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Plasma levels of protein C pathway proteins and brain MRI volumes in multiple sclerosis

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Abstract

Background and purpose: The involvement of protein C (PC) pathway components in multiple sclerosis (MS) has been scarcely explored. The aim was to investigate their levels in relation to clinical and neurodegenerative MRI outcomes in patients.

Methods: 138 MS patients and 42 healthy individuals (HI) were studied. PC, protein S (PS), soluble endothelial protein C receptor (sEPCR) were evaluated by Multiplex assays and ELISA. Regression analysis between 3T MRI outcomes and PC pathway components were performed. ANCOVA was used to compare MRI volumes based on protein level quartiles. Partial correlation was assessed among levels of PC pathway components and with hemostasis protein levels, including soluble thrombomodulin (sTM), heparin cofactor II (HCII), plasminogen activator inhibitor-1 (PAI-1), and factor XII (FXII).

Variation of PC concentration across four time-points was evaluated in 32 additional MS patients.

Results: There was an association between PC concentration, mainly reflecting the zymogen PC, and MRI measures for volumes of total gray matter (GM, p=0.003), thalamus (p=0.007), cortex (p=0.008), deep GM (p=0.009), and whole brain (p=0.026). Patients in the highest PC level quartile were characterized by the lowest GM volumes. Correlations of PC-HCII, PC-FXII, and sEPCR-sTM values were detectable in MS patients, while PC-PS and PS-PAI-1 correlations were present in HI only.

Conclusions: PC plasma concentrations might be associated with neurodegenerative MRI outcomes in MS. Several differences in correlation among protein plasma levels suggest

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dysregulation of PC pathway components in MS patients. Stability of PC concentration over-time supports PC investigation in relation to GM atrophy in MS.

Keywords

multiple sclerosis; coagulation; coagulation inhibitors; MRI

INTRODUCTION

Increased blood-brain barrier (BBB) permeability characterizes multiple sclerosis (MS) pathogenesis, leading to the irruption of hemostasis factors into the central nervous system (CNS), which sustain inflammatory responses and immune activation [1]. FXII, the initiator of intrinsic coagulation, was found to be involved in adaptive immune responses via urokinase receptor (uPAR) activations [2] and showed increased expression in MS lesions [3]. In an animal model, the immunotherapy targeting specific cryptic epitopes of fibrin, produced by thrombin cleavage of fibrinogen, has shown to avoid microglia activation, infiltration into CNS of immune cells, and to reduce the neurotoxicity caused by oxidative stress [4].

Considering these observations, proteins inhibiting thrombin formation with anti-inflammatory properties are candidates to play a protective role in MS progression. Protein C (PC) is a plasma zymogen whose activation is mediated by the complex of thrombin with thrombomodulin (TM) (Figure S1), favored by the binding of PC to its endothelial receptor (EPCR). Activated protein C (aPC) with the cofactor protein S (PS) displays an anticoagulant function inactivating factors Va and VIIIa, which in turn affect thrombin generation and the final production of fibrin (Figure S2).

As a signaling molecule, EPCR-bound aPC cleaves proteinase-activated receptors (PARs), producing several cytoprotective, anti-apoptotic, and anti-inflammatory pleiotropic effects, thus favoring endothelial barrier stabilization, neurogenesis, and angiogenesis [5].

The involvement of PC pathway components in the neurodegenerative process in MS patients, is heavily supported by proteomic profiles within chronic active plaques, which detected the presence of PC inhibitor (PCI) [6]. Moreover, a number of experimental observations in cells and animal models might foster investigation of PC pathway proteins in MS.

EPCR is largely expressed by endothelial cells, and in addition, it has been shown to be present on membranes of immune cells, among them T helper 17 (Th17) cells, where it inhibits their proinflammatory actions [7, 8]. In particular, in the experimental autoimmune encephalomyelitis (EAE) animal model of MS, EPCR deficiency promoted exacerbation and pathogenic Th17 cells response through reduced activation of the aPC-mediated pathway [8]. Moreover, shedding of EPCR and TM, which increases the soluble circulating pool (sEPCR and sTM), reduces their availability at the membrane level for protein C activation, thus contributing to a shift in activities of thrombin towards pro- thrombotic and inflammatory actions [9–11]. Noticeably, treatment of EAE with recombinant aPC significantly ameliorated the pathological condition, which provided evidence that both aPC

anticoagulant and signaling functions are required for improving the disease condition [6]. Moreover, impaired TM ($TM^{Pro/Pro}$)-mediated PC activation perturbed myelination and mitochondrial function, and aggravated the induced EAE pathology [12]. Administration of aPC or recovery of TM expression [12] relieved EAE in the $TM^{Pro/Pro}$ mice.

Besides the cofactor role as inhibitor of hemostasis [13], PS can activate tyrosine kinase TAM receptors expressed by endothelial cells, astrocytes and immune cells, including macrophages and dendritic cells, thus regulating the innate immune response, inhibiting inflammation, and stimulating both apoptotic cell clearance and synapse remodeling as well as promoting blood-brain barrier integrity [14–17].

Overall, based on the aforementioned experimental evidence, PC pathway components could mediate in MS the crosstalk between coagulation, inflammation, and immunity.

