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**GELATINASES, NEUROFILAMENTS AND N-ACETYL  
ASPARTIC ACID  
AS BIOMARKERS IN DIFFERENT PATHOLOGIES**

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# INTRODUCTION

## **Biomarker Concept**

A biomarker is defined as a quality objectively measured and evaluated as indicator of physiological and pathological processes or pharmacologic responses to a therapy (Biomarkers Definitions Working Group, 2001). In the past, biomarkers were primarily physiological indicators, for example body temperature rise is a biomarker for fever or blood pressure is used to determine the risk of stroke. More recently, biomarker is becoming a synonym for molecular marker, namely chemical molecules, genes, proteins and enzymes that can be measured in body fluids and are related to a specific pathology (i.e. transaminases levels as markers for liver functions). Also, biomarkers can be specific cells or complex organ functions and characteristic changes in biological structures.

We can distinguish between two strictly-related kinds of biomarkers: *disease-related* and *drug-related* biomarkers. *Disease-related* biomarkers give an indication for a threat of disease (risk indicator or predictive biomarkers i.e. Anti-citrullinated protein/peptide antibody as marker for Rheumatoid Arthritis), if a disease already exists (diagnostic biomarkers i.e. prostate specific antigens for prostate cancer), or how a disease may develop in a patient (prognostic biomarker i.e. cancer biomarkers). In contrast, *drug-related* biomarkers indicate whether a drug will be effective in a specific patient and therefore, they should vary with a drug treatment.

A number of diseases, such as Alzheimer's disease, rheumatoid arthritis or Multiple Sclerosis, often begin with an early, symptom-free phase. In

these cases, biomarkers may help identifying high-risk individuals to develop a disease, so they can be treated before onset of the disease or either as soon as possible thereafter.

In order to use a biomarker for diagnostic purposes, it should have some well-defined characteristics:

- the sample material to use for the biomarker detection must be as easy to obtain as possible, with a low invasiveness. For example, this may be a blood sample, a urine or saliva sample, or even a drop of blood;
- the assay for a biomarker should be as quick as possible. In fact, for rapid initiation of treatment, the speed with which a result is obtained from the biomarker test is critical. A test which delivers results only in few minutes is optimal.
- the detection method for a biomarker must be accurate and as easy to carry out as possible. The results from different laboratories may not differ significantly from each other, and the biomarker must have proven its effectiveness for the diagnosis, prognosis and risk assessment of the disease in independent studies.

Together with molecular biomarker assayed in biological fluids there are also other kinds of biomarkers referred as imaging biomarkers, developed using imaging technologies like coronary angiography, computed tomography, magnetic resonance imaging, positron emission tomography.

Imaging biomarkers have many advantages: usually they are non-invasive and produce intuitive, multidimensional results. Yielding both qualitative and quantitative data, they are usually relatively comfortable for patients and combined with other sources of information, they can be

very useful to help clinicians in a diagnosis. In contrast to these advantages, often the required analyses are expensive and may require the use of radiations or contrast liquids which can lead to allergies. For these reasons, molecular biomarkers are essential to develop large scale screenings on patient populations and can give, together with imaging biomarkers, more complete information.

In the present study, I aimed to evaluate the possible role of active Matrix Metalloproteinase-9 (MMP-9) and Matrix Metalloproteinase-2 (MMP-2) as biomarkers in inflammatory pathologies, since their involvement in inflammation is well documented but is still unclear (Opdenakker G et al., 2003; Cunningham et al., 2005; Yong VW, 2005; Fainardi et al., 2006). Moreover, I also investigated the role of Neurofilament light and heavy subunits and N-Acetyl aspartic Acid as biomarkers of axonal damage in a neurodegenerative disorder like Primary Progressive Multiple Sclerosis (PPMS), as a lack of reliable data remains on the role of these markers in such MS subtype (Norgren N et al., 2003; Jasperse B et al., 2007; Teunissen CE et al., 2009).

### **Gelatinases in inflammatory conditions**

Matrix Metalloproteases (MMPs) were discovered in 1962, and since then more than twenty five members of this family have been identified, cloned and sequenced (Lijnen HR. et al., 2001).

The MMPs are endopeptidases zinc and calcium dependent which are classified on the basis of the recognized substrates and sequence homology in: collagenases, gelatinases, stromelysins, membrane-type metalloproteases (MT-MMPs) and matrilysins.

Collectively, they are capable of degrading essentially all the Extracellular Matrix (ECM) components, playing an important role in

ECM remodeling during both physiological and pathological conditions. In fact they are expressed during embryonal development, tissue regeneration and wound repair (Van den Steen PE et al., 2002). Also, they play a role in pathological conditions such as rheumatoid arthritis, autoimmune disorders of the skin, chronic ulcerations, malignant tumors and metastasis (Van den Steen PE et al., 2002).

One of the most complex MMPs family is the gelatinases family, that includes MMP-9 (Gelatinase B) and MMP-2 (Gelatinase A). Among these, MMP-9 has been associated with a large number of pathological conditions, such as acute respiratory distress syndrome, destructive lung disease, Sjögren's syndrome, peripheral nerve injury, Guillain-Barré syndrome, blood-brain-barrier damage, Multiple Sclerosis, Alzheimer's diseases, cancers and brain ischemia (Lacraz S et al., 1995). On the contrary, MMP-2 has been associated to both tissue repair and destruction in inflammatory pathologies (Yong VW, 2005).

The objective of my study was to analyse in detail the role of gelatinases in pathological inflammatory conditions such as Multiple Sclerosis or Spontaneous Intra Cerebral Haemorrhage (SICH). Moreover, to better understand the real involvement of MMP-9 in inflammatory processes, I tried to assess *in vitro* the release of MMP-9 from neutrophils during environmental pH changes because an acidic pH in tissues is related to inflammatory conditions.

## Structure of Gelatinases

Gelatinases (gelatinase A or MMP-2 and gelatinase B or MMP-9) are organized into six main domains (**Figure 1**) which will be explained in detail below:

1. *Propeptide*: it maintains the enzyme into inactive form due to the coordination of a cysteine residue with the catalytic zinc ion;
2. *Catalytic domain*: it contains the catalytic site with the zinc, the Zinc Binding Domain with an high conserved Methionine and a calcium ion which is essential to maintain the correct tridimensional structure;
3. *Hemopexin-like domain*: placed in the Carboxy-terminal end, it is important for the binding of substrates and for the interaction with tissue specific inhibitors TIMPs;
4. *Hinge region*: a proline rich region with a variable length, it has a flexible structure, connecting the catalytic domain with the hemopexin-like domain;
5. *Fibronectin II-like domain*: composed by three fibronectin II repeats, it is placed into the catalytic domain between the zinc binding domain and the active site, and it confers the substrate specificity for gelatin, laminin, type I and IV collagens;
6. *Collagen V-like domain*: Rich in serine/proline/threonine, it is present only in MMP-9 between the hinge region and the hemopexin-like domain and it is heavily O-glycosylated. The high glycosylation confers to this region a rigid structure.

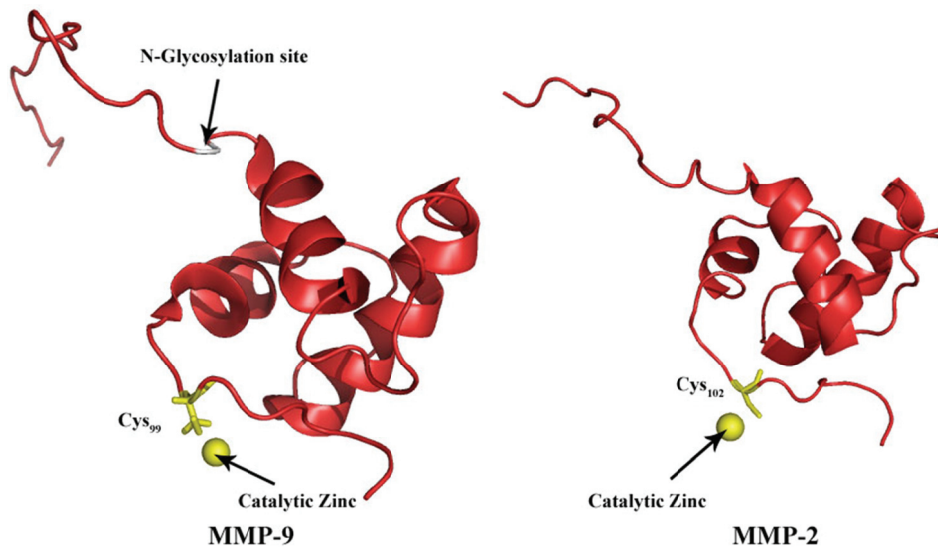


**Figure 1. Domain structure of gelatinases.**

*C5*, Collagen V-like domain; *Fn*, fibronectin repeat; *Pro*, propeptide; *SP*, signal peptide; (Parks W.C. et al., 2004).

## Propeptide

The propeptide (**Figure 2**) consist of 86 amino acids in MMP-9 and 80 amino acids in MMP-2, containing a cysteine residue, the **Cys<sub>99</sub>** in the MMP-9 and the **Cys<sub>102</sub>** in the MMP-2, which interacts with the catalytic zinc atom via its side chain thiol group. A highly conserved consensus sequence (...PRCGXPD...) is present in the propeptide (Massova I. et al., 1998). The interaction of the cysteine with the zinc maintains the enzyme into the inactive form (zymogen). Removal of the propeptide by proteolysis results in zymogen activation, as all members of the MMPs family are produced in a latent form. This activation mechanism is called “Cysteine Switch” (Massova I. et al., 1998).



