a significantly faster rate than the less inflammatory subtype, PPMS or HCs. In addition, the speed of thrombin generation was significantly correlated with time from clinical diagnosis in both subtypes. However, in RRMS the rate of thrombin generation was increased with increased time from clinical diagnosis, while in PPMS the rate of thrombin generation decreased with increased time from clinical diagnosis.

**Conclusions:** These data likely reflect the differential active proinflammatory states in each MS subtype and provide novel mechanistic insights into the clinically relevant prothrombotic state observed in these patients.

**Keywords:** Multiple sclerosis, relapsing–remitting, progressive, thrombin generation, calibrated automated thrombography, platelet-poor plasma

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## Introduction

Multiple sclerosis (MS) is a demyelinating disease of the central nervous system (CNS) and is the most common cause of non-traumatic neurological disability in young adults.<sup>1</sup> A relapsing–remitting (RRMS) course, characterised by active inflammatory plaques and clinical acute exacerbations, is typical in the majority of patients following initial diagnosis. A primary progressive course (PPMS) is recognised in up to 15% of patients who experience accumulating disability without distinct inflammatory relapses.<sup>1,2</sup> Patients with MS have a significantly

elevated risk of cardiovascular disease and venous thromboembolism (particularly in the first year following diagnosis), when compared to the general population.<sup>3–5</sup> This is likely to reflect the proinflammatory state that accompanies MS, particularly during periods of disease activity. Coagulation activation promotes inflammation and conversely proinflammatory cytokines stimulate coagulation activation through multiple pathways.<sup>6,7</sup> Therefore, it is not surprising that levels of both coagulation factors including prothrombin and factor X (FX)<sup>8</sup>

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and procoagulant microparticles (MPs) are increased in MS compared with healthy individuals.<sup>9</sup> Moreover, proteomic analysis of post-mortem MS plaques identified coagulation factors in chronic active plaques which were not present in acute or active plaques.<sup>10</sup>

Thrombin is a multifunctional enzyme that plays a central role in the coagulation cascade by converting fibrinogen to insoluble fibrin. In MS, fibrinogen crosses the blood-brain barrier due to increased endothelial permeability.<sup>11</sup> There, it is cleaved to fibrin which initiates an inflammatory response and demyelination.<sup>12</sup> In perivascular plaques, fibrin deposition is observed even before the onset of clinical symptoms.<sup>13</sup> Interestingly, fibrin depletion was shown to decrease inflammation and delay demyelination in a murine model of MS, suggesting a role in disease pathogenesis.<sup>14</sup> In addition to its role in cleaving fibrinogen, thrombin initiates proinflammatory signalling pathways in a variety of cell types by activation of protease inhibitor receptors (PARs). In MS, thrombin-mediated, PAR1-dependent signalling on leucocytes promotes increased cytokine expression and leucocyte recruitment.<sup>15</sup> Moreover, in an experimental autoimmune encephalomyelitis (EAE) murine model, *in vivo* inhibition of thrombin-dependent signalling with hirudin or treatment with activated protein C, which mediates anti-inflammatory, PAR1-dependent signalling, ameliorated disease severity and reduced CNS inflammatory foci compared with sham-treated mice.<sup>10</sup> Collectively, these data highlight the critical role played by coagulation activation in MS.

Calibrated automated thrombography (CAT) is an assay that permits global characterisation of blood coagulation potential in real time. The combined effects of multiple parameters that have the potential to influence blood clotting capacity are simultaneously assessed. Thrombin generation is initiated with calcium in the presence of either added phospholipids and tissue factor (TF), or endogenous procoagulant MPs, and measured by determining the cleavage rate of a fluorogenic thrombin substrate using specialised software.<sup>16</sup> Of clinical relevance, this assay is sensitive both to hypo- and hypercoagulable states and has been reported to predict both bleeding and thrombotic complications in clinical cohorts.

The aim of this study was to characterise CAT parameters in patients with RRMS and PPMS in comparison to HCs. Moreover, we aimed to characterise the relationship between thrombin generation

and time from clinical diagnosis and disability in MS.