In this study, we investigated plasma levels of several components of the PC pathway and correlated protein concentrations with clinical features and neurodegenerative magnetic resonance imaging (MRI) outcomes.

MATERIALS AND METHODS

Study Population

The observational, cross-sectional study included individuals who were part of the cardiovascular, environmental and genetic (CEG) study [18–21]. The study participants underwent MRI examination within 30 days from the neurological visit where Expanded Disability Status Scale (EDSS) scores was obtained. Inclusion and exclusion criteria, demographic and clinical information are summarized in Supplement Material and Table S1. Secondary-progressive (SP, n=46) and primary-progressive (PP, n=7) MS patients were categorized as progressive MS (P-MS).

A second study population, including 32 P-MS patients (Table S2) who participated in the RAGTIME study [22, 23 [24]], was investigated.

The study protocols were approved by the institutional review boards of University at Buffalo, State University of New York (USA) (CEG study ID:MODCR00000352) and of University/Hospital of Ferrara (Italy) (CEG study ID:170585; RAGTIME study ID:101–2012).

Assays for antigen levels

In the CEG study, EDTA plasma samples were collected at the time of the MRI examination and stored at−80 °C until protein analysis.

In the RAGTIME study, blood samples were collected in sodium-citrate at four time points [23, 25]) and plasma aliquots were stored at −80 °C until use.

sEPCR levels were determined by a commercial ELISA (Human EPCR DuoSet, DY2245, R&D Systems Inc., Minneapolis, MN, USA).

Samples were diluted 1:50 and assayed in duplicate for both PC and sEPCR antigen quantification.

A sandwich ELISA was developed for PS detection. Polyclonal sheep anti-human PS antibody (H.T.I., Vermont, USA) was coated to microtiter plate (Nunc, MaxiSorp®, Denmark). Bound PS, from 1:4000 diluted plasma samples, was detected with polyclonal rabbit anti-PnmS antibody (Dako, Glostrup, Denmark) and with a polyclonal goat peroxidase-conjugated antibody.

A bead-based array was used to measure PC levels (Luminex R&D Systems Inc., Minneapolis, MN, USA). The data analysis occurred through Bioplex Manager Software 6.0 (Biorad Laboratories, Hercules, CA).

The inter-assay coefficients of variability for EDTA plasma were: PC 11.4%, sEPCR 6.6%, PS 1.7%. The intra-assay and the inter-assay coefficient of variations for PC evaluated in sodium citrate plasma were 5.8%, and 9.9%, respectively.

Methods for sTM, heparin cofactor II (HCII), plasminogen activator inhibitor-1(PAI-1) and FXII have been published [18].

Spike-and-recovery PC assay

Since the PC concentrations according to the internal standard curve of the assay were far from the physiological values of zymogen PC and aPC (70nM and 40pM, respectively [5]), additional standard curves for zymogen PC (Human Protein C, HCPC-0070, Hematologic Technologies), and for aPC (activated human PC-DEGR, HCAPC-DEGR, Hematologic Technologies) were obtained.

EDTA plasma samples with low PC levels were spiked by increasing concentrations of zymogen PC (40 ng/mL to 120 ng/mL) or aPC (2,5 ng/mL to 50 ng/mL) to compare the relative ability of the assay to recognize either zymogen PC or aPC.

MRI Acquisition and Image analysis

Brain MRI examination was performed as previously reported [18, 20] and summarized in Supplement Material.

MRI-derived lesion, global, and regional brain volumes of the cohort are reported in Table S1.

Statistical Analysis

Analyses were performed using IBM® SPSS® Statistics version 24 software (IBM Corp. Armonk, NY, USA) and GraphPad Prism version 6.01 (GraphPad Software, Inc. La Jolla, CA, USA). Detailed statistical analysis is reported in Supplement Material.

RESULTS

Plasma levels of PC, PS, and sEPCR

Levels of PC, sEPCR, and PS are reported in Table 1. Individual PC values are shown in Figure S2.

In order to compare circulating levels among groups, the natural logarithm values of PC and sEPCR were extrapolated and ANCOVA was adjusted for age and sex. No significant difference between MS and HI were detected for PC (p=0.095), PS (p=0.403), and sEPCR (p=0.979). Similarly, no differences among HI, relapsing-remitting MS (RR-MS) and P-MS were observed for PC (p=0.211), PS (p=0.689) and sEPCR (p=0.689).

Association of plasma levels of PC pathway proteins and selected hemostasis components

The estimate of the correlations among levels of PC pathway proteins showed differences between MS patients and HI (Table 2).

PC and its cofactor PS concentrations were positively associated (rho=0.434, P=0.005) in HI. Differently, in patients this correlation was not detectable (rho=0.076, P=0.383).

The analysis of correlations was extended to TM [18], a key protein for PC activation by thrombin. In MS group, higher levels of sEPCR correlated to lower levels of sTM (rho= −0.213, P=0.014). To note, this association, not detectable in HI, was mostly supported by the P-MS group as indicated by the higher coefficient of correlation (rho=−0.367, p=0.008).