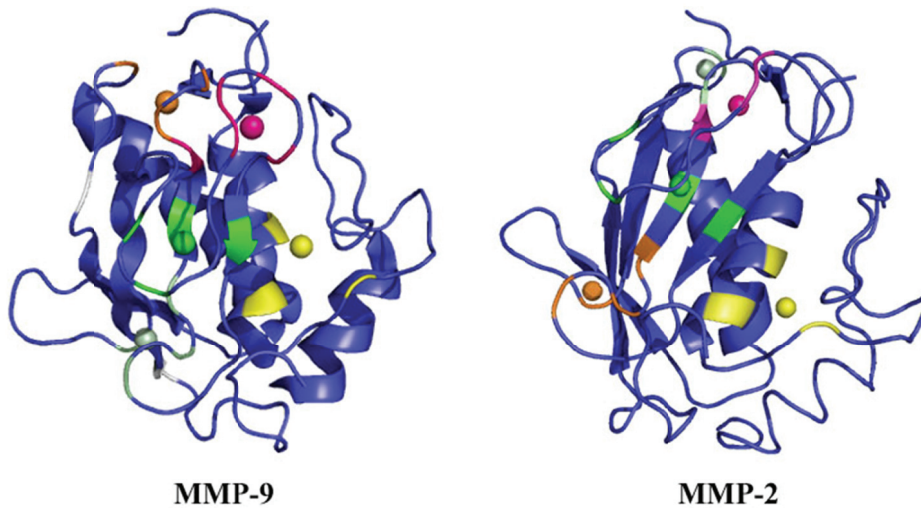
**Figure 2. Tridimensional structure of the MMP-9 and MMP-2 propeptide.** In yellow is marked the cysteine coordinated to the catalytic zinc. In grey is marked the N-Glycosylation site.

The secondary structure of the propeptide in MMP-9 and MMP-2 is organized in three  $\alpha$ -helices linked by several flexible loops, one of which in the MMP-9 has a N-glycosylation site (Asn<sub>38</sub>).

## Catalytic Domain

The catalytic domain consist in 190 amino acids in MMP-9 and 205 amino acids in MMP-2, and contains an highly conserved domain called Zinc Binding Domain, which coordinates a zinc ion through three histidines and is essential for the enzymatic activity (...**AH<sub>401</sub>EXGH<sub>405</sub>XXGXXH<sub>411</sub>**... in MMP-9 and ...**AH<sub>403</sub>EXGH<sub>407</sub>XXGXXH<sub>413</sub>**... in MMP-2) (Van den Steen PE. et al., 2002). Within the catalytic domain there are also one other zinc, coordinated by three histidines (His<sub>175</sub>, la His<sub>190</sub> and His<sub>203</sub> in MMP-9; His<sub>178</sub>, His<sub>180</sub> and His<sub>193</sub> in MMP-2), and two calcium ions; all these ions are essential to maintain the correct spatial structure for the interaction of the substrates with the active site (Massova I. et al., 1998). The secondary structure of the catalytic domain (**Figure 3**) consists in five  $\beta$ -sheets that wrap around three  $\alpha$ -helices. One important amino acid close to the catalytic zinc is the Glu<sub>402</sub> in MMP-9 and Glu<sub>402</sub> in MMP-2, which binds water molecules and is essential for the hydrolysis of the substrates. In addition, there are also two N-glycosylation sites in the Asn<sub>120</sub> and Asn<sub>127</sub> residues only in the catalytic domain of MMP-9.





**Figure 3. Catalytic domains of MMP-9 and MMP-2.**

*In different colors are showed the metal ions and the residues which coordinate them. Yellow, catalytic zinc; Green, structural zinc; Pink, Orange and Light Green, calcium ions.*

### **Hemopexin-like domain**

The hemopexin-like domain, placed at the C-terminal end of the protein (from the amino acid 513 to 707 in MMP-9 and from amino acid 466 to 660 in MMP-2), is present in all the MMPs (except in the matrilysins) and shows a sequence similar to the hemopexin of the plasma.

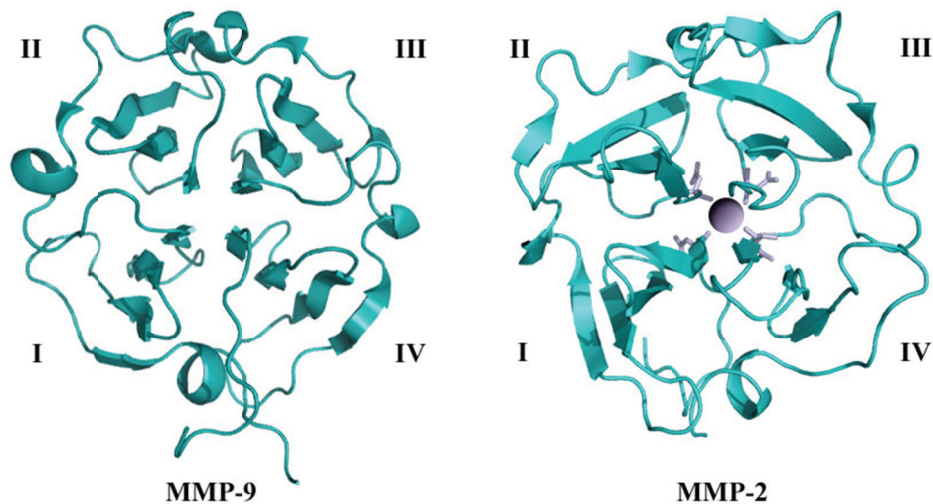
This domain has several functions:

- binds the substrates;
- interacts with the specific inhibitors TIMPs (TIMP-1 for MMP-9 and TIMP-2 for MMP-2): the C-terminal end of the inhibitor has an elevated affinity for the hemopexin-like domain of the respective gelatinase (Massova I. et al., 1998).

The secondary structure of this domain is organized in a  $\beta$ -propeller: a barrel superstructure composed of four blades, each blade consists of one

$\alpha$ -helix and four anti-parallel  $\beta$ -strands; all is organized around a central axis (**Figure 4**).

The first blade is bound to the fourth through a disulfide bridge; the other two blades are relatively away from the catalytic domain and probably interact with the C-terminal end of the inhibitor (Van den Steen PE et al., 2002). Only in MMP-2 there is one calcium ion in the middle of the  $\beta$ -propeller structure.

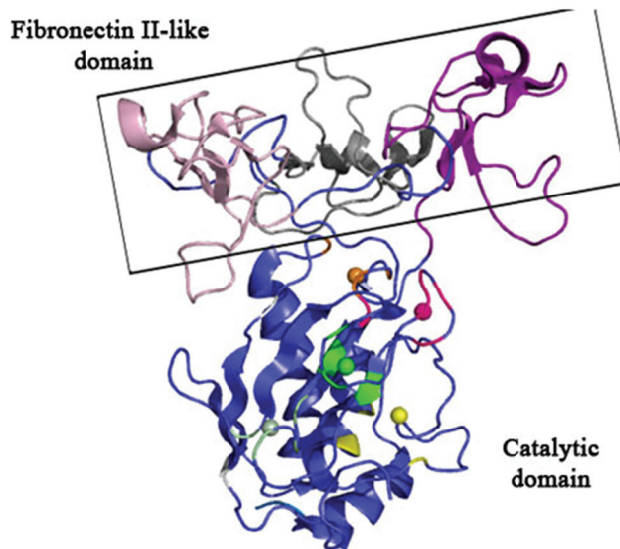


**Figure 4. 3-D structure of the hemopexin-like domain.**  
From I to IV are marked the four  $\beta$ -sheets that compose the  $\beta$ -propeller structure.

Another important function of the hemopexin-like domain is the binding to the LPR-1 receptor (LDL Receptor-related Protein-1), expressed in hepatocytes and macrophages. The binding of gelatinases to this receptor leads to the internalization of the enzyme and the subsequent degradation causing a down-regulation of the enzymatic levels.

### **Fibronectin II-like domain**

This domain is characteristic of gelatinases and is composed by three repeats, each of one of 49 amino acids length, homologue to fibronectin II. These three repeats belong to the catalytic domain and are placed in an external position (**Figure 5**).



**Figure 5. Placement of the fibronectin II-like domain respect to the catalytic domain.** In pink, grey and purple are marked the three fibronectin II repeats, in blue is marked the catalytic domain.

This domain confers the specificity for type I, type IV (gelatin) and type V collagens, elastin, and native type I collagen (Massova I. et al., 1998). The typical collagenolytic and elastinolytic activities of this enzyme depend exclusively on the presence of this domain.

### **Hinge-region and collagen V-like domain**

The hinge-region is a sequence composed by 16 amino acids in MMP-9, whereas in MMP-2 it has a length of 20 residues. It is placed between the catalytic domain and the hemopexin-like domain. Only in MMP-9, after

this sequence there is another region of 54 amino acids called collagen V-like domain. It is rich in Ser/Thr/Pro and is heavily O-glycosylated: as the O-glycosylation confers to this region a rigid structure, probably the role of this sequence is to maintain the correct distance between the hemopexin-like domain and the other domains.

### **Substrate specificity**

The specificity depends on primary sequence of the substrate: the endoproteases prefer the sequences that bind to the pocket of the catalytic site. However, also the three dimensional conformation of the substrate and the accessibility of the cleavage sequence are important features. Finally, the presence of exosites in the catalytic domain which can bind distant residues on the substrate can have a key role promoting the hydrolysis.

Probably, the fibronectin II-like domain of gelatinases plays a role as exosite that binds collagens increasing the hydrolysis efficiency.

The substrate specificity for MMP-9 and MMP-2 has been analyzed using synthetic short peptides as substrates, resembling the sequence Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln which correspond to the P4-P3-P2-P1-P1'-P2'-P3'-P4' sites in the substrate. Mutagenesis studies showed that MMP-9 has a preference for small amino acids (Gly, Ala, Pro) in the P1 site, and hydrophobic residues (Ile, Leu, Tyr, Met) in the P1' and P2 sites. On the contrary, MMP-2 cleaves the substrate between the Glycine in the P1 site and a hydrophobic amino acid in the P1' site, although in this last site hydroxylated and acid residues are tolerated (Van den Steen PE et al., 2002).

Up to now, only a small number of physiologic substrates belonging to the extracellular matrix and cleaved by MMP-9 were described; the most

known are denatured collagens or gelatins. Moreover, also the type II and type V collagens can be cleaved by this enzyme. Other substrates that belong to the ECM include aggrecans, the link protein and elastin (Van den Steen PE et al., 2002) (**Table 1**).

MMP-2 has a similar substrate specificity as MMP-9 for ECM compounds, although differences were observed in substrate type, catalytic efficiency and cleavage pattern of the same substrate for both gelatinases (i.e. gelatin). Among the ECM substrates, there are collagens I, IV, V, VII, X, XI, XIV, gelatin, elastin, fibronectin, laminin-1, laminin-5, galectin-3, aggrecans, decorin, proteoglycan link protein and osteonectin (Van den Steen PE et al., 2002).

