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„Investigation of biomarkers associated with the fibrinolytic system in plasma and CSF of patients with MS“

verfasst von / submitted by

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Declaration of all contribution to the thesis

The original concept of this research project, the aims, hypothesis and experimental designs where developed by Prof Robert Medcalf. All data of laboratory analysis documented in this thesis represents my own work under the guidance of my supervisors.

The work written is my own work and to the best of my knowledge has never been published by anyone else before. Authors used for the literature review are cited in the reference section. When using pictures I tried my best to find the author not to violate copyrights. Tables and figures used to present laboratory analysis data have been created by myself under the guidance of my supervisors using Graphpad Prism 7.

Samples were collected prior to my arrival and I stayed blinded throughout the experimental analysis to avoid bias.

Renate Jauernegger

Author

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List of abbreviations

BBB: blood brain barrier

CIS: clinically isolated syndroms

CNS: central nervous system

CSF: cerebrospinal fluid

CNBr-fibrinogen: cyanogen bromide-digested human fibrinogen

EAE: experimental autoimmune encephalomyelitis

EC: endothelial cells

ECM: extracellular matrix

FDP: fibrin degradation products

LDL: low-density lipoprotein

LAM: leukocyte adhesion molecule

MMP: matrix metalloprotein

MRI: magnetic resonance imaging

MS: multiple sclerosis

PA: plasminogen activation

PAI: plasminogen activator inhibitor

PRMS: primary progressive multiple sclerosis

RRMS: relapsing remitting multiple sclerosis

SPMS: secondary progressive multiple sclerosis

TAFI: thrombin activatable fibrinolysis inhibitor

TJ: tight junction

tPA: tissue plasminogen activator

uPA: urokinase

uPAR: urokinase receptor

Abstract

Multiple sclerosis is one of the major causes for neurological diseases in young adults and still its pathogenetic mechanisms are not completely understood. Disruption of the blood brain barrier seems to occur at an early stage and allows both blood proteins such as fibrinogen and plasminogen and immune cells, especially T-cells, to enter the brain and promote neurodegeneration. Deposition of fibrin around active MS lesions has been detected around MS lesions and seems to inhibit neuronal regeneration. Thus literature suggests a role for the two proteolytic systems PA and MMP in MS pathogenesis. However, whether increased tPA activity plays a beneficial role or promotes disease onset is still controversial.

Hence our project focused on investigating biomarkers associated with the fibrinolytic system in plasma and CSF of patients with multiple sclerosis compared to reference subjects with other neurological diseases. High protein levels were expected in the MS patient group since blood brain barrier disruption is one of the prominent features in MS. Significant differences in plasma or CSF protein levels between MS and other neurological diseases would facilitate easier pre-screening tests in order to diagnose MS.

We used enzyme linked immunosorbent assay (ELISA) to quantify plasminogen, fibrinogen, a2-antiplasmin and tPA:PAI1 levels in human plasma and CSF samples. Contrary to previous studies there was no significant difference found when comparing fibrinogen, plasminogen, tPA:PAI1 and a2-antiplasmin levels in our patient groups. Our hypothesis of elevated protein levels in plasma and CSF in the MS patient group has not been confirmed. However, interestingly the plasminogen-a2-antiplasmin-ratio, with plasminogen usually being double the plasmin inhibitor a2-antiplasmin in plasma, was found to be reversed in the CSF. Whether this reversed ratio, found in both our patient groups has any implications in the pathogenetic process of neurological diseases needs to be further investigated.

In conclusion, proteins associated with the fibrinolytic system were not found to be elevated in plasma or CSF in the MS patient group. However, detectable protein levels in the CSF of both patient groups seem to confirm a disrupted blood brain barrier in neurological diseases. The reversed plasminogen-a2-antiplasmin ratio in the CSF might also suggest a role for the fibrinolytic system in neurological diseases. However, further investigation is needed to elucidate the role of the fibrinolytic system and blood brain barrier disruption in multiple sclerosis.

Abstract German

Multiple Sklerose ist eine der häufigsten Ursachen für neurologische Störungen in jungen Erwachsenen und trotzdem ist der pathogenetische Prozess der Erkrankung noch nicht ganz geklärt. Man nimmt an, dass ein Zusammenbruch der Blut-Hirn-Schranke zu Beginn der Erkrankung und demzufolge der Eintritt von Plasma Proteinen wie Fibrinogen, Plasminogen und Immunzellen, insbesondere T-Zellen, für die Neurodegeneration verantwortlich ist. Fibrin Ablagerungen sind typisch für aktive MS Herde und scheinen die Regeneration der Nervenzellen zu stören. Die zwei proteolytischen System in unserem Körper, Plasminogen-Aktivator-System und Matrixmetalloproteinase-System, scheinen daher eine wichtige Rolle in der Beseitigung dieser Fibrin Ablagerungen zu spielen. Ob ein erhöhte tPA Aktivität positive Auswirkungen auf den Krankheitsverlauf oder schädliche Effekte haben kann ist kontrovers.

Unser Projekt untersuchte Biomarker die in Zusammenhang mit dem fibrinolytischen System stehen in Plasma und CSF Proben von Patienten mit MS oder anderen neurologischen Störungen. Hohe Protein Konzentrationen wurden erwartet, da ein Einbruch der Blut-Hirn-Schranke und Eintritt von Proteinen ins Gehirn eine der Hauptmerkmale für die Erkrankung sind. Signifikante Unterschiede zwischen den Proteinkonzentrationen von MS Patienten und Patienten mit anderen neurologischen Erkrankungen würde die Abgrenzung von MS zu anderen neurologischen Störungen in Pre-screening-Untersuchungen wesentlich erleichtern.

ELISAs wurden in diesem Projekt verwendet, um Fibrinogen, Plasminogen, a2-antiplasmin und tPA:PAI1-Komplex in Plasma und CSF Proben zu quantifizieren. Im Gegensatz zu vorherigen Studien konnten wir keine signifikanten Unterschiede in der Proteinkonzentration zwischen den MS Patienten und der Kontrollgruppe feststellen. Die Hypothese, dass Plasma Proteine im CSF von MS Patienten erhöht sind, konnte nicht bestätigt werden.

Interessanterweise jedoch ist das Verhältnis von Plasminogen:a2-Antiplasmin, mit normalerweise doppelt so hohen Plasminogen Konzentrationen im

Plasma, genau umgekehrt im CSF. Ein 2:1 Verhältnis von a2-Antiplasmin:Plasminogen im CSF wurde sowohl in der MS Patientengruppe als auch in der Kontrollgruppe gefunden und scheint daher eine Rolle in neurologischen Erkrankungen zu spielen. Welche Rolle genau das umgekehrte a2-Antiplasmin:Plasminogen Verhältnis im CSF spielt ist aber noch ungeklärt und bedarf weiterer Untersuchungen.

Zusammenfassend wurden keine erhöhten Proteinkonzentrationen in der MS Patientengruppe gefunden. Quantifizierbare Mengen an Fibrinogen und anderen Proteinen, die normalerweise nicht die Blut-Hirn-Schranke passieren, weisen aber jedenfalls auf einen Zusammenbruch der Blut-Hirn-Schranke in neurologischen Erkrankungen hin. Das umgedrehte Verhältnis von Plasminogen:a2-Antiplasmin lässt außerdem darauf schließen, dass das fibrinolytische System eine Rolle in neurologischen Erkrankungen spielt. Weitere Untersuchungen sind notwendig um genaue Aussagen über die Rolle des fibrinolytischen Systems und dem Zusammenbruch der BHS in neurologischen Störungen, insbesondere MS, zu treffen.

1 Literature review

1.1 Introduction

Multiple sclerosis, a demyelinating disease of the CNS, is one of the major causes for neurological disorders in young adults (1). Mechanisms of disease onset and MS pathology are still not completely understood. However blood brain barrier breakdown, fibrinogen deposition and T-cell infiltration have been detected in MS lesions. Growing literature suggests a role for PAs and MMPs in brain injury, stroke and neurological inflammatory diseases such as multiple sclerosis. However results on whether these effects are beneficial or deleterious are still controversial. The plasmin dependency of MMP-9, meaning the ability of plasmin to activate MMP-9, thus linking the two proteolytic systems PA and MMPs together, may also suggest a correlation between high tPA levels and MMP activity.

