

# Association of plasma levels of Protein S with disease severity in multiple sclerosis

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## Abstract

**Background:** The TAM family of receptor tyrosine kinases (TYRO3, AXL and MERTK) play important roles in modulating innate immune responses and central demyelination. The TAM receptor ligand Protein S (PROS) has also been shown to modulate innate immune cell responses.

**Objectives:** We assessed whether plasma levels of PROS are changed in multiple sclerosis (MS) patients and whether changes are associated with disease severity.

**Methods:** Plasma levels of total and free PROS were measured using enzyme-linked immunosorbent assay in a discovery cohort (MS: 65, control: 14) and an independent replication cohort (MS: 29, control: 29). The Multiple Sclerosis Severity Score (MSSS) was used to evaluate associations between plasma PROS levels and disease severity.

**Results:** We found plasma levels of total, but not free PROS, were decreased in MS patients compared with controls. In female MS patients, we observed decreases in total and free PROS levels compared with controls. In addition, we also observed higher MSSS in patients with very low levels of plasma free PROS.

**Conclusions:** These data suggest PROS may represent a potential marker of disease severity in MS.

**Keywords:** Multiple sclerosis, TAM receptors, Protein S

## Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) of unknown cause. One hallmark of MS is the presence of multifocal lesions throughout the CNS, characterised by demyelination, oligodendrocyte death, axonal degeneration and the accumulation of immune cells. Although adaptive immune cells have been shown to be important in the pathogenesis of MS,<sup>1</sup> innate immune cells such as dendritic cells (DCs) and macrophages are also important, as they play crucial roles both in initiation and progression of central demyelination. Antigen presentation by DCs shape autoimmune T cell responses, while macrophages have been shown to be essential in mediating both CNS damage and repair.<sup>2</sup> Therefore, factors involved in the modulation of innate immune cell responses could prove to be important in the pathogenesis of MS.

The TAM receptors (TYRO3, AXL and MERTK) comprise a family of structurally related receptor

tyrosine kinases that have two identified ligands, growth arrest-specific 6 (Gas6) and Protein S (PROS). Signalling via TAM receptors has been implicated in processes that are crucial during central demyelination, including promoting oligodendrocyte survival, facilitating macrophage/microglial phagocytosis of apoptotic cells and most importantly, limiting the extent of innate immune responses. Specifically, the MERTK receptor has been shown to be crucial in mediating phagocytosis of apoptotic cells by the regulatory M2c macrophage subtype.<sup>3</sup> In addition, TAM signalling has been shown to induce interleukin-10 secretion and subsequent induction of tolerance in M2c macrophages.<sup>3</sup>

To date, the role of PROS as a TAM ligand has often been overlooked. This is most likely due to the better-known, TAM-independent role of PROS as an inhibitor of the coagulation cascade. Protein S is an abundant circulating glycoprotein, of which 60% forms a non-covalent complex with the  $\beta$ -chain of the C4b binding protein (C4BP) in 1:1 stoichiometry

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(bound PROS), while 40% is unbound and in free circulation (free PROS), and it is only free PROS which is biologically active.<sup>4</sup> Early studies debated the role of PROS as an agonist for the TAM receptors.<sup>5</sup> However, PROS has since been implicated in several processes attributed to TAM signalling,<sup>6,7</sup> and has been shown to be an effective agonist both for TYRO3 and MERTK.<sup>8</sup> Furthermore, it was recently shown that activated T cells produce PROS, which is able to signal through TAM receptors present on DCs to limit their activation.<sup>9</sup> Finally, the apparently normal phenotype of mice lacking Gas6 under physiological conditions<sup>10</sup> provides some evidence that PROS is able to compensate for Gas6 as a bona fide TAM receptor ligand.

The TAM family has been implicated in the pathogenesis of several models of autoimmune diseases, including autoimmune hepatitis,<sup>11</sup> psoriasis<sup>12</sup> and rheumatoid arthritis.<sup>13</sup> Most notably, the role of TAM signalling has been investigated extensively in systemic lupus erythematosus (SLE). Studies have shown that plasma concentration of free PROS is lower in SLE patients with active haematologic involvement.<sup>14</sup> Furthermore, plasma PROS concentration was lower in SLE patients with a history of serositis, neurologic disorder, haematologic disorder and immunologic disorder.<sup>14</sup>