## Participants and methods

### Study participants

Fifteen patients with RRMS, 15 with PPMS and 19 HCs were recruited from an MS specialist clinic in a tertiary referral centre. MS diagnosis was confirmed using McDonald criteria<sup>17</sup> and clinical phenotype determined as per recently published guidelines.<sup>18</sup> Patients were included if their disease was stable with no history of relapse in the preceding three months and were treatment naïve or had not been on disease-modifying therapy for the preceding six months. Patients with MS and HCs could not be taking any antiplatelet or other anticoagulant drugs. Full written informed consent was taken prior to any study procedures being carried out. Baseline demographics and disease characteristics are as outlined in Table 1. As expected, patients with PPMS were older, had a significantly longer time from clinical diagnosis and higher Expanded Disability Status Scale (EDSS) than those with RRMS.

### Blood sample collection

Forty-four millilitres of blood was drawn into vacutainers containing acid citrate dextrose. The first 4 mls of blood was discarded and platelet-poor plasma (PPP) was prepared from the remaining 40 mls of blood by differential centrifugation. Plasma was separated from whole blood by centrifugation for 10 minutes at 200 × g. Further contaminating red blood cells and leucocytes were removed by

**Table 1.** Demographics, disability status and disease duration in those with PPMS, RRMS and HCs.

	PPMS (n = 15)	RRMS (n = 15)	HCs (n = 19)
Female			
Number (%)	8 (53)	12 (80)	13 (68)
Age			
Mean (SD)	55 (6.8)	38 (7.9)	45 (13.3)
EDSS			
Mean (SD)	4.2 (2)	1.3 (0.9)	N/A
Disease duration (years)			
Mean (SD)	13 (9)	6 (5)	N/A

PPMS: primary progressive multiple sclerosis; RRMS: relapsing–remitting multiple sclerosis; HCs: healthy controls; EDSS: Expanded Disability Status Scale; SD: standard deviation.

centrifugation for 7 minutes at  $150 \times g$ . PPP was then isolated by centrifugation for 10 minutes at  $600 \times g$  to remove platelets. All samples were stored at  $-80^\circ\text{C}$  until analysis.

### CAT

To characterise blood coagulation in RRMS, PPMS and HCs, thrombin generation in PPP was assessed by CAT using a Fluoroskan Ascent Plate Reader (ThermoLab System, Helsinki, Finland) in combination with Thrombinoscope software (Thrombinoscope BV, Maastricht, The Netherlands). PPP (80  $\mu\text{l}$ ) was incubated with either 20  $\mu\text{l}$  “platelet-poor plasma (PPP) reagent” containing 1 pM tissue factor (TF) and 4  $\mu\text{M}$  phospholipid vesicles (60% phosphatidylcholine, 20% phosphatidylserine, 20% phosphatidylethanolamine), or 20  $\mu\text{l}$  “PRP reagent”, which contains TF with minimal phospholipids, or 20  $\mu\text{l}$  “MP reagent”, which contains phospholipids only. Thrombin generation was initiated by automatic dispensation of fluorogenic thrombin substrate (Z-Gly-Gly-Arg-AMC.HCl) and 100 mM  $\text{CaCl}_2$  into each well (final concentrations, Z-Gly-Gly-Arg-AMC.HCl, 0.42 mM and  $\text{CaCl}_2$ , 16.67 mM). Thrombin generation was determined using a thrombin calibration standard and characterised by measurement of specific parameters, including lag time to initiation of thrombin generation, peak thrombin generated, time to peak thrombin generation, the velocity index, and the area under the thrombin generation curve (endogenous thrombin potential, ETP).

### Data analysis

Data were analysed using R<sup>19</sup> and R Studio.<sup>20</sup> All results are expressed as median and upper and lower 95% confidence intervals. All data was tested for normality by Q-Q plot and Shapiro-Wilk test. Welch *t*-tests were performed to determine differences between means for all parametric pairwise comparisons of groups. Mann-Whitney *U* tests were performed to determine group differences for all pairwise comparisons where at least one group was non-parametrically distributed. Strength of correlations was determined by Pearson correlation, and significance of correlations was determined by linear regression. *P* values of less than 0.05 were considered significant.

### Ethical approval

Full ethical approval was granted by St Vincent's University Hospital Ethics and Medical Research Committee and the Office of Research Ethics, University College Dublin.