1.2 Multiple sclerosis

Multiple sclerosis is a chronic inflammatory autoimmune disease with its aetiology still unknown. It affects the CNS by degrading the myelin sheaths and therefore causes neurological disorder (2). Affecting 2.5 million people worldwide, it is the major cause for neurological disability in young adults (1). The prevalence varies from >100/100.000 inhabitants in Europe and North America to 2/100.000 inhabitants in Eastern Asia and sub-Saharan Africa (3). Most of the patients get diagnosed in their 20s and 30s, with 50% of them being no longer able to perform household and employment responsibilities 10 years after disease onset (1).

1.2.1 Types of MS

Multiple sclerosis features 4 courses: Relapsing-remitting (RRMS), primary progressive (PPMS), secondary progressive (SPMS) and clinically isolated syndromes (CIS) (4). The RRMS is the most prominent disease course and is diagnosed in around 85% of the patients. It features relapses with the return of old symptoms or exacerbations with new neurological symptoms caused by acute neurological inflammation, followed by remission with partial or complete recovery. In some cases the RRMS evolves into SPMS with progressive worsening of the symptoms over time. A disease course of worsening symptoms from the beginning, without preceding relapses/remissions is typical for the PPMS. Patients with CIS experience neurologic symptoms caused by

inflammation and demyelination that last for at least 24 hours, but still don`t fulfil the criteria to be diagnosed for MS (4, 5).

1. 2. 2 Differences between relapse-remitting and progressive types

In MRI scans, patients with RRMS show more brain lesions (e.g. plaques, scars) with a high amount of inflammatory cells, whereas the progressive MS courses tend to show more spinal cord lesions with less inflammatory cells (2, 4). Women are twice as likely to be diagnosed with RRMS than men but rates are similar for PPMS (1). For RRMS the average disease onset is much earlier (29 years) than for PPMS (39 years) (1).

1.2.3 Symptoms of multiple sclerosis

The most common symptoms include fatigue, visual problems, coordination and balance difficulties, muscle spasticity or stiffness, bowel and bladder problems and problems in learning, memory and information processing. Symptoms vary depending on the part of the brain that is affected by the demyelination process (2).

1.2.4 Pathogenesis of multiple sclerosis

BBB breakdown, demyelination, inflammation, loss of oligodendrocytes, axonal degeneration and gliosis are the main features of the pathological MS process (1). There are two hypothesis about the disease onset. The more prominent extrinsic hypothesis suggests peripheral activation of autoreactive lymphocytes that then infiltrate the CNS via breached parts of the BBB (2, 6). Alternatively the intrinsic hypothesis suggests CNS intrinsic events being responsible for the disease onset, with the infiltration of lymphocytes as a secondary phenomenon (2). T-cell entry into the CNS is one of the earliest events in the pathogenesis of MS and other immune-mediated neurological diseases (7). However, fibrin deposition and microglial activation have been reported prior to T-cell infiltration thus suggesting a prominent role for the fibrinolytic system in MS pathogenesis (8, 9).

1.3 The blood brain barrier

The vasculature of the CNS comprises a couple of features to tightly regulate the traffic of ions, molecules, proteins and cells between the brain and the blood (10). Endothelial cells inside the blood vessels, sealed with tight junctions, form a dense layer that eliminates free exchange of solutes between blood and brain (11). Assisted by pericytes, astrocytes and microglia, this so called BBB not only maintains

the sensitive neuronal milieu inside the brain, but also helps to protect the CNS from entering toxins, pathogens and the body´s own immune system (10, 11). Only small lipid-soluble molecules <400 Da can cross the BBB unassisted via lipid-mediated diffusion (12). To import nutrients and export waste, degradation products and other unwanted molecules, the brain uses highly selective transporters. They can be classified into 5 categories: ion transport, carrier-mediated transport, active efflux transport, receptor mediated transport and caveolae-mediated transport (11). Furthermore ECs support the BBB on a metabolic level, by expressing a number of enzymes that alter endogenous and exogenous molecules, which would otherwise be able to bypass the physical barrier (11). Future research is needed to completely understand the BBB, its transport systems and its role in physiological and pathological processes. This will contribute to develop new treatments and better understanding of CNS diseases such as MS, Alzheimer disease (AD) or epilepsy (11).

1.3.1 BBB dysfunction

The neuronal milieu is very sensitive to changes in its chemical composition. Therefore BBB breakdown and extravasation of ions and other blood components leads to neuronal dysfunction (11). In most cases BBB breakdown occurs secondary to a traumatic event such as brain injury or stroke. However, studies suggest that for some neurological disorders such as MS, AD or epilepsy, it may also play a role in disease onset (13). Breakdown of the BBB results in an influx of ions, molecules and also extravasation of immune cells. Opening the brain to the body`s immune system after traumatic events may help to repair damage and clean up remnants, but can also cause unwanted neuronal dysfunction, inflammation and degeneration (10). The most prominent features for BBB dysfunction are disruption of TJs, alteration in transport systems, increased leukocyte infiltration and increased transcytosis. Also a number of molecules, including vasoactive proteins (VEGF), ROS, cytokines, tumor necrosis factor alpha (TNFa), proteases (in particular MMP2 and MMP9) and leukocyte adhesion molecules (LAMs) have been identified to contribute to BBB breakdown (10). Nevertheless to completely understand the interplay of all these features further research is needed.

1.3.2 The brain and the immune system

Our brain is known to take up a privileged site within the body's constant immune surveillances. Meaning that the immune cells patrolling the body via the bloodstream are not able to enter the CNS (11). The presence of the BBB, the lack of a lymphatic system within the CNS and extremely low expression of leukocyte adhesion molecules (LAMs) in the CNS endothelial cells (ECs) greatly limit the amount of entering immune cells into the brain (11, 14). Hence, the myelin and it's antigenic epitopes are usually protected from the immune system by an intact BBB (15). Interestingly the expression of LAMs is much lower in CNS ECs than peripheral ECs and is elevated during neuroinflammatory diseases such as stroke and MS (14, 16). Adhesion is the first step for leukocytes and other cells to infiltrate tissues. Natalizumab, a drug used to treat active MS, prevents adhesion of T-cells via binding and inactivating their LAM binding site (17). Patients treated with natalizumab have shown significant reduction of BBB breakdown and 60% reduction of leukocyte count in the CNS (17). These findings support the immune mediated manner of MS pathogenesis and the importance of an intact BBB for a functioning CNS.

1.4 Fibrinogen and it's multiple performances

1.4.1 Fibrinogen in the coagulation cascade

Fibrinogen is a soluble glycoprotein synthesized by the liver and like other blood proteins, secreted into the plasma. It is not known to be produced by cells of the nervous system (18). Known as the blood-clotting protein, the major role of fibrinogen as part of the blood coagulation cascade is to occlude blood vessels and stop excessive bleeding after tissue or vascular damage (18). After initiation of the coagulation cascade, fibrinogen is cleaved by thrombin into fibrin which elicits polymerisation of fibrin and formation of an insoluble fibrin clot (18). Interestingly the usual plasma concentration for fibrinogen ranging from 1.5 to 4.5 g/l far exceeds the minimum concentration of 0.5-1.5 g/l necessary for haemostasis and increases under pathological conditions (19).

1.4.2 Fibrinogen and inflammation

Among the blood factors, fibrinogen is unique due to its ability to interact with a variety of integrin and non-integrin receptors expressed on hematopoietic, immune

or neuronal cells (20). Therefore it's not only an important regulator in the coagulation process but also plays a role in tissue repair and inflammation. Studies report interactions of fibrinogen and integrin receptors on the surface of leukocytes promoting proinflammatory changes in them. These proinflammatory changes include increase in phagocytosis, antibody-mediated leucocyte toxicity and delay in apoptosis (21, 22).