In terms of CNS demyelination, we have previously shown that in the cuprizone model of CNS demyelination, mice lacking Gas6 displayed greater demyelination compared with wild-type mice. This phenotype corresponded with greater oligodendrocyte loss and potentiated microglial activation.<sup>15</sup> Subsequent to this work, several studies have shown that Gas6 plays important roles in promoting remyelination following cuprizone withdrawal,<sup>16,17</sup> as well the involvement of the receptor Axl in promoting macrophage/microglial phagocytosis both in the cuprizone and experimental autoimmune encephalomyelitis models of central demyelination.<sup>18,19</sup> Recently, by conducting an association study of single nucleotide polymorphisms within TAM receptor and ligand genes, we identified the *MERTK* gene to be a novel MS risk gene,<sup>20</sup> a finding that has since been replicated by the International Multiple Sclerosis Genetics Consortium in their genome-wide association study.<sup>21</sup> Previous studies of TAMs in MS have also shown that levels of soluble forms of MERTK and AXL are increased in chronic MS lesions,<sup>22</sup> and that levels of GAS6 in cerebrospinal fluid are inversely correlated with relapse severity in patients with relapsing–remitting MS.<sup>23</sup>

The increasing body of data showing the importance of TAM signalling during central demyelination and autoimmune diseases, as well as the role of PROS in regulating the innate immune response, prompted us to look for a direct link between PROS and MS. We found that plasma levels of total, but not free, PROS were decreased in MS patients compared with healthy controls (HCs) in two independent cohorts. In female MS patients, both total and free plasma PROS levels were decreased compared with HCs. In addition, we also showed an association of lowered PROS levels with worse MS disease severity, demonstrating the potential of PROS as a novel marker of disease severity for MS.

## Methods

### Participants

The Eastern Health Research and Ethics Committees granted approval for this research. Written informed consent was obtained from all individuals participating in this study. Plasma samples from 65 MS cases and 14 HCs collected between 2008 and 2010 from the Eastern Clinical Research Unit, Box Hill Hospital, Victoria, Australia, were used as an initial discovery cohort (Table 1). An age- and sex-matched replication cohort consisting of 29 MS cases and 29 HCs from the Eastern Clinical Research Unit, Box Hill Hospital, Victoria, Australia, was assessed between 2010 and 2012 (Table 2). All MS cases had at least two confirmed attacks of MS, and met both the 2005 and 2010 McDonald criteria for diagnosis of relapsing–remitting MS, unless otherwise stated. All MS patients were in remission and were not on immunomodulatory therapy at the time of collection. Neurological outcome was assessed using the Kurtzke Expanded Disability Status Scale (EDSS). For MS patients, the EDSS score was used in combination with disease duration from first reported symptom to generate the Global Multiple Sclerosis Severity Score (MSSS).<sup>24</sup> Peripheral blood was collected by standard venepuncture into vacuum tubes containing citrate as an anticoagulant.

### Enzyme-linked immunosorbent assay (ELISA)

PROS levels were measured using a commercially available sandwich ELISA kit (Helena Laboratories, TX, USA) according to the manufacturer's instructions. Briefly, samples were treated with polyethylene glycol (PEG) to precipitate the PROS-C4b binding protein complex, then diluted 1:26 for free PROS determination. For total PROS determination, samples were diluted 1:52 without precipitation. Binding of PROS was detected by a horseradish peroxidase (HRP)-conjugated anti-human PROS

**Table 1.** Discovery cohort participant summary.

	MS	HC
<i>n</i>	65	14
Age: mean (SD)	43.36 (11.15)	47.67 (13.11)
Female	39	7
Male	22	5
Unknown sex	4	1
MSSS: mean (SD)	4.331 (2.543)	—

MS: multiple sclerosis; HC: healthy control; SD: standard deviation; MSSS: Global Multiple Sclerosis Severity Score.

**Table 2.** Replication cohort participant summary.

	MS	HC
<i>n</i>	29	29
Age: mean (SD)	34.76 (7.003)	34.07 (7.2)
Female	19	19
Male	10	10
CIS	2	—
MSSS: mean (SD)	3.342 (2.526)	—

MS: multiple sclerosis; HC: healthy control; SD: standard deviation; CIS: clinically isolated syndrome; MSSS: Global Multiple Sclerosis Severity Score.

antibody. Tetramethylbenzidine (TMB) and hydrogen peroxide were used for colorimetry. Optical density was measured spectrophotometrically at 450 nm. Relative values of PROS were then determined from the appropriate standard curve and expressed as a percentage of normal levels of PROS by comparing with reference plasma provided by the manufacturer. Each sample was tested both for free and total PROS on the same plate, in duplicate.