Correlations of the PC pathway members were studied in relation to selected hemostasis components (Table 2), previously investigated in the same cohorts of patients and HI [18]. PC levels positively correlated with those of PAI-1 both in HI group (rho=0.408, P=0.009) and MS patients (rho=0.269, P=0.002). The association was detected both in RR-MS (rho=0.221, P=0.045) and in the P-MS groups (rho=0.360, P=0.010). PS levels correlated with those of PAI-1 only in HI (rho=0.412, P=0.008). Only in the MS group, higher levels of PC showed association with lower levels of HCII (q = −0.271, P = 0.001, also in RR-MS, q = −0.274, P = 0.012) and higher levels of FXII (q = 0.281, P = 0.001), which was observed in both MS clinical phenotypes (PC-FXII q = 0.248, P = 0.024; P-MS, PC-FXII, q = 0.323, P = 0.021). In P-MS only, PS levels were positively associated with those of FXII (rho=0.301, P=0.032).

Overall, three correlations (PC-HCII, PC-FXII, sTM-sEPCR) were detectable only in MS patients. Conversely, two correlations (PC-PS and PS-PAI-1), present in HI, were not observed in MS patients.

Association of PC, PS and sEPCR plasma levels with clinical findings

Only plasma levels of PS were associated with EDSS (Rho=0.172, p=0.049, Spearman) and disease duration (Rho=0.197, p=0.021, Spearman).

Levels of PC, PS, and sEPCR in MS patients according to DMTs are summarized in Table S3. No significant differences among DMT subgroups were present for PC (p=0.866), PS (p=0.381), and sEPCR (p=0.333) (Table S3).

Association of PC, PS, and sEPCR levels with MRI measures

Linear regression analysis of MRI measures with PC, PS and sEPCR levels was conducted (Tables 3, S4, and S5). Several significant associations were observed only for the PC levels (Table 3). The analysis by multiple regression produced five significant equations, F(3, 128), for PC: whole brain (WBV; F=12.6, p<0.001, adjusted R^2=0.21), cortical volume (CV; F=20.6, p<0.001, adjusted R^2=0.31), gray matter volume (GMV; F=21.1, p<0.001, adjusted R^2=0.315), deep GMV (DGMV; F=7.8, p<0.001, adjusted R^2=0.134), and thalamic volume (F=9.3, p<0.001, adjusted R^2=0.16).

In these models, PC was a significant predictor of WBV (p=0.026), CV (p=0.008), GMV (p=0.003), DGMV (p=0.009), thalamic volume (p=0.007) (Table 3), and was able to account for an additional 2.5%, 3.3%, 4.3%, 4% and 4.2%, respectively, of the variance in volumes, compared to age and sex alone.

A sporadic association by multiple regression analysis was detected in the MS cohort with the entry of sEPCR natural logarithm (T1-lesion volume (T1-LV) equation, F(3, 126)=2.8, p=0.039, adjusted R^2= 0.042). In this model, sEPCR was a significant predictor (p=0.010) and was able to account for an additional 4% of the variance in T1-LV, compared to age and gender alone.

Differently, no association by multiple regression analysis was found in the MS cohort with the entry of PS levels (Table S4). In the HI cohort, no significant associations were found for any of the protein levels in the multiple regression analyses (Tables 3, S4, S5).

MRI volumes associated with PC levels in the multiple regression analysis were further explored by comparison of PC values quartiles. Significant differences between the first and fourth quartile were observed for thalamus volume (18.8±2.0 mL vs 16.8±2.4 mL, p=0.006), DGMV (56.5±5.6 mL vs 50.8±6.8 mL, p=0.008), GMV (751.3±53.1 mL vs 701.1±58.0 mL, p=0.014), WBV (1468.7±79.6 mL vs 1399.7±82.1 mL, p=0.026) and CV (608.8±42.2 mL vs 571.2±44.4 mL, p=0.030), as indicated by ANCOVA adjusted for age and sex.

The GM volumes in patients grouped by PC quartiles are reported in Figure 1. Of note, in the fourth quartile the RR and P-MS patients were balanced.

PC levels over time

To assess the stability of PC levels over time, they were investigated in the RAGTIME-study cohort of P-MS patients over a 4-month period (Figure 2). PC levels were normally distributed and Individual values were highly correlated among time points (Figure 2). No significant difference over time was observed by ANOVA for repeated measures (p=0.564).

Spike-and-recovery PC assay

The ability of the assay to recognize zymogen PC or aPC was compared. The linear relationship between fluorescent intensities and the increased concentration, following the addition of zymogen PC or aPC in two plasma samples, are shown in Figure 3. The assay was able to detect aPC more efficiently than zymogen PC, as indicated by the slopes of fluorescence values in spiked plasmas (aPC, 7.34 and 7.38; zymogen PC 2.91 and 2.43, Figure 3).

Considering that physiological amounts of aPC form have been estimated to be approximately 1/100 of all circulating PC, even if the assay recognized the aPC 2.75 times better than the zymogen PC, the concentration of aPC minimally influences the total amount of PC detected in plasma. Therefore, the protein concentration detected by the assay is essentially that of zymogen PC.

DISCUSSION

We evaluated plasma levels of several proteins, which participate in the anticoagulant, anti-inflammatory and endothelial barrier-stabilizing PC pathway, in relation to ongoing CNS alterations in MS, an investigation that has not previously been reported [26–28].