There are also several non-matrix proteins degraded by MMP-9 and MMP-2. MMP-9 is able to degrade myelin basic protein (which degradation can lead to the release of encephalitogenic peptides),  $\alpha$ 1-antitrypsin and  $\alpha$ 1-antichymotrypsin, P substance, IL-2 receptor-  $\alpha$ , TGF- $\beta$ ,  $\beta$ -amyloid peptide (A $\beta$ 1-40), IL-1 $\beta$ , proTNF-  $\alpha$ . Recently, has been observed that MMP-9 can cleave six N-terminal amino acids from IL-8 (1-77), generating another IL-8 form (7-77) which is more bioactive. Moreover, it can generate a positive feedback signal as IL-8 can stimulate the release of MMP-9 from neutrophils (Van den Steen PE et al., 2002). The non-ECM substrates degraded by MMP-2 include IL-1 $\beta$ ,  $\alpha$ 1-P1, prollyl oxidase fusion protein, MMP-1, MMP-9, MMP-13.

**Table 1. ECM and non-ECM substrates for MMP-9 and MMP-2.**  
(Chakraborti S. et al., 2003)

<b>Protease</b>	<b>ECM Substrates</b>	<b>Non-ECM substrates</b>
<b>MMP-9</b>	Collagens (IV, V, VII, X, XIV), gelatin, elastin, galectin-3, aggrecans, fibronectin, proteoglycan link protein, entactin, osteonectin.	Plasminogen, IL-1 $\beta$ , MBP, ET-1 (endotelin-1), amyloid peptide A $\beta$ <sub>1-40</sub> , IL-8, GRO- $\alpha$ (growth-related oncogene- $\alpha$ ), SDF-1 (stromal-cell derived factor-1), $\alpha$ <sub>1</sub> -antitrypsin, $\alpha$ <sub>1</sub> -antichymotrypsin, P substance, IL-2 receptor- $\alpha$ , TGF- $\beta$ , proTNF- $\alpha$ .
<b>MMP-2</b>	Collagens (I, IV, V, VII, X, XI, XIV), gelatin, elastin, fibronectin, laminin-1, laminin-5, galectin-3, aggrecans, decorin, proteoglycan link protein, osteonectin.	IL-1 $\beta$ , $\alpha$ <sub>1</sub> -PI, prolysin oxidase fusion protein, MMP-1, MMP-9, MMP-13.

## Regulation of Gelatinases

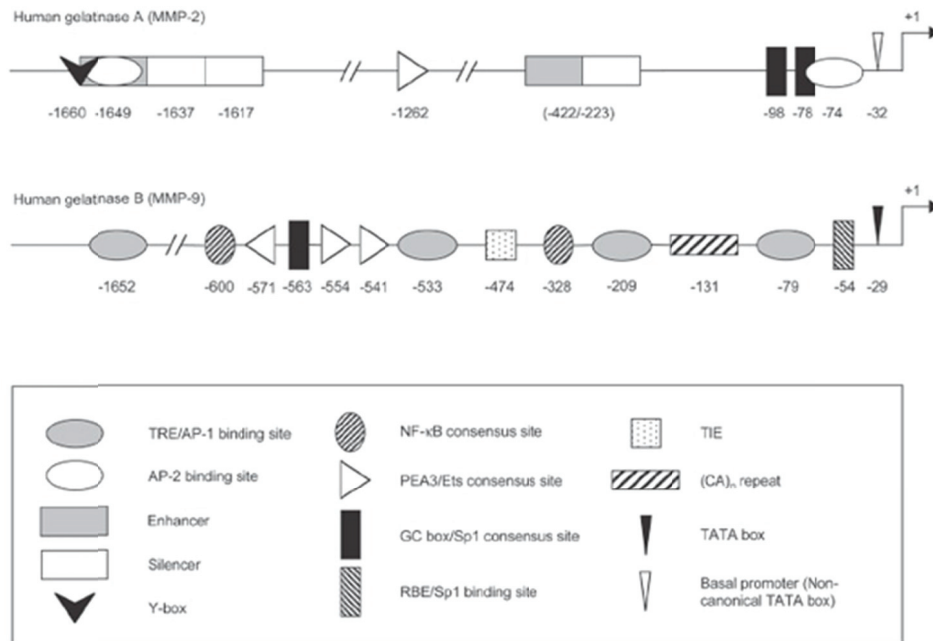
The regulation of gelatinases occurs on different levels:

- Transcription;
- Glycosylation;
- Activation of the pro-enzyme;
- Inhibition by specific inhibitors (TIMP-1 and TIMP-2);
- Uptake and internalization by membrane receptors.

### Transcription

Usually, MMPs expression in tissues is low whereas it increases when ECM remodeling is required.

The different response of the genes of MMP-2 and MMP-9 is due to differences in the promoter region (**Figure 6**). In fact, the promoter region of MMP-9 gene contains regulatory elements recognized by transcription factor induced by growth factors and cytokines, whereas MMP-2 has not. For this reason, the MMP-2 gene is considered more refractory to modulation by growth factors and cytokines produced during inflammation, while MMP-9 can be up-regulated.



**Figure 6. Main regulatory cis elements in the promoter/enhancers region of MMP-2 and MMP-9 genes.**

(Van den Steen et al., 2002)

All the elements present in the promoter region are essential for the regulation of MMP-9 expression, as in many cell types it is expressed at low levels while it can also increase 100 times when induced; moreover, the transcription can be regulated in a tissue specific manner (Frisch and Morisaki., 1990). In the cells where MMP-9 synthesis can be regulated, the pro-enzyme is released after stimulation: often it is secreted together with variable amounts of the inhibitor TIMP-1 and MMP-2 (Van den Steen et al., 2002). Neutrophils are the only cell type where MMP-9 is not synthesized: it is stored within tertiary granules and released after stimulation with different agonists.

Instead, MMP-2 is expressed constitutively by several cell types and only in certain neoplastic cells its expression may increase.



There are several factors that control the transcription of MMP-9: we can distinguish between suppressing factors and inducing factors of the enzyme synthesis and they can vary depending on the examined cell type (Table 2).

**Table 2. Some inducing factors and suppressing factors of MMP-9 synthesis.**  
(Van den Steen PE et al., 2002)

Inducing factors	Suppressing factors
EGF (epidermal growth factor), PDGF (Platelet derived growth factor), bFGF (basic fibroblast growth factor), TGF- $\alpha$ , amphiregulin, TNF- $\alpha$ , IL-1 $\alpha$ , IL-1 $\beta$ , IFN- $\alpha$ (interferon- $\alpha$ ), IFN- $\gamma$ , TGF- $\beta$ , phorbolic esters.	IFN- $\beta$ , IFN- $\gamma$ , IL-4, IL-10.  In some cell types also IFN- $\alpha$ , IL-1 $\alpha$ , IL-13, EGF, PMA (phorbol 12-myristate 13-acetate) and TGF- $\beta$ .

While there are numerous cytokines that can induce MMP-9 synthesis, only a small number of them (IL-4, IL-10, IFN- $\beta$  and IFN- $\gamma$ ) have an inhibitory effect on basal or stimulated expression of MMP-9 (Van den Steen PE et al., 2002).

Generally, IFN- $\beta$  and IFN- $\gamma$  have an inhibitory effect on MMP-9 expression, although it has been shown that the treatment of melanoma metastatic cells with these cytokines cause an up-regulation of MMP-9 (Hujanen et al., 1994; Wu et al., 1997).

Concerning IL-4 and IL-10, cytokines produced by lymphocytes Th2, they have an inhibitory effect on MMP-9 production by monocytes and macrophages at pre-translational level: probably this is due to the down-activation of Protein Kinase A (PKA) which normally promotes the MMP-9 synthesis (Van den Steen PE et al., 2002).

Furthermore, IL-4 and IL-10 can act indirectly inhibiting other functions of monocytes and macrophages, such as the production of inducing factors of MMP-9.

In addition to the modulation of MMP-9 expression, cytokines are powerful regulators of collagen metabolism: IL-1 $\alpha$ , TNF- $\alpha$  and IL-6 can decrease directly the collagen synthesis varying the expression of the pro-collagen mRNA (Siwik AD et al., 2004).

Finally, also the oxidative stress can regulate the extracellular matrix acting on MMP-9 activation/transcription and decreasing the collagen synthesis (Siwik AD et al., 2004).

## Glycosylation

MMP-9 is a heavily glycosylated protein, whereas MMP-2 is not glycosylated.

Three possible N-glycosylation sites in MMP-9 have been located in the N-terminal end of the protein: one is in the prodomain (Asn<sub>38</sub>-Leu-Thr), while the others are in the catalytic domain (Asn<sub>120</sub>-Ile-Thr and Asn<sub>127</sub>-Tyr-Ser) (**Figure 7**).



**Figure 7. Glycosylation of MMP-9.**

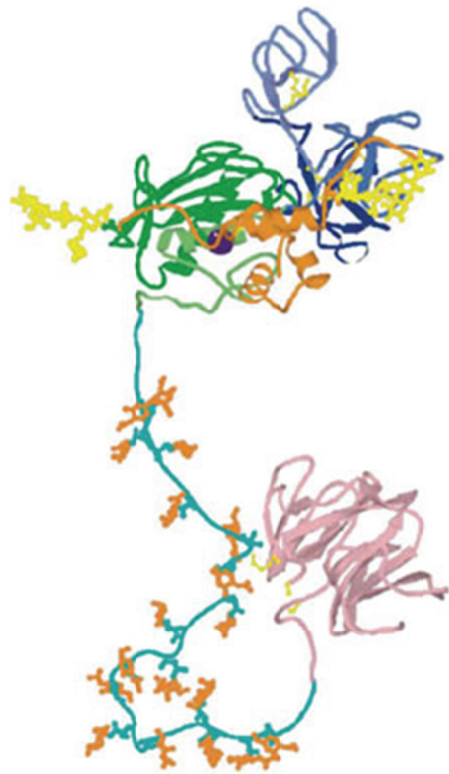
*The N-linked glycans are indicated with Y, while O-glycosylation sites are indicated with vertical lines. Pro, prodomain; Act, active site domain; FN, three repeat fibronectin domain; Zn<sup>2+</sup>, Zn<sup>2+</sup>-binding domain; OG, Collagen V-like domain; Hem, hemopexin-like domain (Van den Steen PE et al., 2006).*

The N-linked glycans in the MMP-9 have a variable composition and can have different functions. For example, during the proteolytic cascade that leads to MMP-9 activation, the glycosylation is important for the specific activity of t-PA and plasminogen. Furthermore, N-linked glycans could influence the MMP-9 stability, the resistance to proteases or they could modulate the interaction with substrates, inhibitors or with enzymatic complexes that degrade the ECM. However, it has been shown that the enzymatic activity of MMP-9 does not change after the removal of N-linked glycans (Rudd PM et al., 1999).