C4b binding protein beta chain (C4BP $\beta$ ) and soluble CD163 (sCD163) levels were measured using commercially available ELISA kits (Cusabio, Hubei Province, PR China) according to the manufacturer's instructions. Plasma samples were diluted 1:4000 (C4BP $\beta$ ) or 1:5 (sCD163) for detection. Antigen binding was measured spectrophotometrically at 450 nm, with optical correction at 540 nm. Each sample was tested in duplicate.

Soluble MERTK (sMERTK) was measured using a sandwich ELISA kit (DYC891, R&D Systems, MN, USA). After coating polystyrene 96-well plates with a monoclonal sMERTK antibody, a blocking solution of 3% fish gelatine in 50 mM Tris, 150 mM NaCl, pH 7.4 containing 0.1% Tween 20 was applied. After plate washing with phosphate-buffered saline, samples were diluted 1:10 in blocking buffer and applied to plates in duplicate. A biotinylated detection antibody was added according to the manufacturer's instructions, followed by streptavidin-HRP. A colorimetric reaction was developed using TMB and absorbance was determined at 450 nm, with optical correction at 540 nm.

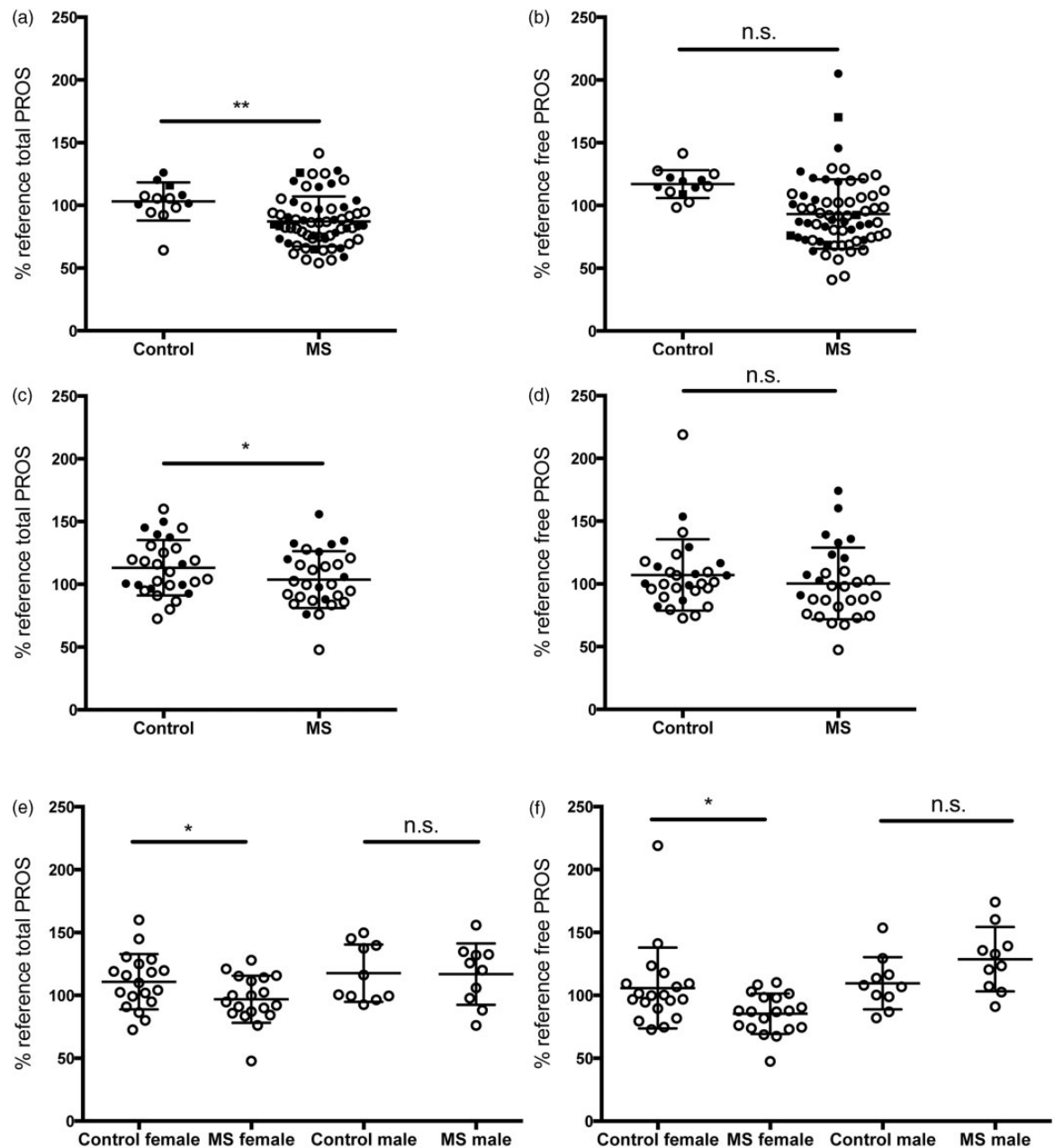
### Statistical analysis

Data were analysed using GraphPad Prism 6, version 6.0 e (GraphPad Software, CA, USA). For PROS analyses in the discovery cohort, unpaired Student's *t*-test was used, whereas the paired Student's *t*-test was used for analyses in the replication cohort. Linear regression was used for correlation analyses. For analysis of data stratified by sex, the Mann-Whitney test was used. For analysis of difference in MSSS, patients were stratified based on their PROS levels. The MSSS of patients with very low and very high PROS were compared with the MSSS of patients whose PROS levels fell within the 95% confidence interval (CI). Patients were deemed to have very low or very high PROS if their PROS levels exceeded the mean PROS  $\pm$  one standard deviation (SD). Unpaired Student's *t*-test with Welch's correction was used to compare each subgroup with the 95% CI. A level of 0.05 was chosen to indicate statistical significance for all tests.