1.4.3 Fibrinogen in multiple sclerosis

Fibrinogen does not occur inside the brain under physiological conditions. But it can enter the CNS after vascular damage through a disrupted BBB and form insoluble fibrin depositions inside the brain (18, 23) (Figure 1.1). Fibrin deposition and microglia activation occurring as an early event in MS lesions and experimental autoimmune encephalomyelitis (EAE) has been detected prior to T-cell infiltration and demyelination (8). Interestingly, injection of soluble fibrinogen into the healthy brain results in fibrin formation, microglia activation and demyelination within 7 days after injection (9). Injection of fibrinogen depleted plasma into the brain, that still contained all the other blood proteins, showed a 82% reduction of demyelination. Furthermore fibrin depletion seems to decrease inflammation and delay disease onset in tumor necrosis factor transgenic mouse models of multiple sclerosis (24). Thus suggesting that fibrinogen plays an important role in MS pathogenesis.

Mechanisms for fibrin induced encephalitogenic adaptive immune responses and peripheral macrophage recruitment are not completely understood, but its seems that interactions with the CD11b/CD18 integrin receptor expressed on monocytes, macrophages and microglia play an important role (9, 25).

Although previous studies have shown a role for fibrinogen in MS pathogenesis, CSF fibrinogen levels were not found to be elevated in MS patients compared to other neurological diseases (26).

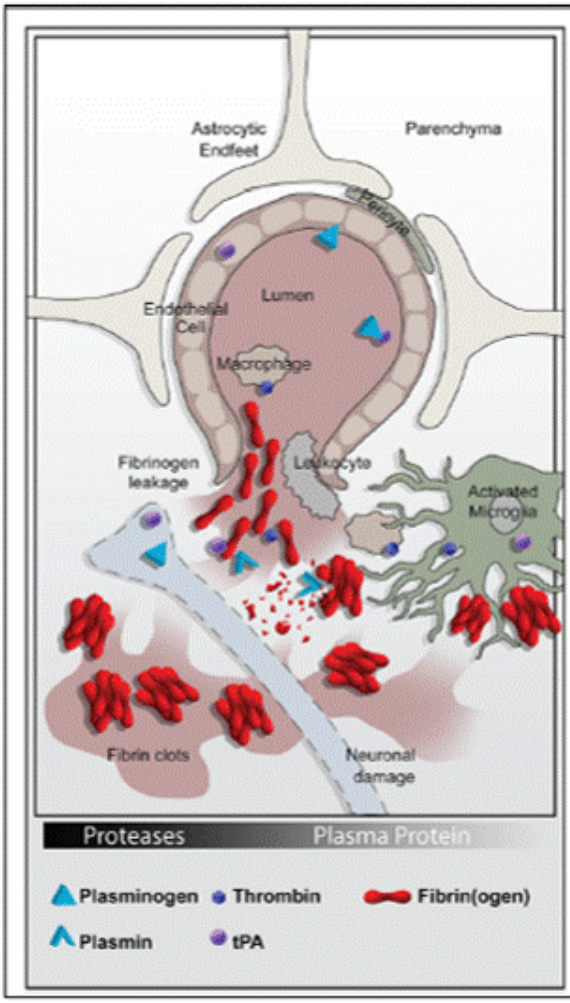

Figure 1.1 The coagulation and proteolytic cascades at the neurovascular interface. *Fibrinogen leakage in the CNS and activation of the PA system after blood brain barrier breakdown. This enables neurodegeneration via activation of microglia, macrophages and leukocytes. Fibrin and fibrin degradation products (FDPs) interact with different receptors to induce inflammation and inhibit regeneration.* *Adapted from Bardehle S, A. Rafalski V, Akassoglou K. Breaking boundaries—Coagulation and fibrinolysis at the neurovascular interface2015.*

1.5 The plasminogen activator system (PA)

The plasminogen activator system comprises the two proteolytic enzymes tissue type plasminogen activator (t-PA) and urokinase plasminogen activator (u-PA), their major inhibitors plasminogen activator inhibitor 1 (PAI-1) and plasminogen activator inhibitor 2 (PAI-2) and of course plasmin, its inactive precursor plasminogen and the plasmin inhibitor a2-antiplasmin (27). Activation of the PA after vascular injury is important to maintain vascular homeostasis. Other than their vital role of removing blood clots, PA proteins are also implicated in CNS functions and MS pathogenesis. Figure 1.2 shows a simplified overview on the fibrinolytic pathways.

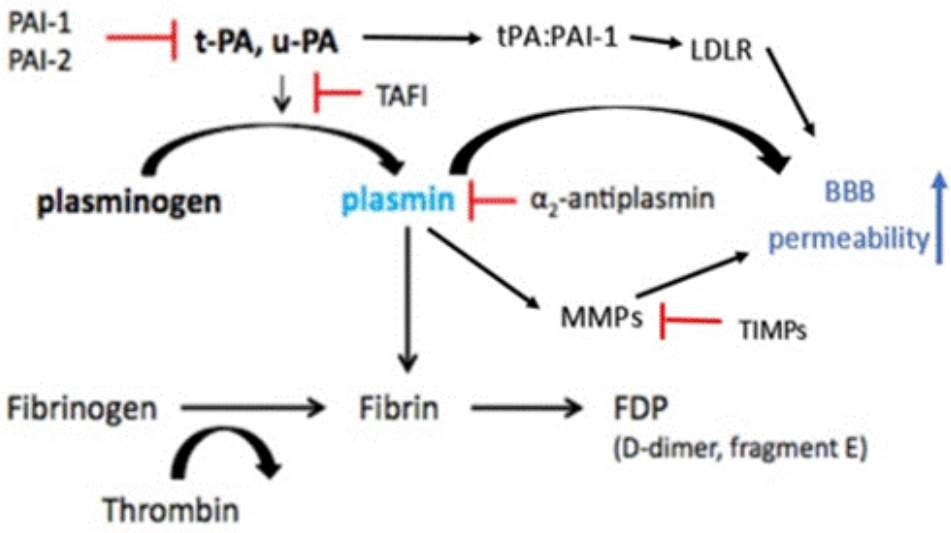

Figure 1.2 ***Schematic overview on fibrinolytic pathways.*** *BBB blood brain barrier; FDP fibrin degradation products; MMP matrix metalloproteinase; PAI-1 plasminogen activator inhibitor 1; PAI-2 plasminogen activator inhibitor 2; t-PA tissue type plasminogen activator; u-PA urokinase type plasminogen activator*

Figure adapted from Fay WP, Garg N, Sunkar M. Vascular Functions of the Plasminogen Activation System. Arteriosclerosis, thrombosis, and vascular biology. 2007;27(6):1231-7.

1.5.1 The plasminogen activators

Tissue type plasminogen activator (tPA) and the less potent urokinase (uPA) are serine proteases that convert the zymogen plasminogen into active plasmin. The glycoprotein tPA is released primarily by endothelial cells in response to stimulation of thrombin, adrenaline, histamine, gonadotropins, acetylcholine, exercise and venous occlusion(28). The generally low activity of tPA increases radically in the presence of fibrin. Formation of a ternary complex with fibrin and plasminogen then promotes the plasmin mediated breakdown of intravascular blood clots and fibrin deposits (28).

1.5.2 Inhibitors of the PA system

Plasminogen activator inhibitor type 1 (PAI1) is the major regulator for the fibrinolytic system. The serine protease PAI1 belongs to the `serpin family` and rapidly inactivates tPA and uPA by forming a 1:1 complex with the target protease (29). Plasma PAI1 is released from endothelial and various other cells, but the majority of

PAI1 is found in blood platelets (30). Inflammatory proteins such as cytokines, lipopolysaccharide, tumor necrosis factor alpha (TNFa), lipoprotein (a) and thrombin have been found to stimulate the release of PAI1, without affecting tPA synthesis (28, 31).