To investigate protein involvement in MS pathophysiology, correlations among their levels were compared between patients and HI with the hypothesis that modified correlations could suggest dysregulated expression. This in turn could help to propose candidate disease biomarkers, among the numerous hemostasis components.

Here, in addition to a weak correlation of PS levels with EDSS and disease duration, we report evidence of correlation between higher peripheral plasma PC levels and more severe neurodegeneration in MS, as indicated by the associations with MRI measures of global, and cortical and DGM brain atrophy during early and advanced MS stages [29, 30]. As a matter of fact, thalamic atrophy is detected as one of the earliest signs of pathologic changes in subcortical GM, and cortical atrophy is a prominent sign of MS disease processes from the earliest disease stages [29, 30]. GM atrophy progresses to brain regions that are both functionally and/or structurally related to each other [29, 30]. The association between PC levels and brain volumes was highlighted by PC level quartiles analysis, showing that patients with the highest levels were characterized by the lowest GM volumes. The correlations between PC levels and GM volumes were particularly detectable in the group of female patients (data not shown). These observations support further investigation in relation to age, disease clinical phenotype and sex. Similarly, additional investigation is required to define DMTs influence, particularly the relation between oral DMTs-PS levels and natalizumab-sEPCR levels.

Our findings, and the observation that the inhibitor of activated PC, PCI, accumulated within chronic active plaques [6], jointly support the involvement of PC in MS pathophysiology. The observation of rather stable and highly correlated values of PC concentration over a 4-month period in patients, at least in a population of severely disabled P-MS patients, would favor further investigation of the contribution of PC plasma levels to the disease.

Prompted by the functional importance of the activated form of PC (aPC), which inhibits coagulation, activates protease-activated receptor (PAR) 1 and provides neuro- and vasculoprotection in experimental neuroinjury models [31], we investigated the relative ability of our assay to detect the inactive zymogen and aPC forms. Our results indicate that the assay is able to detect more efficiently the aPC, which however is masked by the 2-orders of magnitude higher concentration of inactive, zymogen PC in plasma.

Of note, the association of protein levels with MRI outcomes was not paralleled by differences either in PC levels or PS, sEPCR, and sTM between patients and HI. In particular, regarding PC levels, neither antigen serum levels [28] nor plasma activity [32] have been previously found dysregulated in the disease, in line with our plasma antigen evaluation.

Instead, finding in patients different correlations among protein levels led us to hypothesize that the relative amounts of functionally coordinated proteins are modified in MS. This study provides novel information about disease-associated dysregulation of PC pathway protein expression:

- **i.** The inverse correlation of sEPCR with sTM, detectable only in MS patients and particularly in P-MS, might reflect disease-related endothelium alteration, differentially affecting the release mechanisms from membranes of truncated proteins [33–36].
- **ii.** PC and PS levels, noticeably correlated in HI, did not display correlation in patients, suggesting the presence of a physiological association modified by the disease state.

Prompted by both observations, we extended our investigation within this study cohort, supported by the quantification in the same plasma samples of several hemostasis components [18]. We observed correlations, only in MS patients, of PC with i) HCII and ii) FXII:

- **i.** PC and HCII, which inhibit thrombin activation and activity, respectively, were inversely correlated. Of note, glycosaminoglycans increase as cofactors HCII function but decrease PC and PS activity and increase the sTM concentration [37]. Taking into account that glycosaminoglycans are involved in inflammation [38] and pathological CNS conditions [39], this will require further investigation in MS.
- **ii.** the positive association of PC-FXII levels in MS, and of PS-FXII only in P-MS, would favor the hemostatic balance through modulation of anticoagulant proteins (PC, PS) and a procoagulant factor (FXII). On the other hand, the stimulation of adaptive immune responses by FXII, previously found in CNS from MS patients [2] and investigated in MS plasma [23], and the association of PC levels with neurodegeneration would be both congruent with MS disease progression.

In conclusion this study provides novel findings regarding the relation between MS neurodegeneration and PC levels, scarcely investigated in MS patients, as well as hints about dysregulation of hemostasis pathways in the disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

[1]. Ziliotto N, Bernardi F, Jakimovski D, Zivadinov R. Coagulation Pathways in Neurological Diseases: Multiple Sclerosis. Front Neurol. 2019 10: 409. [PubMed: 31068896]

[2]. Gobel K, Pankratz S, Asaridou CM, et al. Blood coagulation factor XII drives adaptive immunity during neuroinflammation via CD87-mediated modulation of dendritic cells. Nat Commun. 2016 7: 11626. [PubMed: 27188843]

[3]. Gveric D, Hanemaaijer R, Newcombe J, van Lent NA, Sier CF, Cuzner ML. Plasminogen activators in multiple sclerosis lesions: implications for the inflammatory response and axonal damage. Brain. 2001 124: 1978–1988. [PubMed: 11571216]

[4]. Ryu JK, Rafalski VA, Meyer-Franke A, et al. Fibrin-targeting immunotherapy protects against neuroinflammation and neurodegeneration. Nat Immunol. 2018 19: 1212–1223. [PubMed: 30323343]

[5]. Griffin JH, Zlokovic BV, Mosnier LO. Activated protein C, protease activated receptor 1, and neuroprotection. Blood. 2018 132: 159–169. [PubMed: 29866816]