## Results

### *PROS is decreased in the plasma of MS patients*

We initially investigated whether levels of circulating PROS were altered in patients with MS. We found that levels of total, but not free PROS were lower in MS patients compared with HCs ( $87.2 \pm 19.8\%$  of normal vs  $103.1 \pm 15.2\%$  of reference,  $p < 0.01$ ) (Figure 1(a), (b)). In order to confirm and extend these findings, we analysed an independent replication cohort of age- and sex-matched MS patients and HCs. In this cohort, consistent with our original data, we found the level of total, but not free, PROS was lower in the plasma of MS patients compared with HCs ( $103.8 \pm 22.6\%$  of normal vs  $113.2 \pm 22.13\%$  of reference,  $p < 0.05$ ) (Figure 1(c), (d)).



**Figure 1.** Plasma levels of Protein S (PROS) are decreased in people with multiple sclerosis (MS) compared with healthy controls. (a), (b) Plasma levels of total and free PROS were measured by enzyme-linked immunosorbent assay (ELISA) in a discovery cohort of MS patients and healthy controls. (Closed circles represent female participants; open circles represent male participants; closed squares represent participants for whom sex was not recorded) (c), (d) Plasma levels of total and free PROS were measured by ELISA in an age- and sex-matched replication cohort of MS patients and healthy controls. (e), (f) Plasma levels of total and free PROS in replication cohort stratified by sex. All dot plots show mean plasma levels of PROS relative to standardised, reference plasma  $\pm$  standard deviation. (\* $p < 0.05$ ; \*\* $p < 0.01$ ; n.s. = not significant. (a)–(d) = Student’s *t*-test; (e), (f) = Mann-Whitney test).

As expression of PROS is known to be regulated by sex hormones, we then stratified our replication data by sex. We found that female, but not male, MS patients had reduced levels both of total

( $96.9 \pm 18.7\%$  of normal vs  $110.8 \pm 22.0\%$  of reference,  $p < 0.05$ ) and free PROS ( $85.4 \pm 16.0\%$  of normal vs  $105.8 \pm 32.2\%$  of reference,  $p < 0.05$ ) compared with HCs (Figure 1(e), (f)).

We also investigated whether the difference in the level of plasma PROS was as a result of differences in circulating levels of C4BP. We used ELISA to determine plasma levels of C4BP $\beta$  in our replication cohort and found no significant differences in levels of C4BP between MS patients and HCs ( $445.3 \pm 524.9$  nmol/l vs  $383.9 \pm 536.2$  nmol/l,  $p > 0.05$ ). We observed no sex differences in plasma C4BP concentration.