The majority of the carbohydrates are O-linked (about 85%), and are located especially in the collagen V-like domain, typical of this metalloproteinase (**Figure 8**). The analysis of these glycans showed that a big variability in its composition exists (Rudd PM et al., 1999).

Different functions have been hypothesized for these O-linked glycans, including protection from proteolysis and increase of the protein stability. Moreover, knowing that O-linked glycan clusters increase the extension and the rigidity of the polypeptide chain, probably they could contribute maintaining the hemopexin-like domain in the correct distance from the other domains and favoring the interaction with its specific inhibitor (Rudd PM et al., 1999).

In fact, it has been shown that the deletion of the collagen V-like domain decrease 4 times the affinity for TIMP-1, suggesting that this domain is essential to maintain the correct space between the C-terminal domain and the catalytic one (Van den Steen PE et al., 2006).



**Figure 8. Possible 3D structure of glycosylated MMP-9.**  
Yellow – N-linked glycans; Orange – O-linked glycans; Green – catalytic domain; Blue – fibronectin domain; Cyan – collagen V-like domain; Light pink – hemopexin-like domain (Van den Steen PE et al., 2006).

### **Pro-enzyme activation**

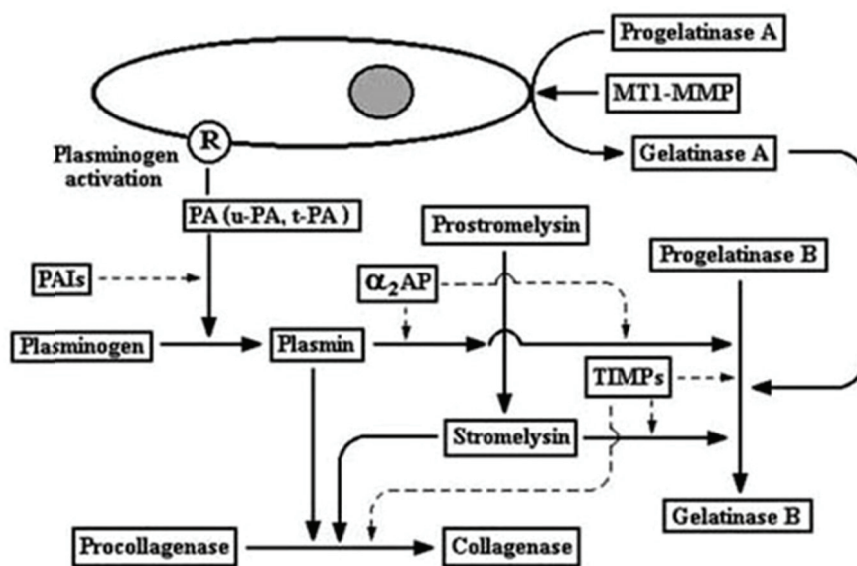
Both gelatinases are synthesized and secreted as zymogens or pro-enzymes, which remain inactive until propeptide removal.

The removal of the propeptide can occur by other proteases including the activation system of plasminogen/plasmin and other MMPs, such as membrane type MMPs (MT-MMPs).

Plasminogen can be converted to plasmin through the tissue plasminogen activator (t-PA) or the urokinase plasminogen activator (u-PA), and then can activate other MMPs. These MMPs can also be activators of other

metalloproteases generating an activating protease cascade which leads to the final conversion of proMMP-9 to active MMP-9 (**Figure 9**).

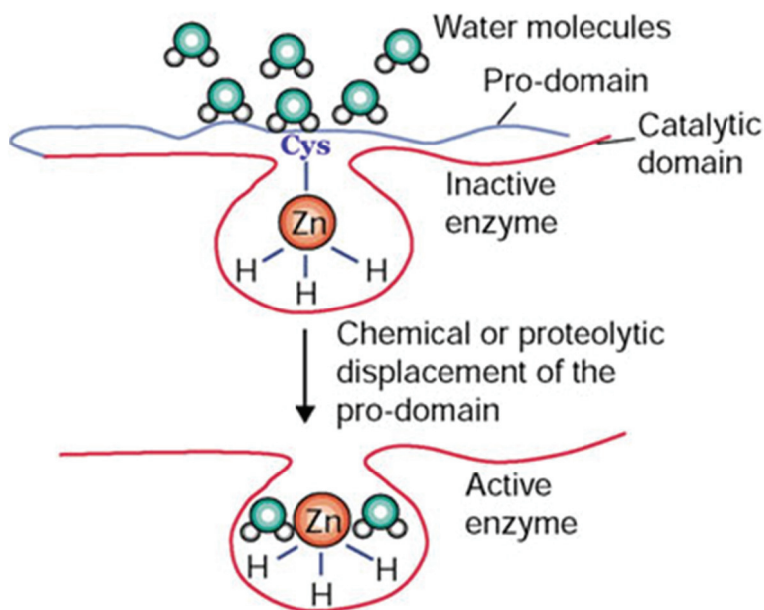
Another activation way involves the MT-MMPs: these proteases have a furin-sensible motif located in the pro-domain and can be activated into the intracellular space. After their exposure on the extern surface of the plasma membrane, they can act on other proteases like MMP-2 or MMP-13, which both can activate the MMP-9 (Van den Steen et al., 2002). Therefore, two important members in this proteolytic activation cascade are MMP-2 and MMP-9.



**Figure 9.** Activation cascade of MMP-2 and MMP-9 (gelatinase B). (Chakraborti S. et al., 2003).

The proposed mechanism for the activation of MMPs is called “Cysteine Switch”, from the cysteine of the propeptide coordinated with the catalytic zinc which maintains inactive the enzyme (Van Wart HE. et al., 1990). When the propeptide has been removed the dissociation of the cysteine from the zinc occurs, with the exposure of the active site that

becomes accessible to the substrate and to the water molecules necessary for the catalysis (**Figure 10**).



*Figure 10. Cysteine switch mechanism for the MMPs activation.*

Therefore, when the cysteine is coordinated to the zinc, the enzyme activity is virtually switched off; thus, the dissociation of the cysteine is seen as the switch that turns on the activity of the enzyme.

In addition to proteases, there are several chemical compounds that can activate the MMPs disrupting the interaction between the cysteine and the catalytic zinc. These can be grouped in different categories: disrupters of the conformation (SDS, NaSCN), reversible modifiers of the sulphidryl group (Hg (II), organomercurial agents), and irreversible modifiers of the sulphidryl group (oxidants and alkylating agents).

Among all, the most widely used are the organomercurial agents (i.e. 4-aminophenylmercuric acetate, APMA); the treatment with these

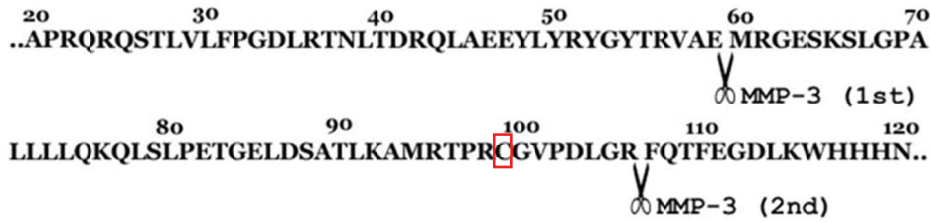
compounds cause the displacement of the sulphidryl group from the zinc leading to an early non-proteolytic activation of the enzyme (Van Wart et al., 1990). Afterwards, it takes place an auto-proteolytic cleavage of the propeptide which leads to the final activation of the enzyme. Furthermore, in the MMP-9 has been observed the loss of the hemopexin-like domain.

In addition, MMP-9 can be activated by reactive oxygen species (ROS) including hypochlorous acid: this is interesting since neutrophils can produce ROS after the respiratory burst activation and they also release MMP-9 (Siwik AD et al., 2004).

### **Activation of proMMP-9**

MMP-9 is synthesized as a 92 kDa proenzyme. As said before, one activation way is through the plasminogen/plasmin system, which converts the proMMP-3 (or pro-stromelysin-1) to active MMP-3, a physiologic activator of proMMP-9 (Ogata Y et al., 1992).

The activation of MMP-9 by MMP-3 occurs in two subsequent steps: the first step is the cleavage of a first portion of the propeptide between the residues Glu<sub>59</sub>-Met<sub>60</sub>, whereas the second step results in the complete removal of the propeptide with a cleavage between the residues Arg<sub>106</sub>-Phe<sub>107</sub> (**Figure 11**). The first cut is necessary because the second takes place: this suggests that probably the residues are not accessible to MMP-3 when MMP-9 is still zymogen.



**Figure 11. Cleavage of MMP-9 propeptide by MMP-3.**

MMP-3 cleaves the propeptide of proMMP-9 in through a two-steps process. The scissors mark the cleavage sites; with a red rectangle is marked the cysteine coordinated with the catalytic zinc (adapted from Ogata Y et al., 1992).

The first cleavage gives a transient 86 kDa form of MMP-9 which is inactive, since the inhibitor  $\alpha_2$ -macroglobulin doesn't bind to it (this nonspecific inhibitor doesn't bind inactive proteases). The second cleavage gives a 82 kDa MMP-9 which is one of the active forms of this enzyme. In fact, there is also another active form that lacks the hemopexin-like domain, produced after the cleavage between the residues Pro<sub>447</sub>-Glu<sub>448</sub> (Okada Y et al., 1992; Saphiro et al., 1995): its molecular weight is 66 kDa and it might be produced only starting from proMMP-9 not bound to TIMP-1. This form is really important because it may skip the normal regulatory mechanism from its specific inhibitor: in this way it may cause incontrolled damages to the ECM.