Plasminogen activator inhibitor type 2 (PAI2), the second serine protease inhibitor in the serpin family is primarily found in the cytosol far away from its target uPA, thus suggesting a different role for this protein than uPA inhibition (32).

Neuroserpin, primarily localized to neurons within the CNS, reacts preferentially with tPA and is therefore thought to be a highly selective inhibitor for tPA (33, 34).

a2-Antiplasmin blocks the fibrinolytic activity on a later stage by forming a 1:1 irreversible complex with plasmin, thus inactivating the protease(35). It circulates the plasma in high concentrations and immediately inhibits plasmin in the bloodstream (28, 36).

Thrombin activatable fibrinolysis inhibitor (TAFI) degrades the tPA and plasminogen binding sides on fibrin clots, thus preventing the formation of the ternary fibrin-plasminogen-tPA complex and decreasing fibrinolytic activity. It circulates the bloodstream as an inactive proenzyme and gets activated by thrombin, linking the coagulative and the fibrinolytic system (28, 37).

1.5.3 The role of tPA outside the fibrinolytic system

Other than performing in the fibrinolytic system, tPA has been reported to be involved in cell migration and tissue invasion of normal and malignant cells (38), morphogenesis and neurogenesis (39), rat brain growth cones (40) and patients with rheumatoid arthritis.(41) Furthermore neurons and microglia in the CNS constantly express the tPA gene, especially in the developing brain, thus suggesting a role for tPA in neuronal development and synaptic remodelling (42, 43).

Although the primary substrate for plasmin is fibrin, the potent protease can also degrade other tissue proteins under pathological conditions. For example, plasmin induced degradation of basement membrane proteins such as laminin is suggested to be one of the mechanisms for neuronal cell death in excitotoxic conditions.(44) In vitro, plasmin has also been found to be able to degrade myelin basic protein (MBP). (45) Nevertheless, in contradiction to these findings, the PA/plasmin system plays a beneficial role in inflammatory brain conditions that involve BBB disruption and

exudation of fibrinogen. In that case, the fibrinolytic activity of plasmin seems to protect from neurodegeneration by removing the fibrin deposits that cause exacerbation of neuronal injury. (46)

1.5.4 The plasminogen activator system in multiple sclerosis

In experimental mouse models of MS (experimental allergic encephalomyelitis EAE), mice deficient in tPA (tPA $^{-/-}$) show a significant delay in disease onset but exhibit more severe symptoms in later points and experience much slower recovery compared to wildtype mice (47). Further EAE studies agree with the development of more severe symptoms and slower recovery in tPA (-/-) mice compared to wildtype mice, but in contrast to Lu et al. report an earlier disease onset for tPA (-/-) mice (23, 48). Therefore tPA seems to play an important role in disease onset and development but whether its role is beneficial or deleterious is still unclear. Also further investigation of the role for the less potent uPA would be interesting.

Along with these findings goes that tissue type plasminogen activator has been found in high concentrations on demyelinated axons in multiple sclerosis together with fibrin deposits (49). Since fibrin deposition interferes with neuronal regeneration, tPA is thought to have beneficial effects in removing fibrin deposits (50, 51).

Furthermore Akenami et al. reported increased activity of tissue type plasminogen activator in cerebrospinal fluid (CSF) of patients with neurological diseases. The highest tPA levels were found in patients with multiple sclerosis.(52) Thus suggesting enhanced fibrinolytic activity. In a follow up study on plasminogen levels, the zymogen plasminogen was found to be diminished in the CSF of the MS group and quantifiable levels of plasmin-a2-antiplasmin complex (PAP)were detected. Thus suggesting increased plasminogen activation in the CSF of the MS patients (53). Furthermore plasminogen in general was found to be elevated in the CSF of patients with neurological diseases (54).

Interestingly the PA inhibitor PAI1 was also found to be elevated in CSF samples of patients with neurological diseases, but not in their plasma (55, 56). Increased PAI1 levels in active lesions and formation of 1:1 tPA-PAI1 complexes seem to decrease the fibrinolytic capacity and thus are suggested to promote neurodegeneration (57). Furthermore tPA:PAI1 complex formation, naturally associated with fibrinolytic

inhibition and tPA clearance, has been reported to open the BBB itself in mouse models of traumatic brain injury via LDL receptor signalling (58). However, further studies are needed to fully understand the role of tPA, PAI1 and their complex in MS pathogenesis.

1.6 Matrix metalloproteinases (MMPs)

Matrix metalloproteinases are a family of proteolytic enzymes comprising over 20 zinc-dependent endopeptidases. Their ability to degrade ECM proteins makes them an important player in tissue and bone remodelling, wound healing, ovulation and angiogenesis (59). Activity of MMPs needs tight regulation, as overexpression and excessive extracellular matrix proteolysis predisposes pathologic events such as inflammation, demyelination and cancer cell invasion (60, 61). The mechanisms for regulation include gene expression, proenzyme activation and specific tissue inhibitors for matrix metalloproteinases (TIMPs) (59).

MMPs are secreted as proenzymes which are activated by cleavage through other proteases including plasmin, trypsin and kallikrein. Their genes are not constitutively expressed. Growth factors such as EGF or PDGF, cytokines, TNFa and oncogene expression have been shown to induce gene expression (62-64).

1.6.1 MMPs and their role in MS pathogenesis

Various members of the MMP family, especially MMP-9, have been detected in human brains of patients with multiple sclerosis (65, 66) and in EAE mouse models (67-69). Moreover MMP-9 deficient mice seem to be less susceptible to the development of EAE (70) and showed reduced MBP degradation in white matter (71). Additionally, injection of MMPs in rat brains has been shown to promote BBB permeability leading to edema and haemorrhage, thus suggesting a role for MMPs in BBB disruption (72, 73).

Since astrocytes, microglia and other neuronal cells are also able to produce MMPs, it is still unclear whether the increase of MMP-9 in the CNS could derive from the leakage through the BBB or intrathecal synthesis (69, 74). However, the proteolytic activity of MMPs synthesized by MBP-sensitized T-cells seems to facilitate extravasation of immune cells into the CNS (75). Furthermore the extracellular proteolytic effects of MMPs may also directly elicit apoptosis of endothelial cells via death receptors, thus contributing to vascular damage (76).

1.7 Positive contribution of the PA-MMP axis

Controversially to their deleterious effects in brain injury and neurological diseases, PAs and MMPs may also contribute in recovery in these conditions. Since both systems are physiologically involved in neuronal plasticity and angiogenesis, their upregulation may play a beneficial role in brain injury and inflammation. Mice deficient in tPA, uPA or plasminogen for example showed delayed recovery after sciatic nerve crush (77). Moreover proteolysis is a necessary event in angiogenesis (78).

Suggesting that fibrin deposits facilitate exacerbation in MS lesions, the fibrin degrading capacity of the PA system may play a beneficial role in disease development. However, overexpression of tPA in EAE mouse models didn't show any significant benefits (48).

Furthermore PAs and MMPs secreted by reactive astrocytes and microglia may help degrade inhibitory ECM proteoglycans that interfere with axonal regeneration (79).

1.8 Conclusion

Though being one of the major causes for neurological disorders in young adults, the aetiology of MS is still not completely understood. Blood brain barrier breakdown, T-cell infiltration and fibrin deposition are discussed for being one of the earliest events in MS pathogenesis and thus suggested to elicit neuronal degeneration. Growing data also proposes a role for the plasminogen activator and MMP axis in onset and progression of the disease. Previous studies found proteins of the fibrinolytic system such as fibrinogen, tPA and PAI-1 to be elevated in the CSF of patients with multiple sclerosis compared to other neurological diseases and EAE mouse models compared to wildtype. Hence the aim of this project was to investigate biomarkers associated with the fibrinolytic system in plasma and CSF of patients with multiple sclerosis.