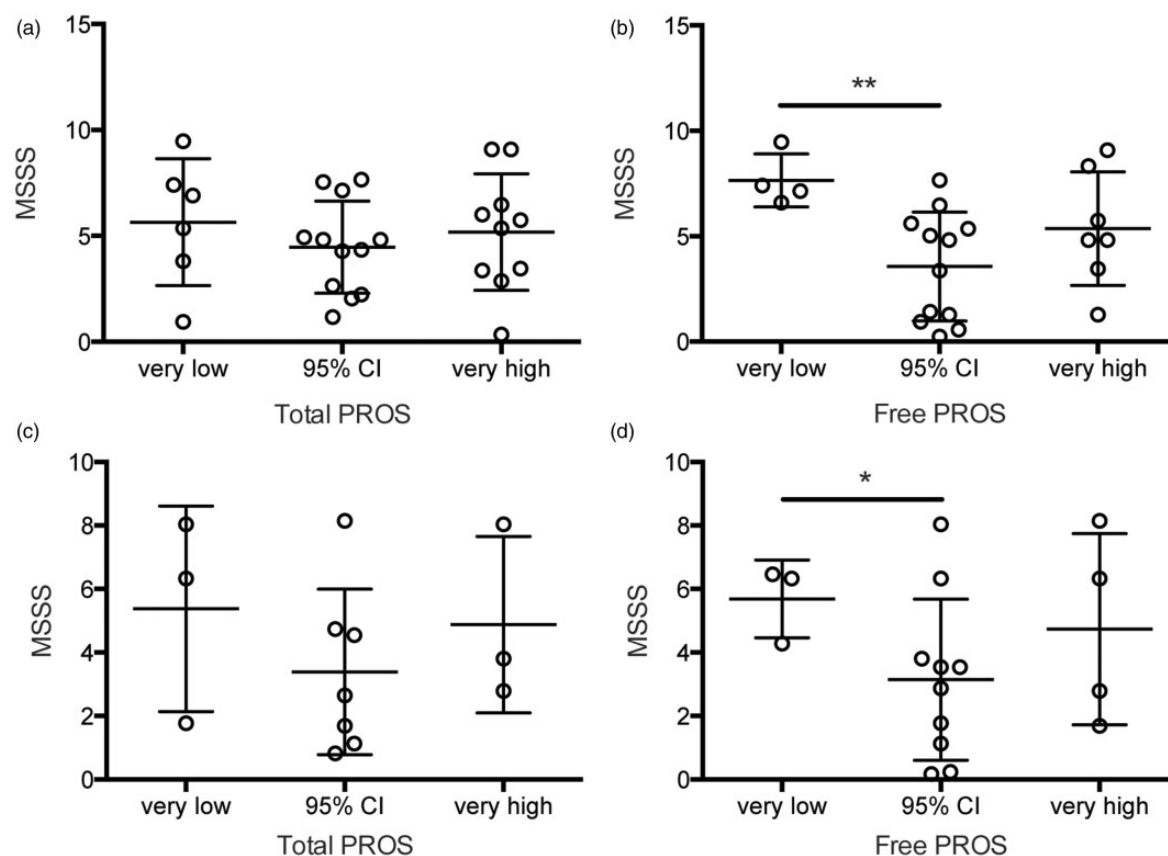
#### *Reduced circulating PROS is associated with increased clinical severity in MS*

In order to determine whether levels of PROS were associated with clinical outcomes in MS patients, we stratified all patients both from the discovery and replication cohorts by their PROS levels into groups based on their PROS levels. We found that discovery cohort patients with very low free PROS

exhibited greater severity, as indicated by higher MSSS scores, compared with patients whose free PROS levels fell within the 95% CI ( $7.7 \pm 1.3$  vs  $3.6 \pm 2.6$ ,  $p < 0.01$ ) (Figure 2(b)). In the replication cohort, patients with very low free PROS also displayed higher MSSS scores ( $5.7 \pm 1.2$  vs  $3.1 \pm 2.5$ ,  $p < 0.05$ ) (Figure 2(d)).

#### *Female MS patients have lower levels of circulating sMERTK*

As previous studies have shown that PROS has high affinity for MERTK,<sup>8,25,26</sup> we investigated in our replication cohort whether lower plasma levels of PROS in MS patients were correlated with differences in plasma concentration of sMERTK between MS patients and controls. We found no significant difference in plasma sMERTK concentration



**Figure 2.** Plasma levels of free, but not total Protein S (PROS) are associated with multiple sclerosis disease activity. (a), (b) Patients in the discovery cohort were stratified into groups based on plasma levels of total and free PROS. (c), (d) Patients in the replication cohort stratified into groups based on levels of total and free PROS. Dot plots show mean MSSS  $\pm$  standard deviation ( $*p < 0.05$ ;  $**p < 0.01$ . Student's *t*-test with Welch's correction).

between MS patients and controls (Figure 3(a)). However, when we stratified our subjects by sex, we found that female MS patients also had lower plasma sMERTK concentration compared with female HCs ( $8.0 \pm 3.9$  ng/ml vs  $9.3 \pm 2.7$  ng/ml,  $p < 0.05$ ) (Figure 3(b)). However, we did not find any correlation between plasma sMERTK concentration and plasma levels of PROS (data not shown).