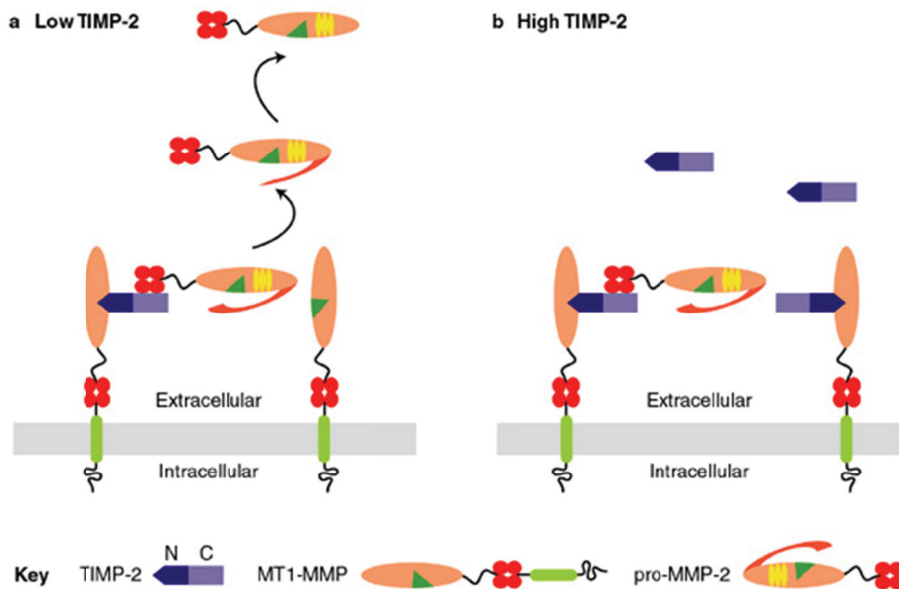
Finally, there is also another form of MMP-9 with a molecular weight of 50 kDa due to the degradation of the 66 kDa MMP-9, which is no longer active: this could be a physiologic mechanism to down-regulate the enzymatic activity of the 66 kDa MMP-9 (Saphiro et al., 1995). We can see a similar pattern of proMMP-9 activation when it is treated with organomercurial compounds, generating various non-active intermediate forms before producing the active 82 kDa and 66 kDa forms.



## Activation of proMMP-2

MMP-2 is synthesized as inactive form (proenzyme) with a molecular weight of 72 kDa.

The activation mechanism is complex and involves the interaction of the enzyme with its specific tissue inhibitor, TIMP-2, and the MT1-MMP (figure 12).

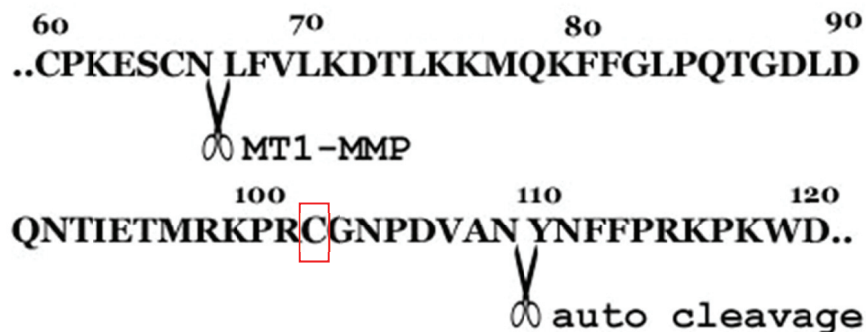


**Figure 12. Activation mechanism of proMMP-2.**

High levels of TIMP-2 (panel b) prevent the activation of proMMP-2, as the inhibition of MT1-MMP occurs (picture adapted from *Expert Reviews in Molecular Medicine*, 2003, Cambridge University Press).

The MT1-MMP on the cell surface acts as a receptor for TIMP-2, which binds to the active site of MT1-MMP through the N-terminal domain. The formed complex acts as receptor for proMMP-2, with the C-terminal domain of TIMP-2 interacting with the hemopexin-like domain of the proenzyme. One free active molecule of MT1-MMP next to the complex cut the propeptide of proMMP-2 between the residues Asn<sub>66</sub> and Leu<sub>67</sub> (figure 13) forming an intermediate species which is released from the

“receptor”. A second autoproteolytic cleavage of MMP-2 between the residues Asn<sub>109</sub> and Tyr<sub>110</sub> (**figure 13**) leads to the complete removal of the propeptide with the formation of the active 67 kDa MMP-2 (Morgunova E et al., 1999).



*Figure 13. The two steps in the activation process of proMMP-2.*

The activation mechanism provided by this model is possible only at low TIMP-2 concentrations, since it can also inhibit MT1-MMP (**figure 12**). High concentrations of TIMP-2 allow the formation of the molecular complex MT1-MMP/TIMP-2/proMMP-2, but the TIMP-2 in "excess" is also linked to the free MT1-MMP preventing the activation of proMMP-2.

ProMMP-2 can also combine with two other inhibitors, TIMP-3 and TIMP-4 with a similar mechanism described for the TIMP-2. It has been observed that the proMMP-2-TIMP-4 complex is able to interact with MT1-MMP without producing active MMP-2 (Nagase H et al., 2006). The biological significance of the complex proMMP-2-TIMP-3 is not known yet.

### **Receptors that mediate the catabolism of MMP-2 and MMP-9**

One mechanism that could be important in the regulation of the levels of these two proteases is the binding to specific receptors, the low density Lipoprotein Receptor-related Protein-1 (LRP-1) and low density Lipoprotein Receptor-related Protein-2/megalin (LRP-2/megalin). These receptors are abundant in hepatic cells and macrophages: these last are one of the major sources of MMP-9 and MMP-2.

It has been shown that these receptors can bind the MMP-2 in complex with trombospondin-2 or TIMP-2, but are not able to bind the free enzyme (Hahn-Dantona E. et al., 2001). On the contrary, the LRP-1 and LRP-2 receptors can bind the free MMP-9 and its complex with TIMP-1, without changing the affinity: this suggests that the site recognized by the receptor is on the MMP-9 molecule (Hahn-Dantona E. et al., 2001; Van den Steen PE. et al., 2006). Mutagenesis experiments showed that the collagen V-like domain is important for the binding to these receptors: its deletion decrease 10 times the efficiency of the binding, suggesting that it is essential for the correct orientation of the molecule. Moreover, these experiments identified in the hemopexin-like domain the binding site to the receptors (Van den Steen PE. et al., 2006). Once internalized, the protease is degraded within the lysosomes.

It is clear that this mechanism of MMP-2 and MMP-9 uptake is important to regulate the extracellular levels of these two enzymes. In fact, it has been observed that many tumours are associated to high levels of gelatinases correlating with a low expression of the LRP-1 and LRP-2 receptors, conferring to the cell a more aggressive behaviour (Hahn-Dantona E. et al., 2001).

## **Inhibition**

Once MMPs have been secreted and activated into the extracellular space, one important mechanism of control on their activity is the inhibition. It can take place through non-specific inhibitors, such as  $\alpha_2$ -macroglobulina, or by the interaction with specific inhibitors namely TIMPs (Tissue Inhibitor of Matrix Metalloproteinase). Up to now, four members of TIMPs family have been identified: TIMP-1, TIMP-2, TIMP-3 and TIMP-4. All these inhibitors share a high structure homology.

In the primary structure there are 12 conserved cysteines forming 6 disulfide bridges (**Figure 14**) essential for the correct folding and inhibitory activity of the protein (Murphy G and Willenbrock F, 1995). These disulfide bridges define 6 loops in the secondary structure of the protein: the first halves are in the N-terminal domain and the second halves in the C-terminal domain. These two domains follow an independent folding: it has been shown that keeping only the N-terminal domain the resulted molecule still has inhibitory activity (Van den Steen et al., 2002). Moreover, this suggests that the inhibition is due to the N-terminal domain.

However, also the C-terminal domain plays important roles: in fact, it is involved in the interaction between the gelatinases and their inhibitors.

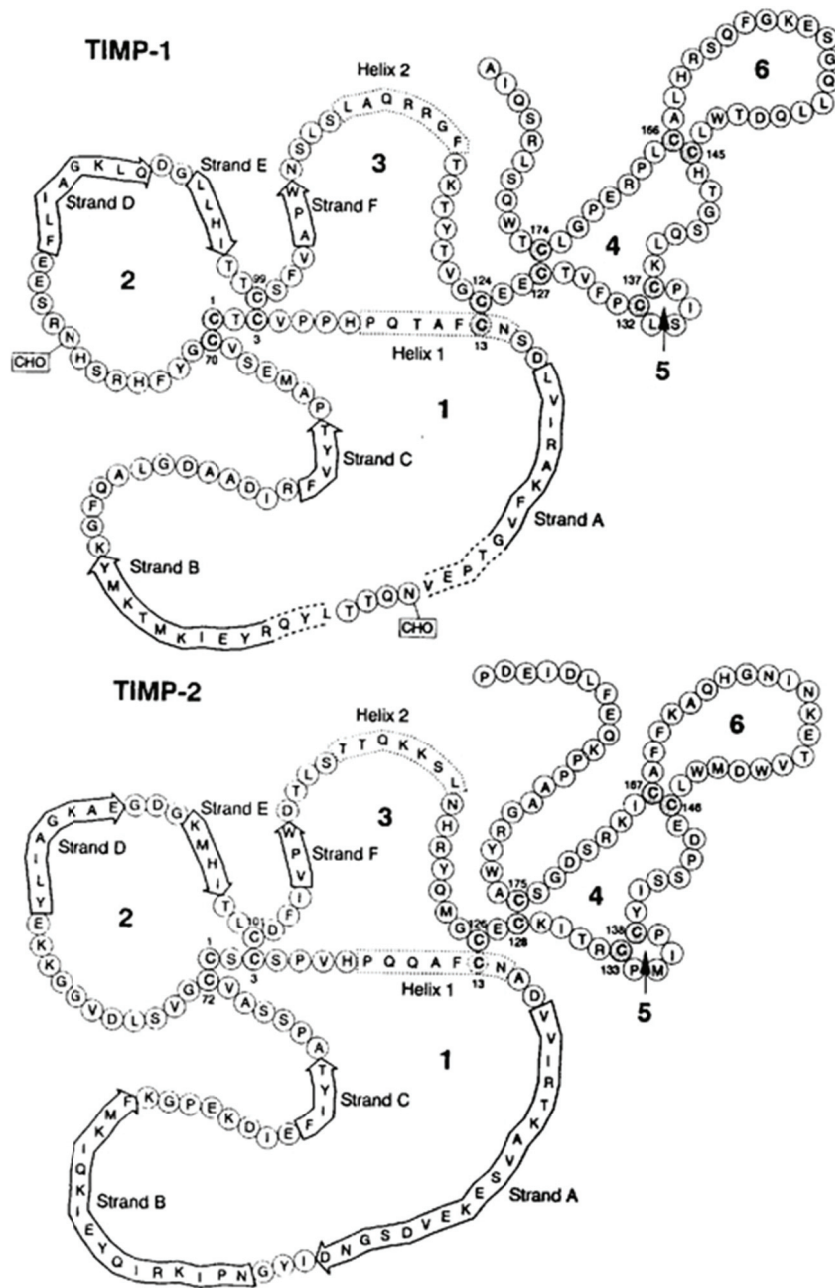
TIMP-1 is a glycoprotein of 28.5 kDa composed by 184 amino acids: the glycosylation sites are placed in the first and second loops (**Figure 14**, CHO mark). Moreover, its content in sialic acid is extremely variable conferring a heterogeneous charge to the molecule.