1.9 Concept and aims of this project

Literature thus far suggests a role for blood brain barrier breakdown and the PA system in the pathogenesis of multiple sclerosis. Increased t-PA activity has been found in the CSF of patients diagnosed with MS and EAE mouse models (48, 52). However it is still controversial whether the increased t-PA activity plays a beneficial role or promotes exacerbation (23, 47, 48). Thus this project tried to further investigate the link between a disrupted blood brain barrier and MS pathogenesis.

1.9.1 Hypotheses

Based on the literature presented, the hypotheses of this project are:

-A disrupted blood brain barrier enables plasma proteins to enter the CSF and thus protein levels are expected to be elevated in the CSF compartment

-Disruption of the blood brain barrier activates the PA system

-Increased t-PA activity facilitates further blood brain barrier breakdown

2. Materials and methods

2.1 Materials

Over a course of 5 years 60 human samples of matching plasma and CSF were collected prior to study onset at the Austin hospital and stored at -80° celcius. Patients suspected with MS were enrolled in this study and underwent lumbar puncture for diagnostical reasons. Of them, 18 were diagnosed with MS and the others had other neurological diseases. Reagents used are presented in table 2.1.

2.2 ELISAs

Enzyme linked immunosorbent assay (ELISA) was used to quantitate fibrinogen, plasminogen, a2-antiplasmin and tPA:PAI1 complex levels in human CSF and plasma samples. All ELISAs were performed under blinded conditions.

2.2.1 ELISA principles

Enzyme linked immunosorbent assay is a research method to either quantitate the amount of antibody in a sample or the amount of protein that can be bound by an antibody. The basic principle of an ELISA is shown in Figure 2.1

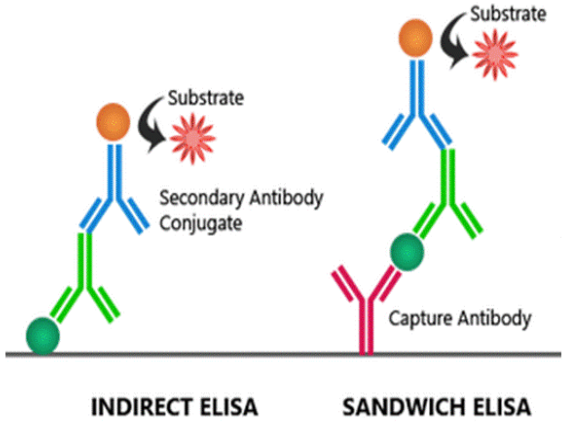

Figure 2.1 ELISA basic principle, adapted from Boster biological technology.

Typically a 96-well plate coated with an antibody is used to capture the protein/antigen of interest. Then a detection antibody (primary antibody) added attaches to the target protein/antigen. A secondary antibody conjugated to an enzyme such as HRP then binds the primary antibody. When substrate such as TNB is added, the enzyme metabolises the colourless substrate into a coloured product. The colour intensity is in direct proportion to the amount of protein/antigen in the sample and can be measured using an absorbance plate reader.

2.2.2 Human fibrinogen antigen ELISA

Human fibrinogen binds the capture antibody coated on the microtiter plate. Fibrinogen bound to the capture antibody then binds the primary antibody in a second step. Excess primary antibody is washed away and the bound primary antibody then reacts with streptavidin conjugated to horseradish peroxidase (HRP).

For assay performance a commercial kit (Human fibrinogen antigen ELISA kit; Molecular innovations, #HFIBKT) was used according to manufacturer`s protocol. We used matching plasma and CSF samples, equally divided in MS and non MS on each plate.

High fibrinogen concentrations were expected both in plasma and CSF. Hence plasma samples were diluted 1:100.000 using 1X diluent prior to adding them to the

plate. CSF samples were diluted 1:100 before adding them to the plate. Standards were prepared as required in the instructions and then added in duplicates à 100 µl to the plate, followed by adding 100µl of the diluted CSF and plasma samples in duplicates.

Plate was shaken for 30 minutes at 300rpm and then washed 3 times with 250µl 1X wash buffer. The primary antibody was added using 100 µl for each well and then shaken again for 30 minutes at 300rpm. The washing step was repeated before adding 100µl of streptavidin-HRP. Again the plate was shaken for 30 minutes at 300rpm and washed 3 times. 100µl of TMB were added and incubated for a change from colourless to blue. Incubation was stopped after ~7 minutes with stop solution (table 2.1) resulting in a change from blue to yellow. The absorbance was then measured at 450nm using a plate reader.

A 4PL sigmoidal standard curve was then generated with the absorbance data using Graphpad Prism 7. Values were then interpolated from the standard curve and dilutions were calculated back to full concentrations.

Table 2. 1

Reagent	Preparation steps
1X wash buffer	**50ml of 10X wash buffer were diluted with 450ml deionized water**
1X diluent	**50ml 5X diluent were diluted with 200ml deionized water**
Horseradish peroxidase-conjugated streptavidin (HRP)	**2,5 µl HRP were diluted in 2,5 ml diluent. This 1:1000 dilution was then further diluted resulting in a 1:50,000 dilution for HRP**
TMB substrate solution	**Tetramethylbenzidin**
Stop solution	**1 N H2SO4**
Blocking buffer (BB)	**3% BSA (w/v) in TBS**
TBS buffer	**0.1M Tris, 0,15M NaCl, pH 7,4**
Standards, primary and secondary antibodies	**Provided by manufacturer and diluted according to potocol**

2.2.3 Human plasminogen ELISA

A commercial kit (human plasminogen total antigen ELISA kit; Molecular innovations; #HPLGKT-TOT) was used to quantify plasminogen levels in human plasma and CSF samples of patients diagnosed with MS and other neurological diseases. Since high levels of plasminogen were expected in both plasma and CSF, plasma was diluted 1:20.000 and CSF was diluted 1:20. The assay was performed according to manufacturer`s protocol. Standards and matching CSF and plasma were added in 100 µl duplicates to the plate. The plate was shaken for 30 min at 300rpm and washed 3 times with 1X wash buffer. The washing and incubation steps were repeated for primary and secondary antibody addition. TMB substrate was then added and incubated for 5 min for a change from colourless to blue. The reaction was stopped by adding 50 µl of 1N H_2SO_4. Absorbance was measured at 450nm using a plate reader.

A 4PL sigmoidal standard curve was then generated with the absorbance data using Graphpad Prism 7. Values were then interpolated from the standard curve and dilutions were calculated back to full concentrations

2.2.4 Human a2-antiplasmin ELISA

A commercial kit (human alpha2-antiplasmin total antigen ELISA kit; molecular innovations; #HA2APKT-TOT) was used to quantify a2-antiplasmin levels in human plasma and CSF samples of patients diagnosed with MS and other neurological diseases. Since high levels of a2-antiplasmin were expected, plasma was diluted 1:250.000 and CSF was diluted 1:250. The assay was performed according to manufacturer`s protocol. Standards and matching CSF and plasma were added in 100 µl duplicates to the plate. The plate was shaken for 30 min at 300rpm and washed 3 times with 1X wash buffer. The washing and incubation steps were repeated for primary antibody and HRP addition. TMB substrate was then added and incubated for 5 min for a change from colourless to blue. The reaction was stopped by adding 50 µl of 1N H_2SO_4. Absorbance was measured at 450nm using a plate reader.

A 4PL sigmoidal standard curve was then generated with the absorbance data using Graphpad Prism 7. Values were then interpolated from the standard curve and dilutions were calculated back to full concentrations.

2.2.5 Human tPA:PAI1 complex ELISA

A commercial kit (human PAI-1/tPA complex antigen ELISA kit; molecular innovations; #HPAITPAKT-COM) was used to quantify tPA:PAI1-complex in human plasma and CSF samples of patients with MS and other neurological diseases. Standards and neat plasma and CSF samples were added in 98 µl duplicates to the plate. The plate was shaken for 30 min at 300rpm and washed 3 times with 1X wash buffer. The washing and incubation steps were repeated for primary and secondary antibody addition. TMB substrate was then added and incubated for 5 min for a change from colourless to blue. The reaction was stopped by adding 50 µl of 1N H_2SO_4. Absorbance was measured at 450nm using a plate reader.