*The level of circulating sCD163 is positively correlated with the level of free PROS*

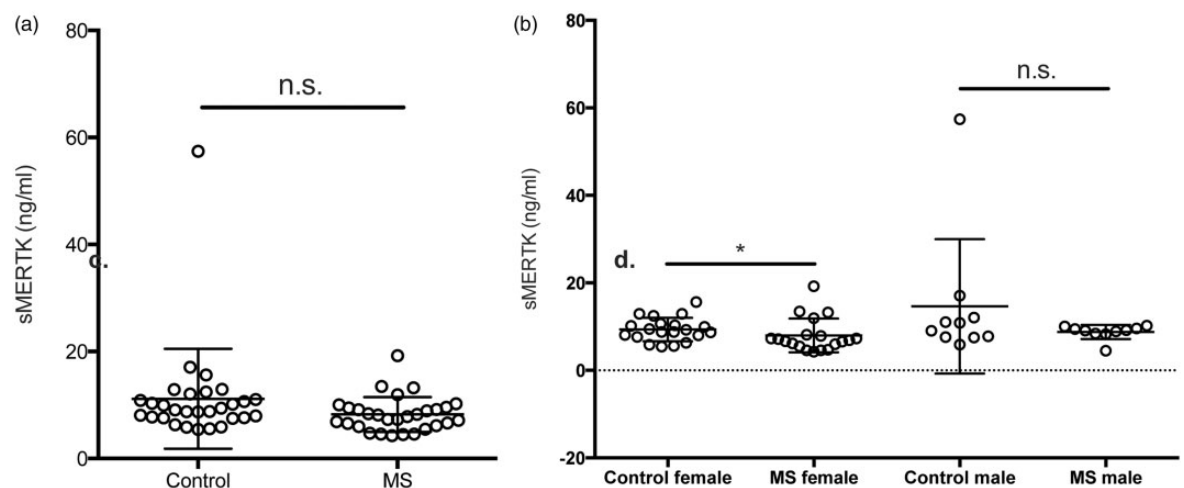
We also searched for a link between PROS levels and plasma concentration of sCD163, a marker of M2c macrophage activation, in our replication cohort. We found a trend towards an increase in circulating sCD163 in the plasma of MS patients compared with HCs ( $63.4 \pm 29.3$  ng/ml vs  $51.5 \pm 33.1$  ng/ml,  $p = 0.067$ ) (Figure 4(a)), with no sex differences (data not shown). We observed a positive correlation between plasma sCD163 concentration and free PROS levels ( $R^2 = 0.17$ ,  $p < 0.05$ ) (Figure 4(c)). No correlation was observed between plasma sCD163 concentration and total PROS levels (Figure 4(b)).