In addition to the inhibitory role on MMP-9 activity, TIMP-1 can also act on the MMP-3-mediated proMMP-9 activation: when MMP-3 concentration is less than TIMP-1, the protease is inhibited and cannot

activate proMMP-9. On the contrary, if MMP-3 concentration is greater than TIMP-1, proMMP-9 can be activated (Bode et al., 1999).

TIMP-2 is a protein of 194 amino acids with a molecular weight of 21 kDa, differing from TIMP-1 for the lack of glycosylation and the different sequence alignment of the loop 1. Moreover, TIMP-2 has a more extended C-terminal end negatively charged. The presence of this negative charge in the C-terminal end seems to be essential for the not-inhibitory interaction with MMP-2 leading to its activation by MT1-MMP (Bode et al., 1999).

Beside the inhibitory effect, TIMPs have also other physiological functions like stimulating the growth of keratinocytes and fibroblasts: this effect could be due to the inhibition of growth factors degradation mediated by MMPs (Van den Steel PE. et al., 2002).



**Figure 14. Primary structure of TIMP-1 and TIMP-2.**  
 The loops are determined by six disulfide bridges. Here are shown the residues that contribute to the formation of the six  $\beta$ -sheets (arrows) and the two  $\alpha$ -helices (dashed lines) (Murphy G. and Willenbrock F, 1995).

### Mechanism of inhibition of TIMP-1 and TIMP-2

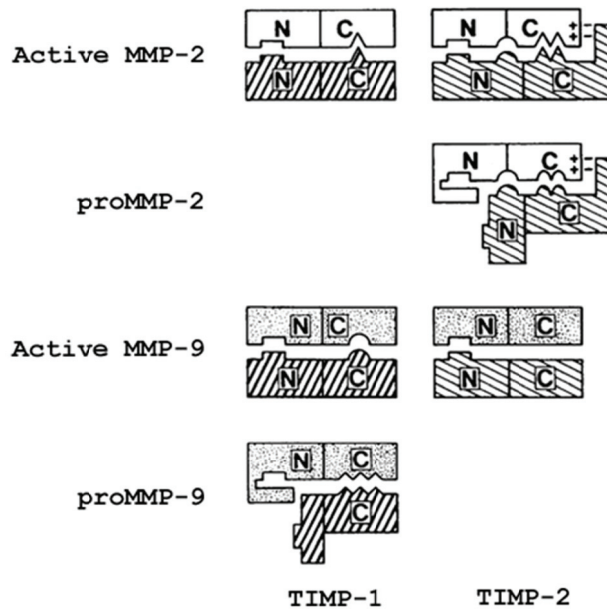
The mechanism of interaction of TIMP-1 and TIMP-2 with the corresponding MMPs (MMP-9 and MMP-2) follows a general pattern, with little variations.

For both gelatinases there are two sites of interaction with the inhibitor: one at high affinity, represented by the hemopexin-like domain with a nanomolar Kd, and one at low affinity in the active site of the enzyme, with a micromolar Kd. As a general mechanism of interaction, the C-terminal domain of the inhibitor binds to the C-terminal domain of the enzyme, while the N-terminal domains of both molecules interact together; this so-defined mechanism provides a 1:1 stoichiometry (Bode et al., 1999).

The forms of MMP-9 and MMP-2 have different dissociation constants from the inhibitors, due to different ways of interaction with them (**Table 3; Figure 15**).

*Table 3. Dissociation constants for active and pro MMP-9. (Olson MW et al., 1997).*

MMP	TIMP-1		TIMP-2	
	Kd (μM)	Kd (nM)	Kd (μM)	Kd (nM)
proMMP-9 (92 kDa)	7.4	35	-	-
MMP-9 attiva (82kDa)	3.1	23.9	12.7	57.9
proMMP-2 (72 kDa)	-	-	0.19	5.2
MMP-2 attiva (67 kDa)	8.5	28.6	2.7	23.1



**Figure 15. Interaction of TIMP-1 with active MMP-9 and proMMP-9 during the formation of the complex.**

*Adapted from Murphy G. and Willenbrock F, 1995.*

Although TIMP-1 binds specifically and with high affinity to MMP-9, it has been observed that it is also able to bind MMP-2; moreover, also TIMP-2 can bind MMP-9, but with low affinity.

Regarding to the active MMP-2, the binding of TIMP-2 is faster and with higher affinity than the binding of TIMP-1. This is probably due to the negatively charged C-terminal tail of TIMP-2 which can specifically bind the C-terminal domain of MMP-2. These interactions between charges are also responsible for the binding of TIMP-2 to proMMP-2, since the interaction between the N-terminal domains are not possible as the proMMP-2 still has the propeptide (Murphy G. and Willenbrock F, 1995).

Even in the case of MMP-9, the enzyme binds faster and with greater affinity to the TIMP-1 compared with TIMP-2, and this seems due to the



interaction between the C-terminal domains of the two proteins. In fact, it has been shown that the TIMP-2 binds only to the catalytic site (with low affinity) of MMP-9, without any interaction between the C-terminal domains (**figure 15**).

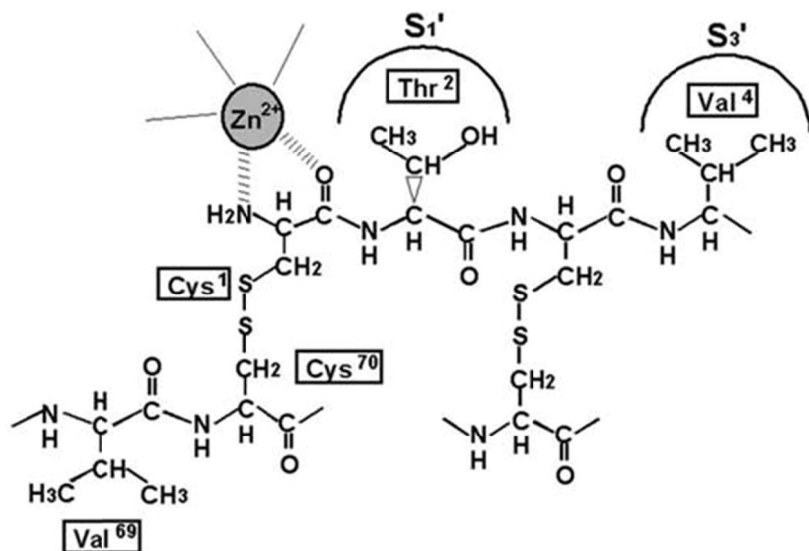
As for the association between proMMP-2/TIMP-2, the ability of TIMP-1 to bind to proMMP-9 is due to the interaction between the C-terminal domains of the two proteins.

The inhibitors can bind both sites on the active form of the enzymes except for the TIMP-2/active MMP-9 interaction, where only the N-terminal domains are involved (**figure 15**).

The inhibitory activity of TIMP-1 and TIMP-2 is due to the interaction of the first five amino acids (from Cys<sub>1</sub> to Pro<sub>5</sub>) and the strand C of the inhibitor with the enzyme (Bode et al., 1999).

The first five residues of TIMP bind the active site of MMP mimicking the P1-P1'-P2'-P3'-P4' sites of a possible substrate.

The Ser<sub>68</sub>/Val<sub>69</sub> residues of TIMP-1 and Ala<sub>70</sub>/Val<sub>71</sub> of TIMP-2 from the strand C interact with the S2 and S3 sites on the enzyme in a reverse way respect to a substrate (Bode *et al.*, 1999) (**Figure 16**).



**Figure 16.** Mechanism of interaction of the N-terminal domains of TIMP-1 and MMP-9.

The TIMP-2 follows a similar mechanism, only the interacting residues change.

From all these interactions, the Cys<sub>1</sub> of the TIMP is placed on the catalytic zinc, avoiding the enzyme to be active. The  $\alpha$ -amino group of Cys<sub>1</sub> occupies roughly the site of water binding in the active site, forming a hydrogen bond with the catalytic residues Glu<sub>402</sub> in MMP-9 and Glu<sub>404</sub> in MMP-2, avoiding any catalytic function. However, mutagenesis experiments have been shown that this interaction is not essential for the inhibition (Bode et al., 1999).

The Thr<sub>2</sub> and Ser<sub>2</sub> residues respectively of TIMP-1 and TIMP-2 interact with the site S<sub>1</sub>' of the MMP-9; the Ser<sub>2</sub> of TIMP-2 forms a hydrogen bond with the catalytic residue Glu<sub>404</sub> of MMP-2.

The Cys<sub>3</sub>, Val<sub>4</sub> (TIMP-1) or Ser<sub>4</sub> (TIMP-2) and Pro<sub>5</sub> interact with the S<sub>2</sub>', S<sub>3</sub>' and S<sub>4</sub>' sites as a substrate should do (**figure 16**).

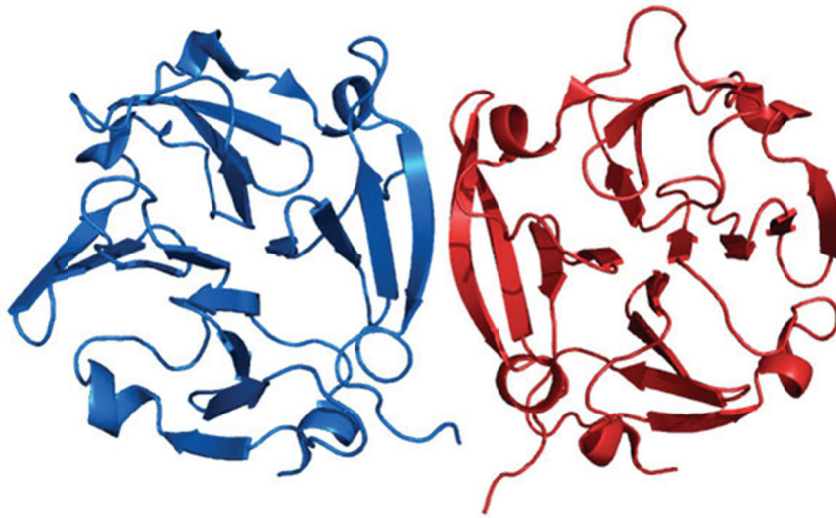
## **Different forms of gelatinases**

We can distinguish different forms of gelatinases, both not active or active.