[6]. Han MH, Hwang SI, Roy DB, et al. Proteomic analysis of active multiple sclerosis lesions reveals therapeutic targets. Nature. 2008 451: 1076–1081. [PubMed: 18278032]

[7]. Gavlovsky PJ, Tonnerre P, Guitton C, Charreau B. Expression of MHC class I-related molecules MICA, HLA-E and EPCR shape endothelial cells with unique functions in innate and adaptive immunity. Hum Immunol. 2016 77: 1084–1091. [PubMed: 26916837]

[8]. Kishi Y, Kondo T, Xiao S, et al. Protein C receptor (PROCR) is a negative regulator of Th17 pathogenicity. J Exp Med. 2016 213: 2489–2501. [PubMed: 27670590]

[9]. Xu J, Qu D, Esmon NL, Esmon CT. Metalloproteolytic release of endothelial cell protein C receptor. J Biol Chem. 2000 275: 6038–6044. [PubMed: 10681599]

[10]. van Hinsbergh VW. Endothelium--role in regulation of coagulation and inflammation. Semin Immunopathol. 2012 34: 93–106. [PubMed: 21845431]

[11]. Loghmani H, Conway EM. Exploring traditional and nontraditional roles for thrombomodulin. Blood. 2018 132: 148–158. [PubMed: 29866818]

[12]. Wolter J, Schild L, Bock F, et al. Thrombomodulin-dependent protein C activation is required for mitochondrial function and myelination in the central nervous system. J Thromb Haemost. 2016 14: 2212–2226. [PubMed: 27590316]

[13]. Castoldi E, Hackeng TM. Regulation of coagulation by protein S. Curr Opin Hematol. 2008 15: 529–536. [PubMed: 18695379]

[14]. Zhu D, Wang Y, Singh I, et al. Protein S controls hypoxic/ischemic blood-brain barrier disruption through the TAM receptor Tyro3 and sphingosine 1-phosphate receptor. Blood. 2010 115: 4963–4972. [PubMed: 20348395]

[15]. Chung WS, Clarke LE, Wang GX, et al. Astrocytes mediate synapse elimination through MEGF10 and MERTK pathways. Nature. 2013 504: 394–400. [PubMed: 24270812]

[16]. Lemke G. Biology of the TAM receptors. Cold Spring Harb Perspect Biol. 2013 5: a009076. [PubMed: 24186067]

[17]. Lumbroso D, Soboh S, Maimon A, Schif-Zuck S, Ariel A, Burstyn-Cohen T. Macrophage-Derived Protein S Facilitates Apoptotic Polymorphonuclear Cell Clearance by Resolution Phase Macrophages and Supports Their Reprogramming. Front Immunol. 2018 9: 358. [PubMed: 29545796]

[18]. Ziliotto N, Bernardi F, Jakimovski D, et al. Hemostasis biomarkers in multiple sclerosis. Eur J Neurol. 2018 25: 1169–1176. [PubMed: 29758118]

[19]. Zivadinov R, Ramasamy DP, Benedict RR, et al. Cerebral Microbleeds in Multiple Sclerosis Evaluated on Susceptibility-weighted Images and Quantitative Susceptibility Maps: A Case-Control Study. Radiology. 2016 281: 884–895. [PubMed: 27308776]

[20]. Ziliotto N, Bernardi F, Jakimovski D, et al. Increased CCL18 plasma levels are associated with neurodegenerative MRI outcomes in multiple sclerosis patients. Mult Scler Relat Disord. 2018 25: 37–42. [PubMed: 30031282]

[21]. Ziliotto N, Zivadinov R, Jakimovski D, et al. Plasma levels of soluble NCAM in multiple sclerosis. J Neurol Sci. 2019 396: 36–41. [PubMed: 30412901]

[22]. Straudi S, Manfredini F, Lamberti N, Martinuzzi C, Maietti E, Basaglia N. Robot-assisted gait training is not superior to intensive overground walking in multiple sclerosis with severe disability (the RAGTIME study): A randomized controlled trial. Mult Scler. 2019: 1352458519833901. [PubMed: 30829117]

[23]. Ziliotto N, Baroni M, Straudi S, et al. Coagulation Factor XII Levels and Intrinsic Thrombin Generation in Multiple Sclerosis. Front Neurol. 2018 9: 245. [PubMed: 29731736]

[24]. Straudi S, Manfredini F, Lamberti N, et al. The effectiveness of Robot-Assisted Gait Training versus conventional therapy on mobility in severely disabled progressIve MultiplE sclerosis patients (RAGTIME): study protocol for a randomized controlled trial. Trials. 2017 18: 88. [PubMed: 28241776]

[25]. Marchetti G, Ziliotto N, Meneghetti S, et al. Changes in expression profiles of internal jugular vein wall and plasma protein levels in multiple sclerosis. Mol Med. 2018 24: 42. [PubMed: 30134823]

[26]. Ma GZ, Giuffrida LL, Gresle MM, et al. Association of plasma levels of Protein S with disease severity in multiple sclerosis. Mult Scler J Exp Transl Clin. 2015 1: 2055217315596532. [PubMed: 28607700]

[27]. Festoff BW, Li C, Woodhams B, Lynch S. Soluble thrombomodulin levels in plasma of multiple sclerosis patients and their implication. J Neurol Sci. 2012 323: 61–65. [PubMed: 22967748]