### Discussion

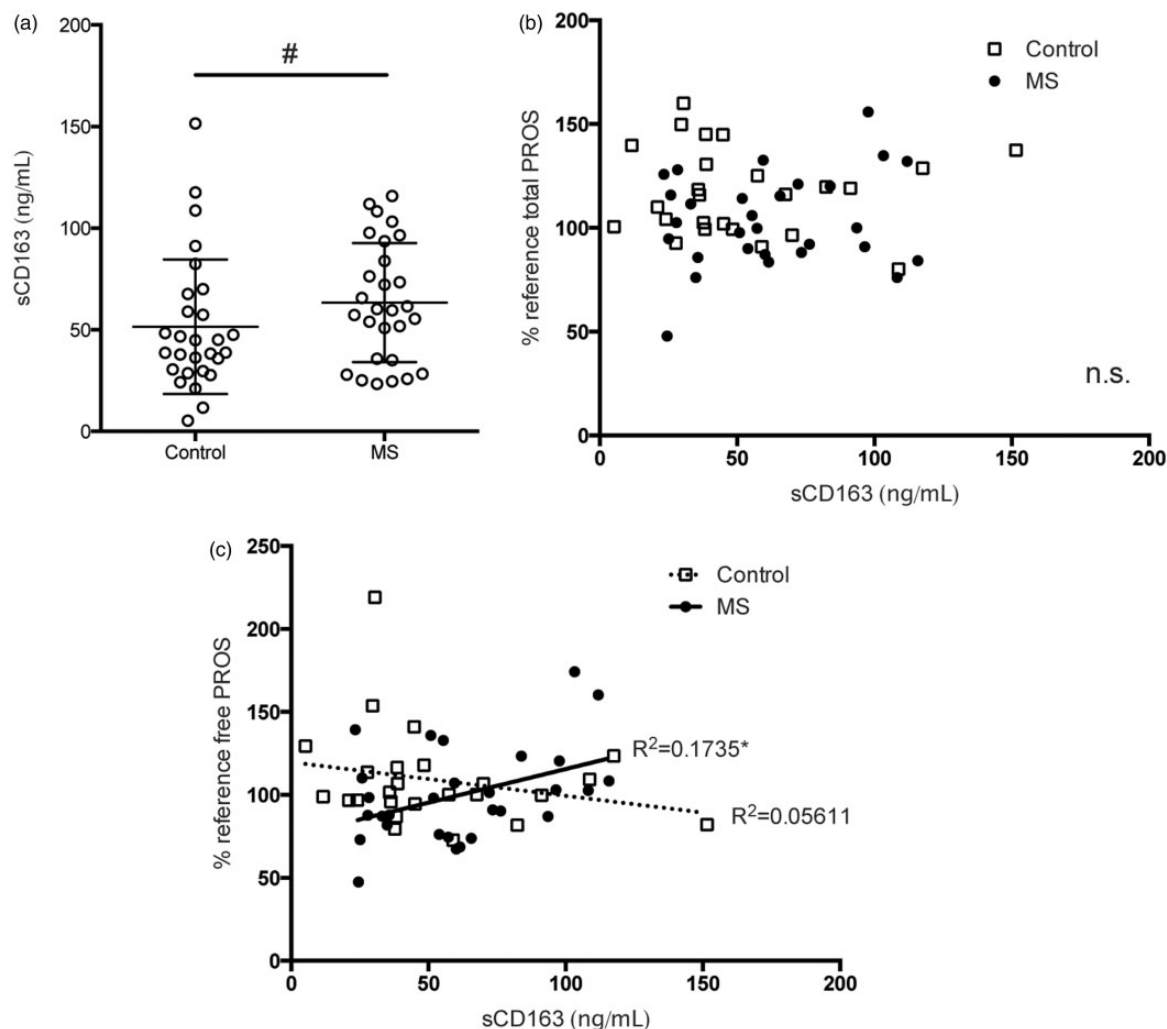
The present study shows that the TAM ligand PROS is decreased in the plasma of MS patients in the absence of acute inflammatory activity. In addition, we also found that low-plasma free PROS is associated with increased MS severity scores, suggesting that low PROS could be associated with worse disease severity. Signalling through TAM receptors has

been shown to play an important role in regulating immune responses, particularly that of innate immune cells.<sup>27</sup> As TAM signalling has previously been shown to limit the activity of innate immune cells in response to inflammatory cues through upregulation of suppressor of cytokine signalling protein expression,<sup>28</sup> a low level of plasma PROS could be directly associated with greater deleterious innate immune cell activity. In particular, the MERTK receptor has previously been implicated in promoting tolerogenic responses of innate immune cells.<sup>3,8</sup> Specifically, wild-type DCs from nonobese diabetic (NOD) mice undergo inhibition upon treatment with apoptotic cells; NOD mice DCs lacking a functional MERTK receptor were resistant to apoptotic cell-induced tolerance.<sup>29</sup> We observed a positive correlation between free PROS and sCD163 in MS patients, such that lower circulating PROS was correlated with lower sCD163, a marker of the homeostatic anti-inflammatory M2c macrophages.<sup>30</sup> Given the previous work showing that TAM signalling induces tolerance in M2c macrophages,<sup>3</sup> these data suggest a potential role for PROS signalling through MERTK to promote tolerance. In particular, the increased MSSS observed in patients with very low levels of PROS may be a reflection of dysregulation of tolerance in innate immune cells, and specifically decreased activation of M2c anti-inflammatory macrophages, leading to increased disease severity.

The role of TAM signalling in regulating innate immune responses is also supported by a recent



**Figure 3.** Plasma levels of soluble MERTK (sMERTK) are decreased in female, but not male, multiple sclerosis patients. (a) Plasma concentrations of sMERTK were measured by enzyme-linked immunosorbent assay (ELISA) both in patients with MS and healthy controls from the replication cohort. (b) Plasma concentrations of sMERTK in replication cohort stratified by sex. Dot plots show mean plasma concentration of sMERTK  $\pm$  standard deviation ( $*p < 0.05$ ; n.s. = not significant. Mann-Whitney test).