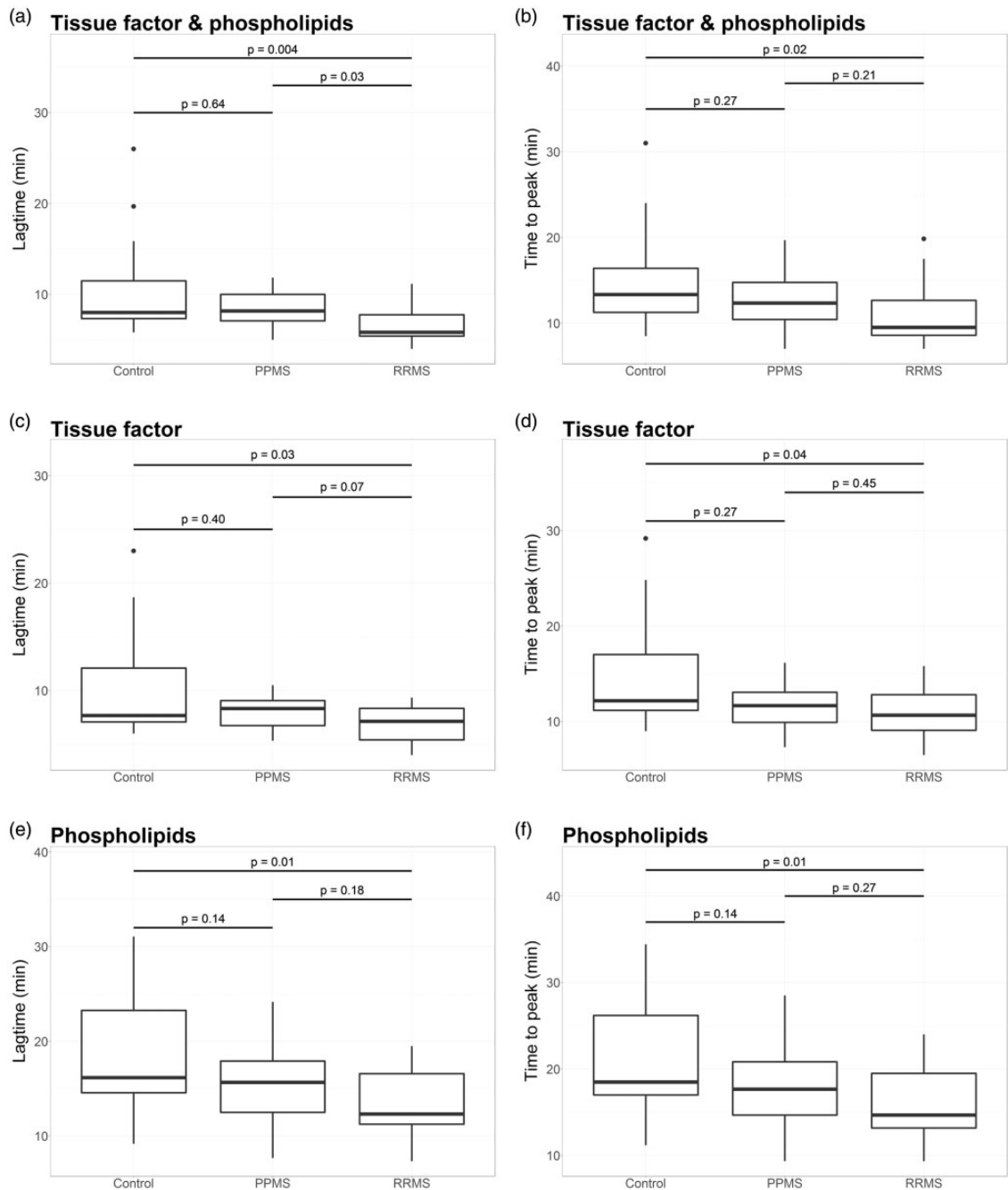
## Results

### *Blood coagulation measured by CAT is significantly accelerated in RRMS patients compared with PPMS patients and HCs*