[28]. Balkuv E, Varoglu AO, Isik N, et al. The effects of thrombomodulin and activated protein C on the pathogenesis of multiple sclerosis. Mult Scler Relat Disord. 2016 8: 131–135. [PubMed: 27456888]

[29]. Bergsland N, Horakova D, Dwyer MG, et al. Gray matter atrophy patterns in multiple sclerosis: A 10-year source-based morphometry study. Neuroimage Clin. 2018 17: 444–451. [PubMed: 29159057]

[30]. Zivadinov R, Jakimovski D, Gandhi S, et al. Clinical relevance of brain atrophy assessment in multiple sclerosis. Implications for its use in a clinical routine. Expert Rev Neurother. 2016 16: 777–793. [PubMed: 27105209]

[31]. Lyden P, Pryor KE, Coffey CS, et al. Final Results of the RHAPSODY Trial: A Multi-Center, Phase 2 Trial Using a Continual Reassessment Method to Determine the Safety and Tolerability of 3K3A-APC, A Recombinant Variant of Human Activated Protein C, in Combination with Tissue Plasminogen Activator, Mechanical Thrombectomy or both in Moderate to Severe Acute Ischemic Stroke. Ann Neurol. 2019 85: 125–136. [PubMed: 30450637]

[32]. Gobel K, Kraft P, Pankratz S, et al. Prothrombin and factor X are elevated in multiple sclerosis patients. Ann Neurol. 2016 80: 946–951. [PubMed: 27774643]

[33]. Menschikowski M, Hagelgans A, Eisenhofer G, Siegert G. Regulation of endothelial protein C receptor shedding by cytokines is mediated through differential activation of MAP kinase signaling pathways. Exp Cell Res. 2009 315: 2673–2682. [PubMed: 19467228]

[34]. Rochfort KD, Cummins PM. Thrombomodulin regulation in human brain microvascular endothelial cells in vitro: role of cytokines and shear stress. Microvasc Res. 2015 97: 1–5. [PubMed: 25250518]

[35]. Qu D, Wang Y, Esmon NL, Esmon CT. Regulated endothelial protein C receptor shedding is mediated by tumor necrosis factor-alpha converting enzyme/ADAM17. J Thromb Haemost. 2007 5: 395–402. [PubMed: 17155946]

[36]. Sugiyama S, Hirota H, Kimura R, et al. Haplotype of thrombomodulin gene associated with plasma thrombomodulin level and deep vein thrombosis in the Japanese population. Thromb Res. 2007 119: 35–43. [PubMed: 16507317]

[37]. Yuan C, Yang S, Wang H, Cui Q. Effects of glycosaminoglycan from Mactra veneriformis on the protein C system and expression of relevant factors in human umbilical vein endothelial cells. Blood Coagul Fibrinolysis. 2016 27: 64–69. [PubMed: 26340457]

[38]. Taylor KR, Gallo RL. Glycosaminoglycans and their proteoglycans: host-associated molecular patterns for initiation and modulation of inflammation. FASEB J. 2006 20: 9–22. [PubMed: 16394262]

[39]. Smith PD, Coulson-Thomas VJ, Foscarin S, Kwok JC, Fawcett JW. “GAG-ing with the neuron”: The role of glycosaminoglycan patterning in the central nervous system. Exp Neurol. 2015 274: 100–114. [PubMed: 26277685]

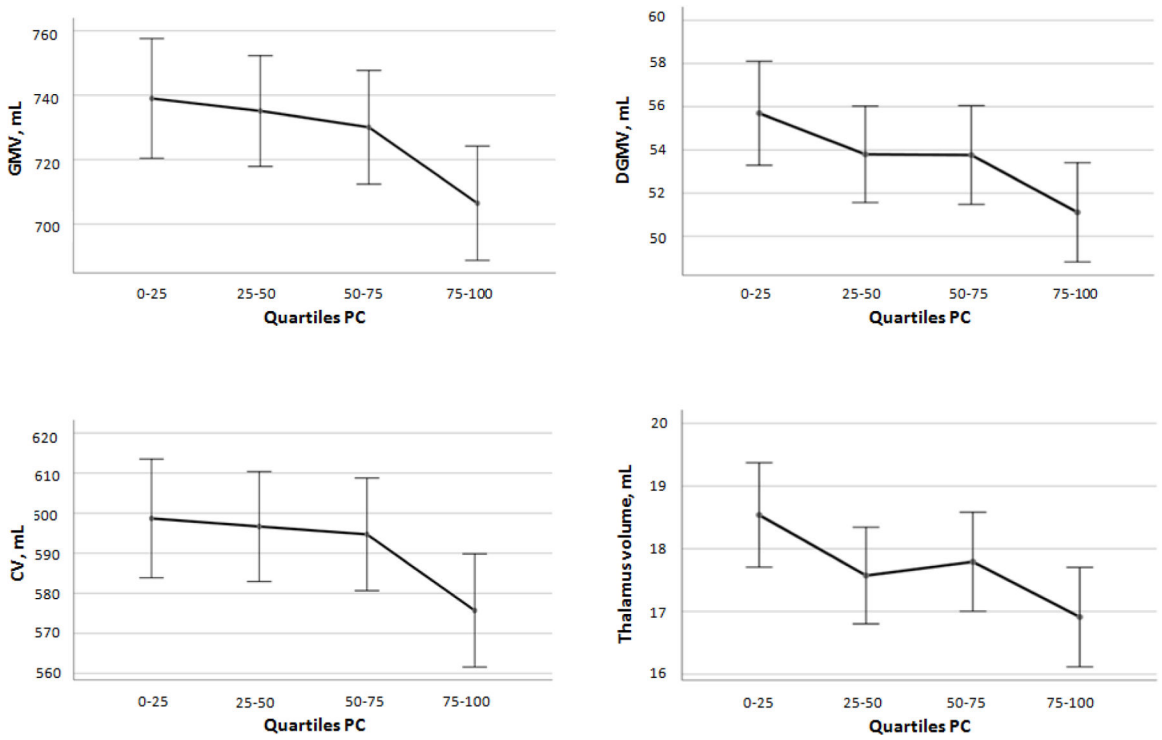
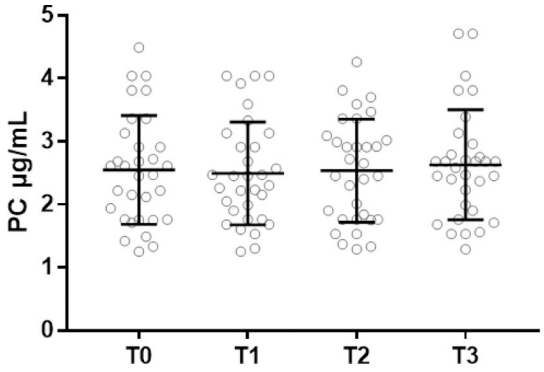