**Figure 4.** Plasma levels of soluble CD163 (sCD163) are positively correlated with free Protein S (PROS) levels in multiple sclerosis patients. (a) Plasma concentrations of sCD163 were measured by enzyme-linked immunosorbent assay (ELISA) both in patients with MS and healthy controls from the replication cohort. (b) Plasma concentrations in the replication cohort stratified by sex. (c) Scatter plot of sCD163 concentration vs PROS levels. All dot plots show mean plasma concentration of sCD163  $\pm$  standard deviation ( $*p < 0.05$ ;  $\#p = 0.067$ , Mann-Whitney test).

study showing that T cell-derived PROS limits the activation of DCs. In this study, the authors showed that transfer of T cells deficient for PROS in a mouse model of induced colitis resulted in exacerbated disease and increased numbers of pro-inflammatory T cells.<sup>9</sup> In addition, it was shown that T cell-derived PROS limited DC activation through engagement of Axl and MERTK on DCs.<sup>9</sup> In this context, it may be that circulating PROS is also available to bind to phosphatidylserine on T cells, and enable immunosuppressive interactions with DCs. As DCs are key in the initiation of adaptive immune cell responses by presenting antigen, a decrease in PROS during MS could enhance DC activity, leading to increased

disease severity. However, further studies are needed in order to replicate the association of very low PROS levels and increased MSSS, and also to examine the relationship between very low PROS and DC capacity to present antigen. Relatively impaired TAM system activation has also been observed in the autoimmune disease SLE, where those patients with a history of serositis, neurologic disorder, haematologic disorder and immunologic disorder had lower PROS.<sup>14</sup>

Interestingly, when we stratified our data by sex, we observed that the decreased plasma PROS in female MS patients was found both for free and total PROS.

In addition, all patients who had very low free PROS and higher MSSS than those that fell within the 95% CI both in the discovery and replication cohorts were females. The level of circulating PROS is known to be regulated by a number of factors, including liver disease, disseminated intravascular coagulation, as well as sex hormones.<sup>31</sup> Therefore, the sex difference we observed may be related to the regulation of PROS expression by female sex hormones, as it was previously shown that PROS expression is upregulated by progesterone<sup>31</sup> and downregulated by 17 $\beta$ -oestradiol.<sup>32</sup> We observed that PROS levels were generally lower in females compared with males, which could reflect the downregulation of PROS by 17 $\beta$ -oestradiol. Due to the lower baseline level of PROS in females compared with males, we speculate that a further decrease in PROS levels could result in more significant activation of deleterious innate immune cell responses in MS. It has long been known that MS has a female predominance,<sup>1</sup> and that pregnancy is accompanied by a decrease in the number of MS relapses, followed by a rebound in symptoms postpartum.<sup>33</sup> It is interesting to note that progesterone levels are increased during pregnancy and decrease following delivery. Since PROS expression is upregulated by progesterone, we hypothesise that some of these effects could be mediated by steroid-hormone-associated PROS regulation.

We also analysed whether decreased PROS levels were a result of changes in proteins that directly interact with it, and found that the decrease was not associated with changes in the C4BP. Although we also observed a decrease in plasma concentration of the decoy receptor sMERTK in female MS patients, the sMERTK concentrations were, in fact, not correlated with PROS levels. However, decreased levels of the decoy receptor sMERTK in the plasma of MS patients could be independently important. Previous work has implicated TAM signalling in promoting leucocyte extravasation and inflammation,<sup>34</sup> and recent work has shown that MERTK expression is induced by tolerogenic stimuli.<sup>8</sup> Furthermore, despite MERTK being important in models of self-tolerance, there has also been work showing that lack of MERTK in a mouse model of spontaneous type 1 diabetes reduces inflammation in pancreatic islets and that the mice do not develop diabetes, implicating MERTK in thymic negative selection of autoreactive T cells.<sup>35</sup> Clearly, further investigation of the functional role of specific TAM receptor and ligand alterations within the range observed in MS is required and justified by our observations.

In conclusion, this study has shown that the TAM receptor ligand PROS is decreased in the plasma of female MS patients compared with controls, and that low levels of circulating PROS are associated with increased MS disease severity. We also identified a positive correlation between sCD163, a marker of anti-inflammatory M2c macrophages, and circulating PROS, potentially linking lowered levels of PROS with immune dysregulation. Taken with previous data implicating TAM signalling in central demyelination and autoimmune responses, these data provide evidence that the TAM system could be dysregulated in MS and suggest that PROS may represent a potential marker of disease severity in MS.

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### Conflict of interest

None declared.

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