CAT was performed in PPP prepared from RRMS, PPMS and HCs. Following initiation of thrombin generation by both TF and phospholipids, lag time to initiation of thrombin generation was found to be significantly shorter in RRMS, 5.83 minutes (interquartile range (IQR) 5.41–7.75 minutes), compared both with PPMS, 8.17 minutes (IQR 7.08–10 minutes) and HCs, 8 minutes (IQR 7.33–11.49 minutes) (Figure 1(a)). In addition, time to peak thrombin was also significantly shorter in RRMS patients, 9.5 minutes (IQR 8.59–12.66 minutes), compared to HCs, 13.33 minutes (IQR 11.26–16.41 minutes), but not when compared to PPMS patients, 12.34 minutes (IQR 10.42–14.75 minutes) (Figure 1(b)). Collectively, these data show that blood clotting begins at a faster rate in response to these stimuli in RRMS patients compared with HCs, suggesting a potential mechanism for the clinically observed prothrombotic state in these patients.

Patients with MS show evidence of platelet activation and a proinflammatory state.<sup>9,21</sup> Microparticle profiles have been shown to be altered in other proinflammatory states accompanied by platelet activation, including inflammatory types of arthritis,<sup>22</sup> systemic lupus erythematosus,<sup>23</sup> and pre-eclampsia.<sup>24</sup> Consequently, we next aimed to determine the potential contribution of phospholipid components of endogenous MPs to thrombin generation by initiating thrombin generation with TF alone. Under these conditions, lag time to initiation of thrombin generation was significantly reduced in RRMS patients, 7.14 minutes (IQR 5.41–8.34 minutes), compared to HCs, 7.67 minutes (IQR 7.08–12.09 minutes), but not compared to PPMS, 8.33 minutes (IQR 6.75–9.07 minutes) (Figure 1(c)). In addition, time to peak thrombin was significantly reduced both in RRMS, 10.67 minutes (IQR 9.09–12.83 minutes), and PPMS, 11.67 minutes (IQR 9.92–13.08 minutes), compared to HCs, 12.18 minutes (IQR 11.17–17.02 minutes) (Figure 1(d)). These data suggest that phospholipid components of endogenous MPs differ in MS patients compared with HCs and may contribute mechanistically to the observed prothrombotic state.

To determine the effect of endogenous TF on thrombin generation, initiation was performed using phospholipids only. Lag time to thrombin generation was



**Figure 1.** Thrombin generation is significantly altered in RRMS compared to healthy control and PPMS. Thrombin generation is significantly altered in RRMS with reduced lag time to initiation of thrombin generation and time to peak thrombin generation compared to healthy controls observed with the addition of ((a) and (b)) both tissue factor and phospholipids, ((c) and (d)) tissue factor alone, and ((e) and (f)) phospholipids alone. No significant difference was observed between RRMS and PPMS apart from in lag time with the addition of both tissue factor and phospholipids (a). No significant difference was observed in lag time of time to peak thrombin between PPMS and healthy controls. RRMS: relapsing–remitting multiple sclerosis; PPMS: primary progressive multiple sclerosis.

significantly reduced in RRMS, 12.33 minutes (IQR 11.26–16.59 minutes), compared to HCs, 16.17 minutes (IQR 14.57–23.27 minutes), but not compared to PPMS, 15.67 minutes (IQR 12.51–17.92