Figure 1.

Gray matter volumes according to PC quartiles of values.

For each quartile group the estimate mean is reported with the 95% confidence interval (bars), using ANCOVA adjusted for age and gender.



		T0	T1	T2
T1	Rho	0.873		
	P-value	<0.0001		
T2	Rho	0.839	0.802	
	P-value	<0.0001	<0.0001	
T3	Rho	0.747	0.735	0.668
	P-value	<0.0001	<0.0001	<0.0001

Figure 2.

PC plasma concentration and correlation in multiple sclerosis patients over four time points. Time points of RAGTIME-study cohort: (T0) baseline; (T1) half month after T0; (T2) after one month after T0; (T3) 4 months after T0.

Mean values ± SD are shown. Pearson's correlation coefficient and P-values are reported for PC concentration over time points.

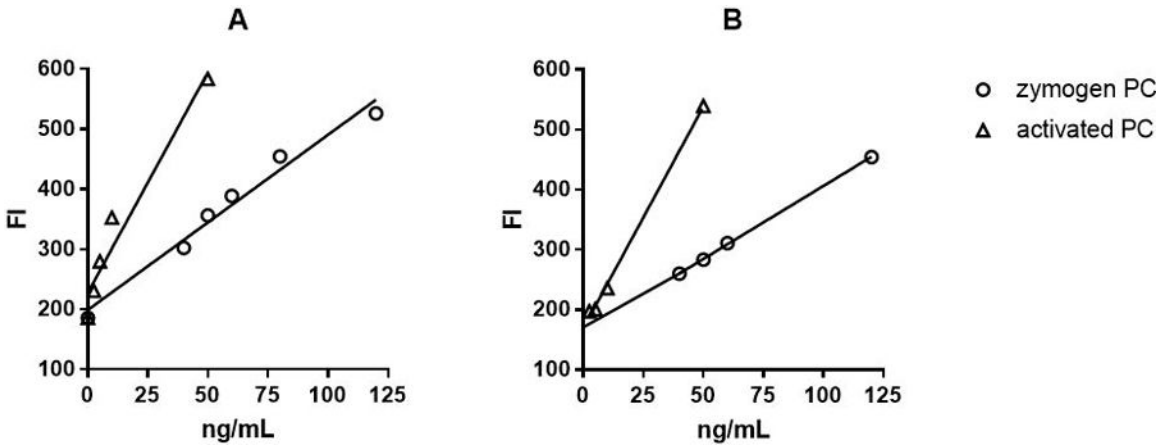


Figure 3.

Spike-and-recovery PC assay.

Increased concentrations of zymogen PC and activated PC were plotted in relation to the respective fluorescent intensity (FI).

A) plasma of a healthy individual, slope zymogen PC 2.915±0.231, R^2=0.976; slope activated PC 7.344±0.982, R^2=0.949.

B) plasma of a MS patient, slope zymogen PC 2.43±0.027, R^2=0.999; slope activated PC 7.387±0.241, R^2=0.998.

Table 1.

Levels of PC, PS and sEPCR.