MMP-2 exists only as monomeric form: a proenzyme of 72 kDa, which is not glycosylated; an active form of 62 kDa generated after plasma membrane activation with the N-terminal sequence starting at Tyr<sub>81</sub>. Moreover, an additional active species of about 45 kDa was reported, with high specific activity (Fridman R et al., 1995). This last form lacks the C-terminal domain, which is important for the interaction with its inhibitor TIMP-2. Thus, the nature of the active species of MMP-2, formed after plasma membrane activation, may regulate enzyme activity and inhibition by TIMP-2 (Fridman R et al., 1995).

Also MMP-9 exists in different forms: a monomer of 92 kDa, corresponding to the inactive enzyme, an homodimer of ~220 kDa and an heterodimer of ~120-130 kDa bound to the lipocalin-2 or NGAL (Neutrophil Gelatinase-Associated Lipocalin; Olson *et al.*, 2000). All these forms were identified in different MMP-9-producing cell types, both normal or pathological, and in various biological fluids and tissues, confirming that both dimers and monomers are physiologically present in the human organism.

Concerning dimerization, it takes place within the cell (Dufour A. *et al.*, 2010) involving the formation of a disulfide bridge between two cysteines of the blade IV of the hemopexin-like domain (Dufour A. *et al.*, 2010).



***Figure 17. Representation of two hemopexin-like domains involved in the dimerization.***

The heterodimeric form of MMP-9 covalently associated with NGAL has been found within the tertiary granules of neutrophils, and it is released in the extracellular space after different degranulation stimuli together with the monomer. However, its physiological role is not yet known although it has been shown that the association between NGAL and MMP-9 can prevent the degradation of the gelatinase (Yan L et al., 2001).

In addition to dimers and proenzyme form, MMP-9 exists in two active forms as previously reported: an 82 kDa and a 66 kDa form, both produced by MMP-3 and APMA activation. The difference between these two forms is the lack of C-terminal domain in the 66 kDa MMP-9, which is necessary to bind with high affinity the specific inhibitor TIMP-1. In fact, it has been proposed that the C-terminal truncation of MMP-9 decreases the rate of formation of the enzyme-inhibitor complex (O’

Connell JP et al., 1994) and might reduce the TIMP-1 binding ability (Roeb E et al., 2000). However, up to now this potentially uncontrolled MMP-9 active form is not yet evidenced in body fluids.

### **Inflammatory conditions**

Several evidences exist on the role of MMP-9 in pathological conditions. When the failure of regulatory mechanisms occurs, it might lead to diminished or excessive production of MMP-9 and subsequently to restricted, extensive, or improperly timed degradation of ECM (Van den Steen PE et al., 2002). Examples of this pathway are premature rupture of amniotic membrane and pathologic bone resorption. Other mechanisms of disease induction are inflammation, infection, vascular pathology, degeneration and malignancy.

In particular, MMP-9 has been associated to inflammatory conditions playing a dual role: direct, by tissue destruction when its activity is not controlled by TIMP-1, or indirect, by generation of an inflammatory signal or recruitment of inflammatory cells (Delclaux et al., 1996).

Its role is well-accepted in neuroinflammatory diseases where the impairment of the Blood Brain Barrier (BBB) occurs, such as Multiple Sclerosis (MS), Intra Cerebral Hemorrhage (ICH), brain ischemia, as the up-regulation of the total enzyme levels were detected, but there are not information on the levels of the active form of MMP-9.

On the other hand, the role of MMP-2 in inflammatory conditions is controversial. In fact, MMP-2 has been associated to both tissue damage and repair (Itoh T et al., 2002; Yong VW, 2005).

One evidence of the role of MMP-2 in the repair of injured tissue after the onset of inflammation is its ability to process a chemokine, the MCP-

3 (Monocyte Chemoattractant Protein-3), resulting in a shorter form with antagonist activity on receptors that bind the entire MCP-3 or other chemokines (McQuibban GA et al., 2000).

The production of this form causes a variation in the chemotactic gradient with a corresponding decrease in the recruitment of other inflammatory cells that may produce lytic enzymes such as MMP-9 (i.e. monocytes and macrophages). Thus, MMP-2 seems to contribute to the resolution of the inflammation, a very important aspect of tissue repair. In addition, the TGF- $\beta$ 1, a growth factor that starts the tissue repair, stimulates the MMP-2 production but inhibits other MMPs: this could contribute to the attenuation of the inflammatory response by decreasing pro-inflammatory stimuli (McQuibban GA et al., 2000).

Therefore, I aimed to evaluate the levels of active MMP-9, active MMP-2, and their inhibitors TIMP-1 and TIMP-2, in inflammatory pathologies in order to clarify the role of the active enzymatic forms as biomarkers of inflammation and resolution of the inflammatory process. Furthermore, I evaluated the influence of pH environmental changes on MMP-9 release from neutrophils, as different pH conditions in tissues are associated with different inflammatory status (Trevani AS et al., 1999; Kellum JA et al., 2004).

### **Multiple Sclerosis**

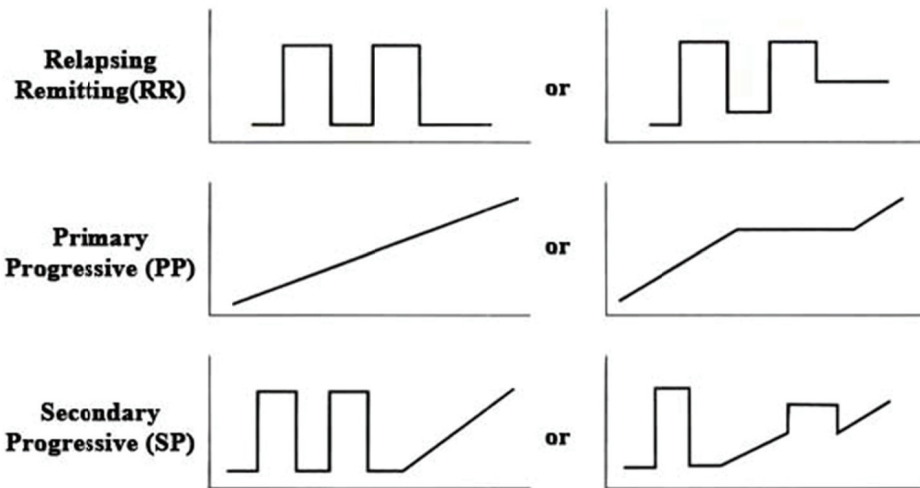
Multiple Sclerosis (MS) is a chronic demyelinating inflammatory disease which affects the Central Nervous System (CNS), in particular the white matter; it is characterized by a progressive loss of myelin and oligodendrocytes and axonal damage (Noseworthy JH et al., 2000). The etiology of MS is still unknown, although it is thought to be on autoimmune base, where the loss of tolerance to one or more unidentified

myelin and oligodendrocytes antigens occurs, starting an immune response (Noseworthy JH et al., 2000).

The progressive myelin degradation in several points of the CNS expresses as clinical variable symptoms, as the destruction of myelin sheaths slows or blocks the transmission of nerve impulses along the fibres of the brain and spinal cord (Noseworthy JH et al., 2000). The areas of the white matter interested in the removal of the myelin are known as demyelination plaques.

Different clinical subtypes of MS are defined on the basis of the disease course (**Figure 18**):

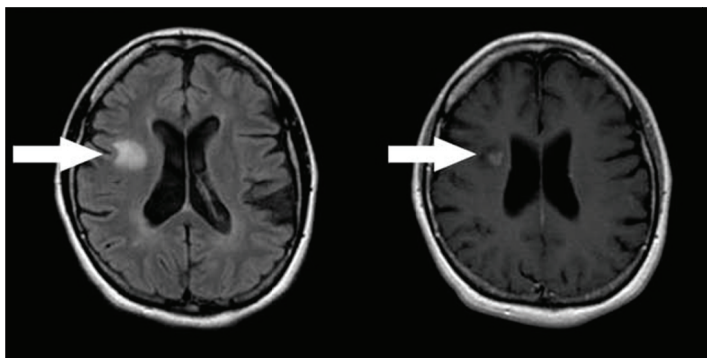
- Relapsing-Remitting (RR), which affects the 80 % of the MS patients, characterized by the abrupt start of symptoms and acute episodes of worsening (exacerbations or relapses), with complete or partial recovery. Between these episodes, patients may be clinically stable, may experience gradual progression or disability, or may undergo to a combination of both (Hauser S et al., 2006);
- Secondary Progressive (SP), in which the RR subtype converts in most cases, characterized by gradual progression of disability with or without superimposed relapses;
- Primary Progressive (PP), that accounts for 10-15 % of all the MS clinical subtypes and it is characterized by gradual progression of disability from disease onset, without superimposed relapses (Hauser S et al., 2006).



*Figure 18. Clinical subtypes of MS.*

The diagnosis of MS is based on the presence of symptoms and signs that characterize the disease (Noseworthy JH et al., 2000), detected with the clinical examination and the Magnetic Resonance Imaging (MRI).

MRI is a powerful instrumental tool that can evidence demyelinating active lesions enhanced after the administration of gadolinium, a paramagnetic contrast agent (Miller DH et al., 1998) (**Figure 19**).



*Figure 19. Demyelinating lesion evidenced by MRI: the first is hyperintense, the second is after the administration of gadolinium.*



As the assumption of gadolinium reflects an alteration of the Blood Brain Barrier (BBB) due to the ongoing inflammation, the foci with contrast enhancement are considered plaques of recent onset where the inflammatory process is still active. On the contrary, the lesions without contrast enhancement are defined as early onset where the inflammatory process is idle (Hawkins et al., 1990). Therefore, MRI is able to detect disease activity as clinical examination do but with more sensitivity, because the appearance of a enhanced lesion may also occur in the absence of relapses or clinical disease activity. This could be due to the appearance of the lesion in a brain area “silent” from a functional point of view, without causing symptoms.