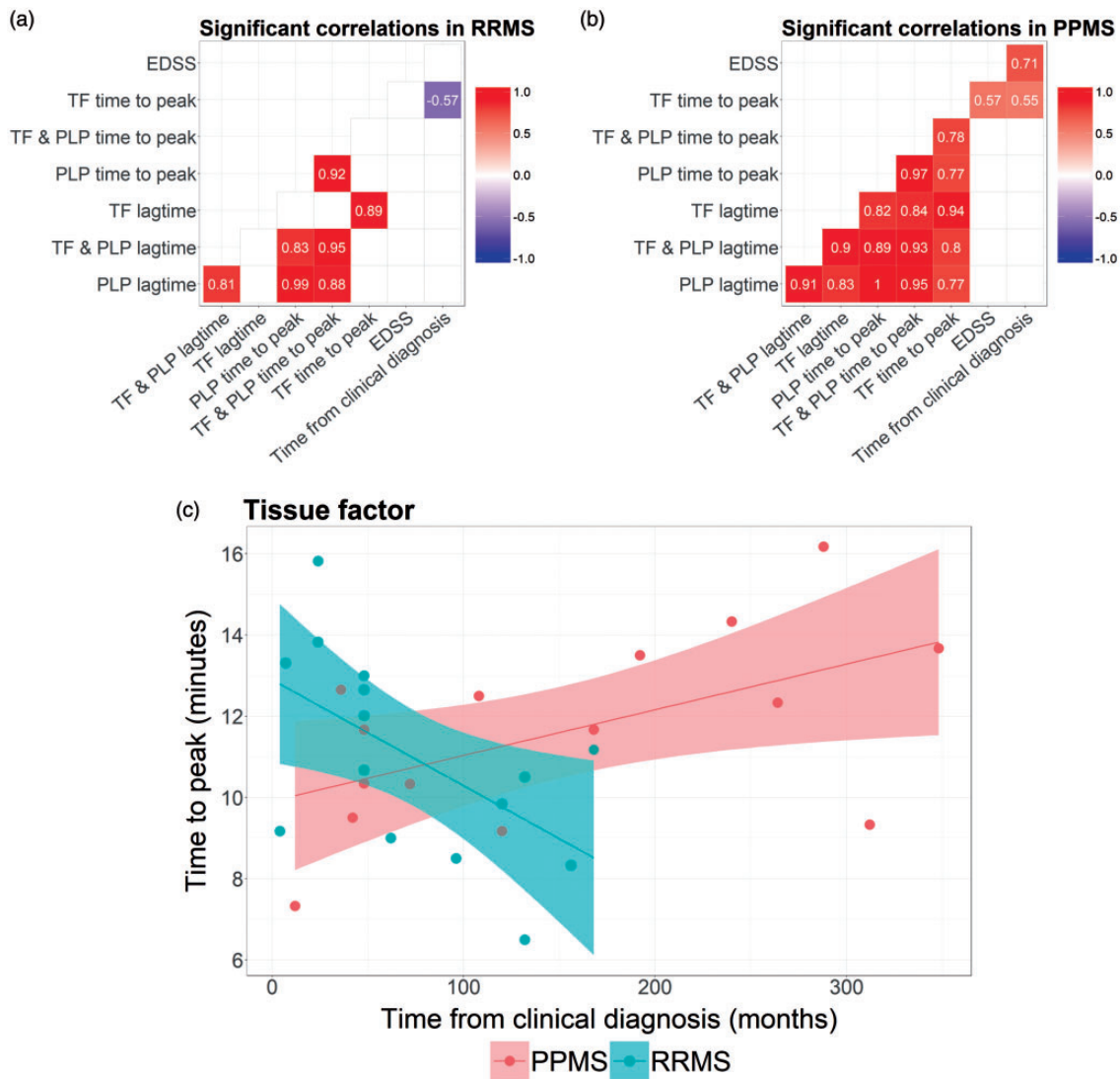
minutes) (Figure 1(e)). Time to peak thrombin was significantly reduced in RRMS, 14.67 minutes (IQR 13.18–19.5 minutes), compared to HCs, 18.5 minutes (IQR 17–26.2 minutes), but not compared

to PPMS, 17.67 minutes (IQR 14.67–20.84 minutes) (Figure 1(f)). Similarly, these data suggest that circulating endogenous TF levels (potentially associated with circulating MPs) differ in MS patients compared with HCs and may also contribute mechanistically to the observed prothrombotic state.

*Time to peak thrombin generation significantly correlates with time from clinical diagnosis in RRMS and PPMS*

To determine whether EDSS or time from clinical diagnosis had a relationship with the observed

significantly altered thrombin generation parameters (lag time to thrombin generation and time to peak thrombin generation), correlation analysis was performed for RRMS (Figure 2(a)) and PPMS (Figure 2(b)). Time to peak thrombin generation initiated with TF and minimal phospholipids significantly ( $p = 0.025$ ) negatively correlated ( $r = -0.57$ ) with time from clinical diagnosis in RRMS. Interestingly, time to peak thrombin generation with the addition of TF with minimal phospholipids significantly ( $p = 0.034$ ) positively correlated ( $r = 0.57$ ) with time from clinical diagnosis in