A 4PL sigmoidal standard curve was then generated with the absorbance data using Graphpad Prism 7. Values were then interpolated from the standard curve and dilutions were calculated back to full concentrations

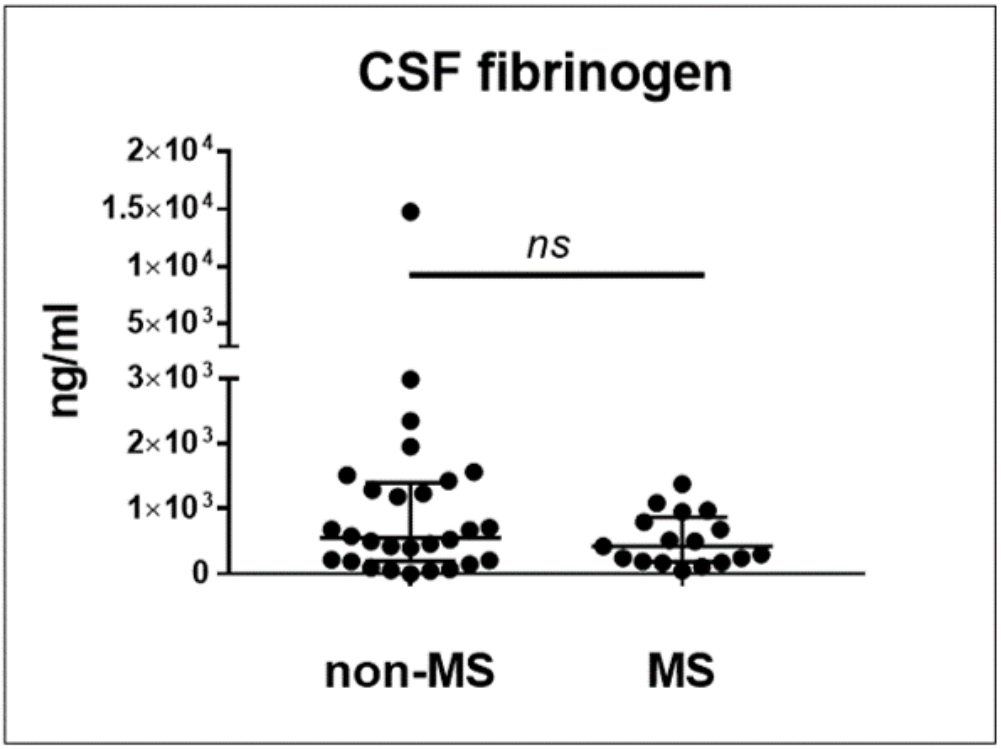

Figure 3.1 *CSF fibrinogen levels. MS-patients diagnosed with multiple sclerosis. Non-MS patients with other neurological diseases. No significant difference between the MS and non-MS group. (bar=median with interquartile range. Mann-Whitney-U-test used to compare data.*

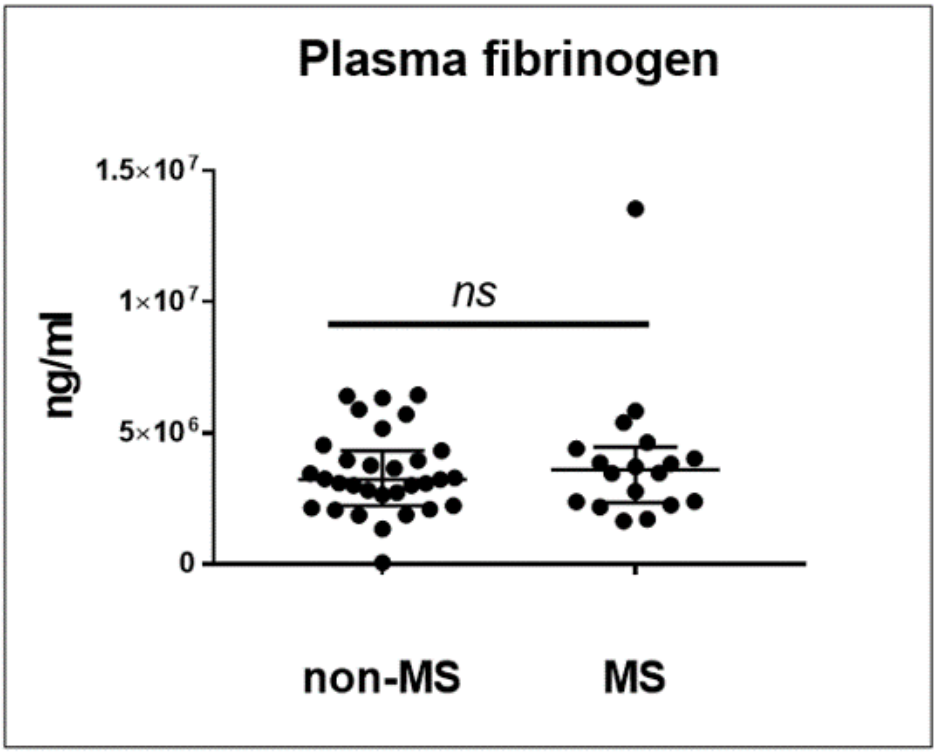

Figure 3.2 *Plasma fibrinogen levels. MS: patients diagnosed with multiple sclerosis. Non-MS: patients with other neurological diseases. No significant difference between the MS and non-MS group. (bar=median with interquartile range; Mann-Whitney-U-test used to compare data.*

3 Results

3.1 Fibrinogen levels

Although previous studies suggest a role for fibrinogen in MS pathogenesis, fibrinogen levels were not found to be elevated in the CSF of the MS patients. Overall levels where lower than in the control group though the difference was not statistically significant (Figure 3.1). Although elevated plasma fibrinogen levels are usually found in acute infectious diseases, most of the samples were in the normal range level of 1,5 to 4,5 mg/ml with no significant difference between the MS and non MS group (Figure 3.2).

3.2 Plasminogen and a2-antiplasmin

Our study confirmed the noted correlation of plasminogen and the plasmin inhibitor a2-antiplasmin in plasma. Plasma plasminogen levels were found to be double or higher than a2-antiplasmin in both the MS patients and the control group (see figure 3.3). Interestingly the correlation found in the CSF was reversed, where a2-antiplasmin doubled plasminogen in most of the MS and non MS patients (see figure 3.4). Though there was no significant difference in plasma or CSF plasminogen and

a2-antiplasmin levels for MS patients compared to the control group (Figure 3.5 and 3.6).

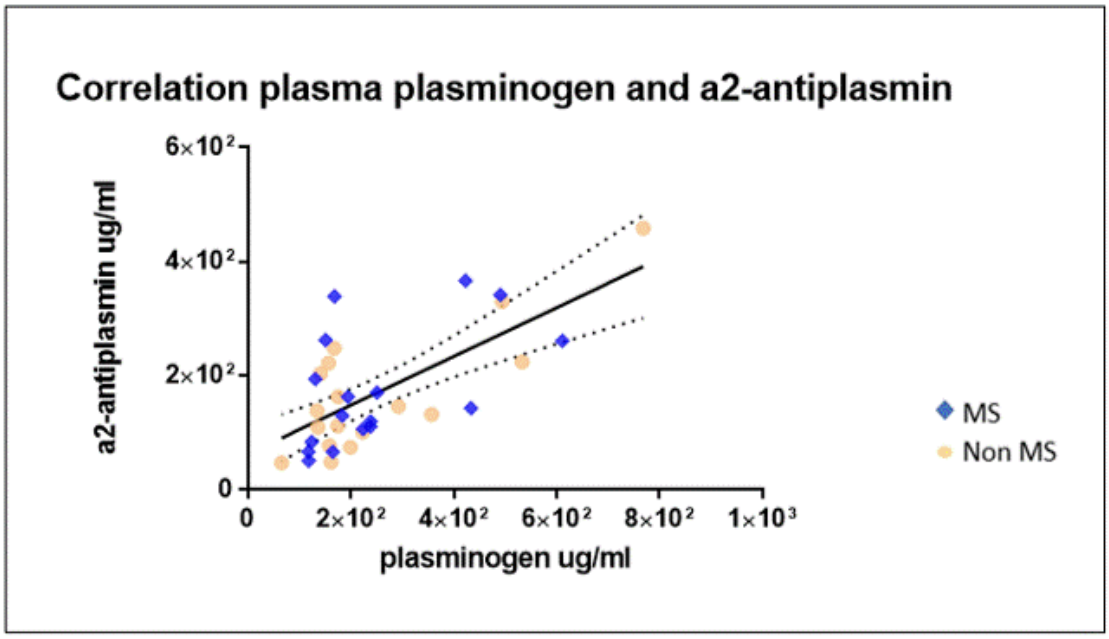

Figure 3.3 *Correlation of plasma plasminogen and a2-antiplasmin ug/ml. p for correlation <0.0001. Pearson r, two tailed used for correlation. Single data points. For MS n=17; for non-MS n=18*