	Multiple Sclerosis						Healthy Individuals		
	Female	**Male**	**All**	**RR-MS**	**P-MS**		**Female**	**Male**	**All**
N	**100**	**38**	**138**	**85**	**53**	***N***	**31**	**11**	**42**
PC (μg/mL)									
Median (IQR)	2.27 (1.82–2.99)	2.46 (1.95–3.63)	2.34 (1.88–3.02)	2.15 (1.80–2.89)	2.50 (2.12–3.19)		2.29 (1.57–2.80)	1.84 (1.4–2.57)	2.28 (1.54–2.69)
PS (mg/L)									
Mean±SD	32.0±7.5	29.3±5.4	31.3±7.0	30.6±7.7	32.3±5.6		31.8±6.6	32.1±7.1	31.9±6.7
sEPCR (ng/mL)									
Median (IQR)	25.9 (23.0–29.9)	28.6 (26.1–32.6)	26.6 (23.3–31.2)	26.6 (23.3–30.1)	27.0 (23.4–32.4)		26.0 (22.4–29.8)	27.9 (23.4–29.6)	26.1 (23.0–29.7)

MS, Multiple Sclerosis; RR-MS, Relapsing Remitting Multiple Sclerosis; P-MS, Progressive Multiple Sclerosis; HI, Healthy Individuals; N, number; PC, protein C; PS, protein S; sEPCR, soluble endothelial protein C receptor.

Table 2.

Correlations among protein levels in multiple sclerosis patients and healthy individuals.

		PC	PS	sEPCR		PC	PS	sEPCR		PC	PS	sEPCR		PC	PS	sEPCR
All Multiple Sclerosis patients						**RR- MS**				**P- MS**				**Healthy Individuals**		
PS	*Rho:* *P value:*	0.076 0.383				0.066 0.555				0.147 0.303				**0.434** **0.005**		
sEPCR	*Rho:* *P value:*	0.111 0.204	−0.011 0.905			0.134 0.236	0.101 0.377			0.048 0.741	−0.180 0.206			−0.030 0.858	0.270 0.097	
sTM	*Rho:* *P value:*	0.099 0.251	0.079 0.365	**−0.213** **0.014**		0.092 0.410	0.042 0.710	−0.159 0.160		0.085 0.554	0.160 0.263	**−0.367** **0.008**		0.013 0.938	0.115 0.478	0.187 0.254
HCII	*Rho:* *P value:*	**−0.271** **0.001**	−0.006 0.947	−0.150 0.084		**−0.274** **0.012**	−0.034 0.762	−0.110 0.331		−0.248 0.080	0.061 0.668	−0.187 0.189		−0.003 0.988	0.263 0.100	−0.020 0.905
PAI-1	*Rho:* *P value:*	**0.269** **0.002**	0.155 0.072	0.100 0.252		**0.221** **0.045**	0.124 0.265	0.135 0.234		**0.360** **0.010**	0.229 0.106	0.070 0.628		**0.408** **0.009**	**0.412** **0.008**	−0.055 0.738
FXII	*Rho:* *P value:*	**0.281** **0.001**	−0.010 0.908	−0.088 0.313		**0.248** **0.024**	−0.164 0.140	−0.078 0.493		**0.323** **0.021**	**0.301** **0.032**	−0.122 0.395		−0.115 0.481	−0.126 0.438	−0.028 0.864

sTM, soluble thrombomodulin; HCII, heparin cofactor II; PAI-1, plasminogen activator inhibitor-1; FXII, factor XII.

Correlation coefficients and p-values of non-parametric partial correlation with age and sex as covariates are reported. Significant associations are in bold.

Correlations both within MS patients and in HI are reported in dark gray cells, and those observed only in MS cohort or HI in light gray cells.

Table 3.

Association of PC with MRI characteristics in multiple sclerosis patients and healthy individuals.

	Multiple Sclerosis				RR-MS		Healthy Individuals	
	Linear regression		Multiple regression		Multiple regression		Linear regression	
	r_p	P	r_p	P	r_p	P	r_p	P
T2-LV	0.116	0.181	/	/	/	/	0.095	0.555
T1-LV	0.025	0.778	/	/	/	/	-	-
WBV	**−0.265**	**0.002**	**−0.195**	**0.026**	**−0.228**	**0.042**	−0.151	0.347
WMV	−0.095	0.277	/	/	/	/	−0.194	0.225
GMV	**−0.330**	**0.0001**	**−0.258**	**0.003**	**−0.292**	**0.008**	−0.096	0.551
CV	**−0.310**	**0.0003**	**−0.230**	**0.008**	**−0.289**	**0.009**	−0.086	0.591
LVV	0.069	0.429	/	/	/	/	−0.039	0.810
DGMV	**−0.279**	**0.001**	**−0.228**	**0.009**	**−0.262**	**0.019**	−0.191	0.232
Thalamus volume	**−0.291**	**0.001**	**−0.235**	**0.007**	**−0.273**	**0.014**	−0.189	0.236

LV, lesion volume; WBV, whole brain volume; WMV, white matter volume; GMV, gray matter volume; CV, cortical volume; LVV, lateral ventricular volume; DGMV, deep grey matter volume.

Partial correlation (r_p) and P-value from regression analysis are shown. Linear regression model: each MRI characteristic was used as the dependent variable while the PC levels, expressed as natural logarithm, were the predictor variable.

Multiple regression model: each MRI characteristic was used as the dependent variable while gender, age, and natural logarithm of PC as predictor variables. The first block included the forced entry of age and gender, and the second block included the stepwise entry of the natural logarithmic of PC levels.

No multiple regression equations were produced (/) in the progressive multiple sclerosis and healthy individuals groups. Healthy individuals have not T1-LV (-). Significant results are in bold.