**Figure 2.** Correlation of thrombin generation with Expanded Disability Status Scale score and time from clinical diagnosis. Time to peak thrombin generation with the addition of tissue factor alone was found to (a) significantly negatively correlate with time from clinical diagnosis in RRMS and to (b) significantly positively correlate with time from clinical diagnosis in PPMS. Linear models were generated to determine the relationship between time to peak with tissue factor alone and time from clinical diagnosis (c). A clear relationship can be observed between time from clinical diagnosis both for RRMS and PPMS and time to peak thrombin generation. RRMS: relapsing–remitting multiple sclerosis; PPMS: primary progressive multiple sclerosis.

PPMS (Figure 2(c)). No other thrombin generation parameter was found to correlate with either EDSS or time from clinical diagnosis. The association between disease status and thrombin generation was still significant in multivariate regression models including age and sex as covariates.

### Discussion

In this study, we reveal *in vivo* coagulation alterations in MS. Despite the small sample size in our groups, our data show that patients with the pro-inflammatory subtype of MS, RRMS, generate thrombin at a significantly faster rate than the less inflammatory subtype, PPMS, or HCs. In addition, the speed of thrombin generation was significantly correlated with time from clinical diagnosis. In the RRMS group longer time from clinical diagnosis was associated with a faster rate of thrombin generation in comparison to the PPMS group where a slower rate was seen. This may reflect the differential active proinflammatory states in each MS subtype<sup>1</sup> and provide novel mechanistic insights into the clinically relevant prothrombotic state observed in these patients.

While the increased risk of cardiovascular disease in MS has been confirmed in several studies, the aetiology underlying the increased thrombotic risk remains elusive. Both the intrinsic and extrinsic coagulation pathways have been found to be altered in MS. Higher levels of factor XII, the initiator of the intrinsic coagulation cascade, are found in MS patients during relapse and FXII-deficient mice are less susceptible to CNS inflammation.<sup>25</sup> Prothrombin and FX (components of the “common” coagulation pathway) are upregulated in RRMS compared both to HCs and PPMS.<sup>8</sup> In accordance with these findings our data indicate that the rate of thrombin generation is significantly increased by activation of the intrinsic coagulation cascade with the addition of phospholipids alone, and by activation of the extrinsic coagulation pathways with the addition of TF with minimal exogenous phospholipids.

In RRMS, clinical disease appears to be driven by new inflammatory demyelinating lesions in the CNS.<sup>26</sup> Profound damage to the blood-brain barrier allows infiltration of inflammatory cells into the CNS, as can be observed in gadolinium-enhanced magnetic resonance imaging (MRI) of lesions.<sup>27</sup> Perivascular fibrin deposition is also observed in plaques and coincides with areas of demyelination.<sup>13</sup> In PPMS demyelination is also observed and

associated with inflammation.<sup>28</sup> However, blood-brain barrier dysfunction is less pronounced and is too limited to detect by gadolinium-enhanced MRI imaging.<sup>26</sup> In contrast to RRMS, plaques in PPMS slowly expand and are less dependent on new inflammatory lesions.<sup>26</sup> Our data show that hypercoagulability increases with time from clinical diagnosis in RRMS but decreases with time from clinical diagnosis in PPMS. We hypothesise that hypercoagulability in RRMS patients compared with PPMS may reflect a more pronounced proinflammatory state and more severe blood-brain barrier dysfunction than is observed in PPMS patients.

In conclusion, we demonstrate that blood coagulation, as measured by thrombin generation, is enhanced in RRMS patients compared to PPMS and HCs. Increased coagulability in RRMS is related to time from clinical diagnosis. These data are of novel translational relevance and may have future potential in exploring risk stratification of MS patients to identify and protect patients who are at highest risk of potentially dangerous cardiovascular and venous thromboembolic events.

### Conflicts of interest

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: PBM is supported by PI award 10/IN.1/B3012 from Science Foundation Ireland. K O’C has received educational grants from Biogen, Merck, Bayer and Novartis. CMcG has received research grants from Bayer, Biogen, Genzyme, Novartis & Teva and honoraria from Biogen, Genzyme, Novartis, Merck & Roche. MEMP, SA, KE, PBS, FNA have nothing to declare.

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