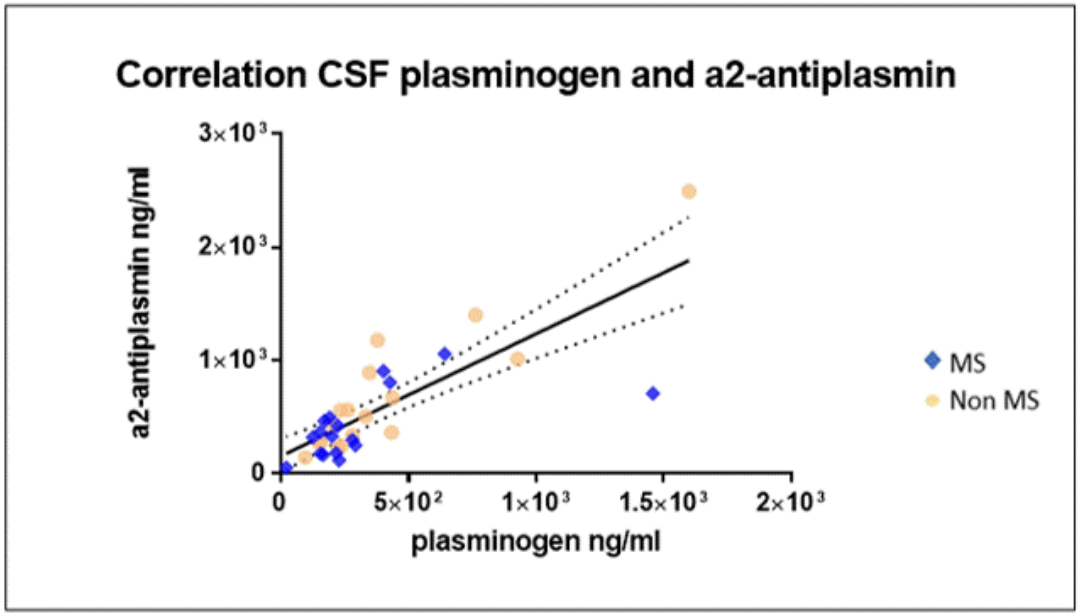

Figure 3.4 *Correlation CSF plasminogen and a2-antiplasmin ng/ml. p for correlation <0.0001. Pearson r, two tailed used for correlation. Single data points. For MS n=17; for non-MS n=18*

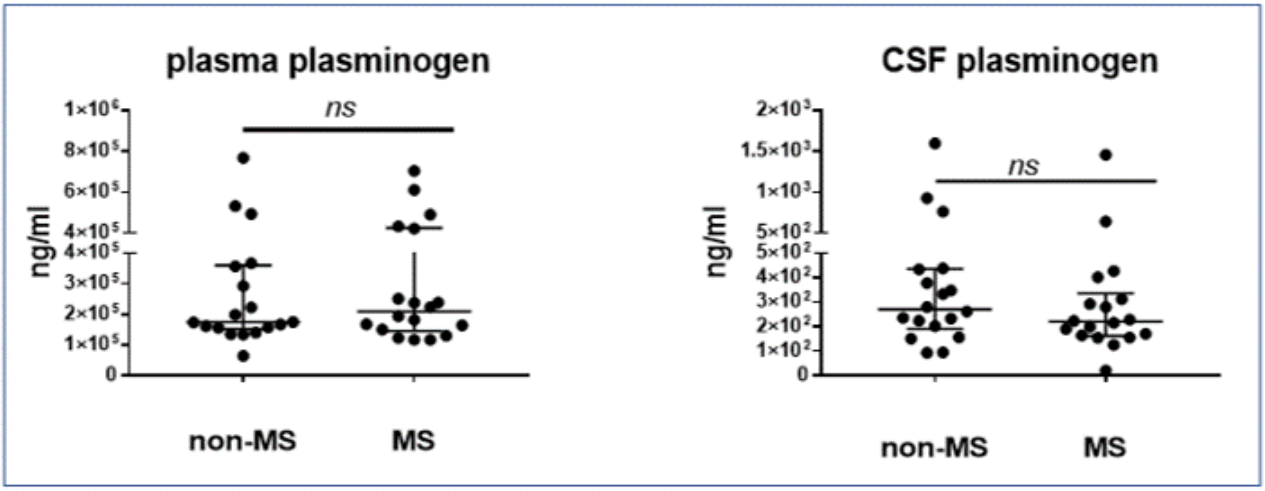

Figure 3.5 *Plasma and CSF plasminogen levels in the MS and non-MS group in ng/ml. No significant difference when comparing the data. Mann-Whitney-U-test used for comparison. Bar=median with interquartile range.*

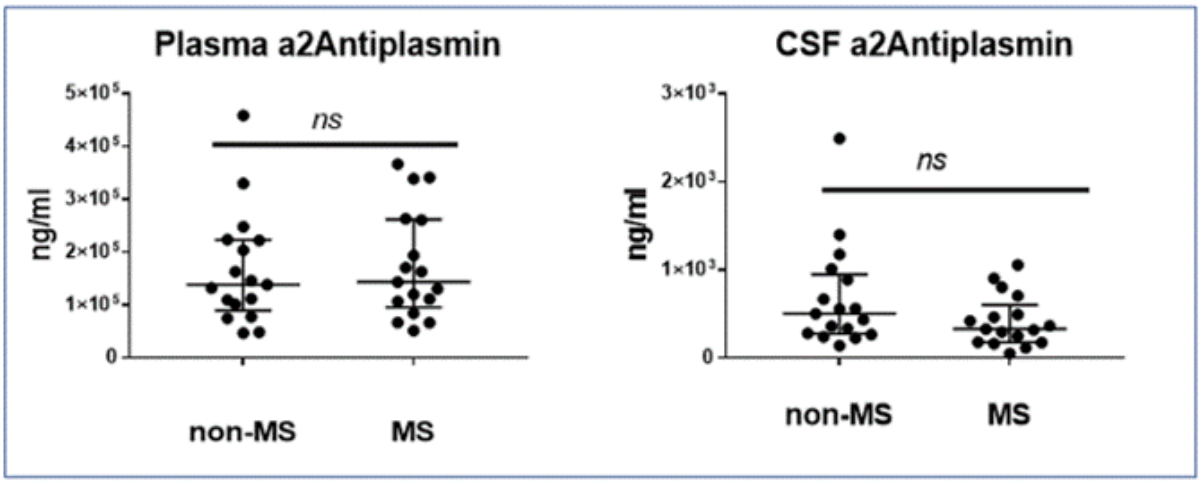

Figure 3.6 *Plasma and CSF a2-antiplasmin levels in the MS and non-MS group in ng/ml. No significant difference when comparing the data. Mann-Whitney-U-test used for comparison. Bar=median with interquartile range.*

3.3 tPA:PAI-1 complex

We could measure tPA:PAI-1 complex levels in some of the plasma samples, with 2 very high levels in the MS group and 5 in the control group (Figure 3.7). CSF tPA:PAI-1 concentrations were close to minimum detection level (Figure 3.8). No correlation was found between high tPA:PAI-1 complex levels and other parameters.

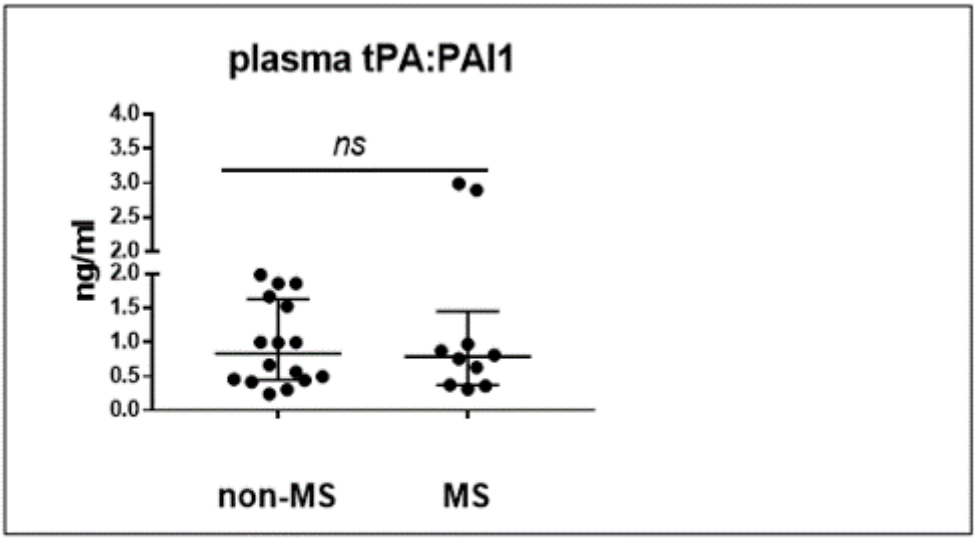

Figure 3.7 *plasma concentration of tPA:PAI1 complex in ng/ml. (bar=median with interquartile range. No significant difference between the MS and non-MS group. Mann-Whitney-U-test used to compare data*

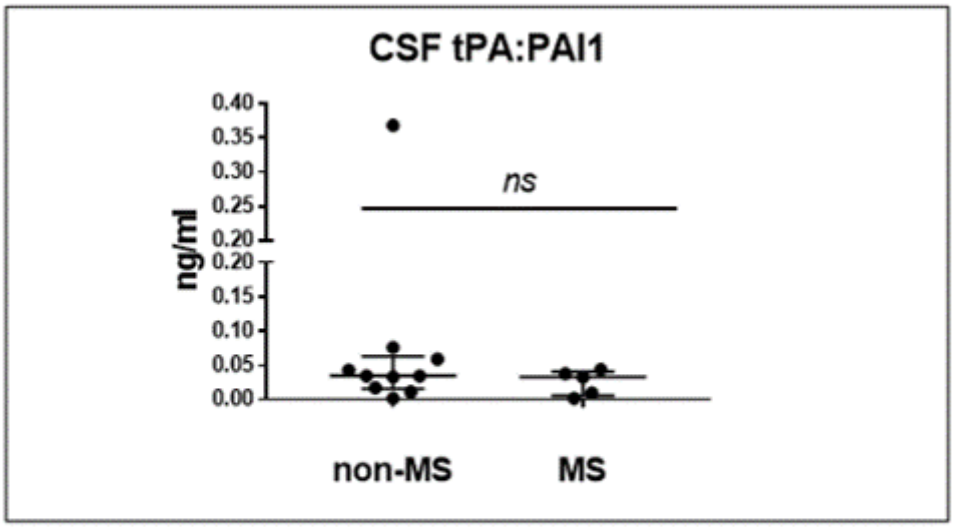

Figure 3.8 *CSF concentration of tPA:PAI1 complex in ng/ml. (bar=median with interquartile range. No significant difference between the MS and non-MS group. Mann-Whitney-U-test used to compare data*

4 Discussion

Despite growing literature, the pathogenetic processes underlying multiple sclerosis are still not completely understood. Blood-brain barrier breakdown and extravasation of blood proteins and immune cells seem to occur as an early event in MS and promote disease onset and exacerbation. Infiltration of blood proteins through a disrupted blood brain barrier may suggest elevated levels in the CSF compartment. Therefore the aim of our study was to investigate biomarkers of the coagulation system in patients diagnosed with multiple sclerosis compared to other neurological diseases.

Unexpectedly fibrinogen levels were not found to be elevated in the CSF compartment of the MS patients when compared to the control group (Figure 3.1).. These findings are in accordance with Ehling et al (26). Hence this may suggest that the role of fibrinogen in MS pathogenesis is not accompanied by elevated concentrations in the CSF. However, since fibrinogen does not occur in the healthy brain and all of our samples had detectable levels of fibrinogen in their CSF compartment, it proofs the contribution of a disrupted blood brain barrier in the course of neurological diseases.

Since MS comes in many different forms and stages, and fibrinogen levels are known to be elevated under acute inflammatory conditions, chronic disease courses in our patient group could be one explanation for the low levels in plasma and CSF. Local restriction of blood brain barrier disruption rather than a widespread leakage in the brain might also contribute to the unexpectedly low levels. Further information on patients treatment and disease course would be needed.

Plasminogen levels were not found to be elevated neither in plasma nor CSF of the MS patient group . One explanation for that might be an increased tPA activity as suggested by Akenami et al. (52, 53). The increased conversion of plasminogen into its active form plasmin may explain low plasminogen levels. Thus further research on tPA activity and quantification of PAP complex in our samples would be necessary.

Intriguingly the plasminogen:a2-antiplasmin ratio was found to be reversed in the CSF compared to plasma in both the MS patients and the control group. Usually plasminogen levels double the plasmin inhibitor a2-antiplasmin in plasma. Elevated a2-antiplasmin levels in the CSF may contribute to decreased fibrinolytic activity. Since fibrin deposition is known to inhibit neuronal regeneration, the roughly 2:1 ratio

for a2-antiplasmin:plasminogen in the CSF in both our patient groups suggest a beneficial role for the fibrinolytic system in neurological diseases. So far there were no previous studies on the plasminogen: a2-antiplasmin ratio to be found. Thus it might be interesting to further investigate the relationship between this ratio and other disease parameters. The reversed ratio was detected in both groups, hence indicating a role in neurological diseases. Further comparison with patients not suffering from neurological diseases might be interesting.

High PAI1 levels and 1:1 complex formation with tPA seem to decrease the fibrinolytic activity and therefore promote neurodegeneration (57). Some of the patients in both groups had very high tPA:PAI1 complex levels in plasma but no correlation was found between elevated plasma tPA:PAI1 complex and other disease parameters. CSF tPA:PAI1 was below detection level in most of the samples, though we could interpolate concentrations for some of the patients. Since the tPA:PAI1 complex itself is not only known for tPA clearance but also interacts with LDL receptors contributing to BBB opening, further investigation of tPA:PAI1 complex levels in CSF might be of interest.

4.1 Conclusion and future perspectives

Our study confirms that disruption of the blood brain barrier seems to play a role in neurological diseases. Although, the use of biomarkers associated with the fibrinolytic system in order to distinguish MS from other neurological diseases still needs further investigation, since we didn`t find any significant differences. However, as suggested by previous studies it seems that the plasminogen activator system plays a role in neurological diseases. We confirmed the correlation of plasminogen:a2-antiplasmin in plasma and found it to be reversed in the CSF of both our patient groups. Since high levels of the plasmin inhibitor decrease the fibrinolytic activity and seem to promote disease symptoms, further investigation in this area might be of interest.

For the future, larger patient groups and more detailed information about patients disease courses and treatments would be necessary for better interpretation of the clinical parameters.

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