Currently, it is thought that MS is caused by the infiltration into the CNS of autoreactive CD4<sup>+</sup> T helper 1 (Th1) lymphocytes, characterized by the production of pro-inflammatory cytokines and directed against myelin components (Prat et al., 2005). In normal conditions, these lymphocytes encounter a hostile and inhospitable anti-inflammatory microenvironment that prevents the starting of any immune reaction. In MS, the presence of a chronic inflammation into the CNS triggered by the tissue damage converts the microenvironment into a pro-inflammatory type, characterized by the massive recruitment of inflammatory cells and blood proteins into the CNS through the BBB. It causes the accumulation of chemical mediators and amplification of the tissue damage (Prat et al., 2005). Such mediators are proinflammatory cytokines, chemokines and metalloproteases, that facilitate the infiltration of other cells like macrophages, CD4<sup>+</sup> Th1 and Th2 lymphocytes, B lymphocytes and CD8<sup>+</sup> T lymphocytes (Opdenakker et al, 2003). These cells can increase the tissue damage directly through the cytotoxic action, as well as indirectly through the release of soluble products toxic for the

myelin and neurons (Opdenakker et al., 2003). Among these molecules, MMPs may act directly causing damage to the myelin and axons and indirectly by favouring the infiltration of immune cells into the CNS through the BBB. Of all the MMPs, an over-expression of MMP-9 not counterbalanced by the action of TIMP-1 seems to be a crucial step involved in the modulation of the immune and inflammatory responses in MS. In fact, an impairment in the MMP-9/TIMP-1 balance towards uncontrolled proteolytic activity of MMP-9 may lead to promotion of the BBB degradation and to the myelin damage (Opdenakker et al., 2003).

Thus, the MMP-9 proteolytic activity can favour the recruitment of inflammatory cells into the CNS enhancing the ongoing inflammation (Opdenakker et al., 2003). Furthermore, the importance of this enzyme in MS pathogenesis was confirmed by neuropathological studies observing that MMP-9 is expressed by all the cells into the demyelinating lesions typical of MS (Lindberg et al., 2001).

Recently, it has been shown that the variation of active MMP-9, which is the only form that exerts the catalytic activity, rather than the total MMP-9 may be a more appropriate biomarker to monitor the status and course of the disease (Fainardi E et al., 2006). In fact, it has been observed that the levels of active MMP-9 in serum and cerebrospinal fluid (CSF) of MS patients were increased during active state of disease evidenced by both clinical examination and MRI investigation (Fainardi E et al., 2006). Furthermore, it has been found a decrease in TIMP-1 levels in patients with clinical and MRI active state of disease: this is in line with the hypothesis of an imbalance towards the proteolytic activity of MMP-9. Therefore, the variation in the active MMP-9/TIMP-1 ratio might be used as biomarker of disease activity, to monitor the disease course even during pharmacological therapy (Fainardi E et al., 2006).

Unlike MMP-9 that is predominantly up-regulated during inflammatory conditions, MMP-2 is constitutively expressed in the brain (Rosenberg GA, 2002). An over-expression of MMP-2 could be both protective and harmful in MS (Yong VW, 2005; Yong VW et al., 2007) since MMP-2 seems to promote tissue repair as well as penetration of CD4<sup>+</sup> Th1 cells and macrophages across the BBB and myelin and/or axonal injury. Contradictory findings have also been found in previous neuropathological, CSF, and blood studies in which MMP-2 levels in acute and chronic demyelinated lesions as well as in CSF, in serum and in peripheral blood mononuclear cells (PBMCs) were increased, decreased, or represented in equivalent amounts in MS patients when compared to controls (Anthony DC et al., 1997; Lindberg LR et al., 2001; Avolio C et al., 2003; Kouwenhoven M et al., 2003).

### **Spontaneous Intra Cerebral Hemorrhage (SICH)**

Within the various forms of stroke, spontaneous cerebral intraparenchymal hematoma (Spontaneous Intra Cerebral Hemorrhage, SICH) is a relatively common disease which accounts for 10-15% of all cerebrovascular strokes, with a worldwide incidence from 10 to 20 cases each 100.000 inhabitants increasing with age (Qureshi et al., 2001; Xi et al., 2006). Currently, the spontaneous intraparenchymal hematoma is the main cause of death and disability worldwide (Qureshi et al., 2001).

Spontaneous intraparenchymal hematoma is a leakage of blood into the brain parenchyma due to the rupture of small penetrating arteries that originate from the major intracranial arteries (**Figure 20**).

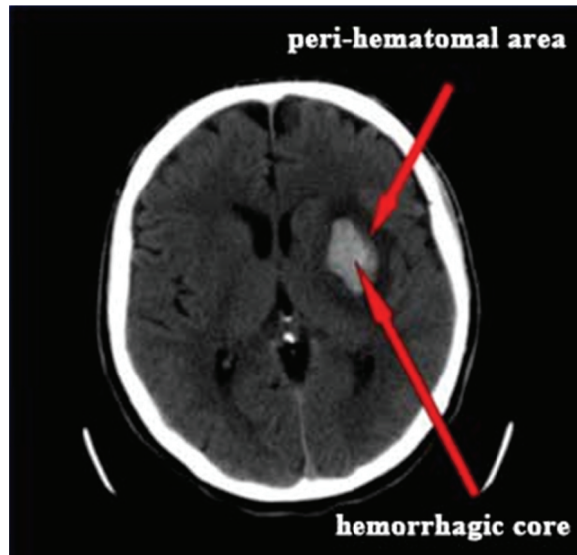


*Figure 20. Spontaneous intraparenchymal hematoma.*

Intraparenchymal hemorrhage is not preceded by premonitory phenomena as happens in brain stroke with transient ischemic attacks (TIA), but begins with acute focal neurological defects that worsen quickly and continuously, reaching the highest level within few minutes or hours (Qureshi et al., 2001).

The worsening of the symptoms in the hours following the cerebral hematoma may be primarily caused by the extension of intraventricular bleeding that increases the possibility of a negative outcome. However, the most common cause of neurologic deterioration is the expansion of the hematoma, which usually occurs within the first 3 hours from the hemorrhage onset (Mayer SA et al., 1994).

The intraparenchymal hematoma is composed by two different areas: the center of the lesion (**hemorrhagic core**) that represents the bleeding area, and the periphery of the lesion (**peri-hematoma area**), consisting of an edematous area surrounding the central core (**Figure 19**) (Parizel PM et al., 2001).



*Figure 21. Hemorrhagic core and peri-hematoma area evidenced by Computed Tomography.*

Different stages can be distinguished in the evolution of the hematoma, characterized by different events in the pathophysiology (Moritani T et al., 2004):

1) **hyperacute stage** (within 6 hours from symptoms onset). In the hemorrhagic core the activation of the coagulation cascade occurs with the appearance of a clot containing blood cells with oxy-hemoglobin, followed by the destruction of the nervous tissue. In the peri-hematoma area starts a compression of the nervous tissue.

2) **acute stage** (7 hours – 3<sup>rd</sup> day from symptoms onset). In the hemorrhagic core appears a blood clot consisting of erythrocytes with intact membrane containing deoxy-hemoglobin. It is followed by the infiltration of macrophages that start phagocytosis of red blood cells. In the peri-hematoma area starts the formation of edema due to the thrombin released in the core.

3) **early sub-acute stage** (4<sup>th</sup> – 10<sup>th</sup> day from symptoms onset). In the hemorrhagic core the clot becomes composed by erythrocytes with intact

membrane containing meta-hemoglobin. In the peri-hematoma area the edema increases and the reactive astrocytosis appears, consisting of a change in the astrocytes shape typical of neuroinflammation.

4) **late sub-acute stage** (11<sup>th</sup> day – 6<sup>th</sup> week from symptoms onset). At hemorrhagic core level the rupture of the erythrocytes's plasma membrane occurs, with the release of meta-hemoglobin. In the peri-hematoma area starts the reduction of the edema and the appearance of gliosis scar in the area next to the hemorrhagic core.

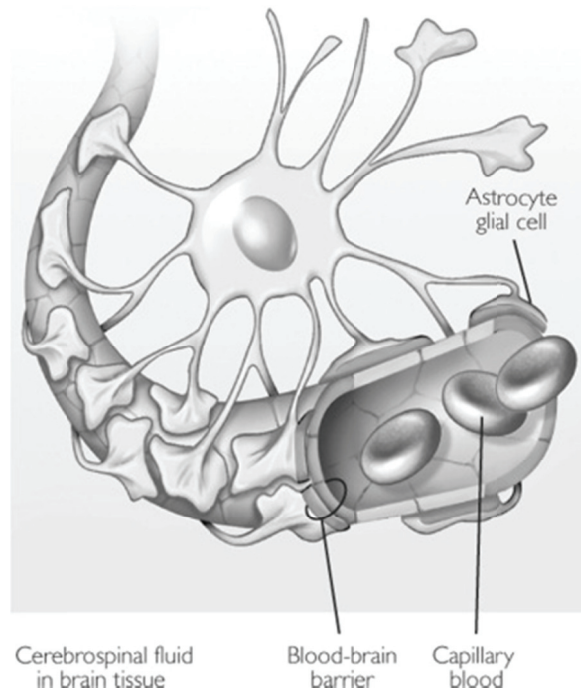
5) **early chronic stage** (7<sup>th</sup> week – 6<sup>th</sup> month from symptoms onset). In the hemorrhagic core starts the accumulation of macrophages containing hemosiderin and ferritin, whereas in the peri-hematoma area starts the resolution of the edema and the formation of a fibrous capsule next to the hemorrhagic core.

6) **late chronic stage** (over the 6<sup>th</sup> month from symptoms onset). In the hemorrhagic core there is the formation of a damaged area containing CSF and occurs the accumulation of macrophages in the surroundings, whereas in the peri-hematoma area starts the retraction of the glial scar.

Summarizing, the hemorrhagic core undergoes cell necrosis and hence the nervous tissue is irreversibly damaged. Instead, the peri-hematoma area is affected by edematous reaction which will be subject to resolution; thus, the nervous tissue does not suffer irreversible damage.

The edema that develops in the peri-hematoma area is accompanied by an intense inflammatory response with the passage of fluids from the blood to the intrathecal space between nervous cells, and the recruitment of inflammatory cells into the brain amplifying the tissue damage.

Both these situations result in an increased permeability of the BBB, which is formed by the tight junctions of brain capillaries and perivascular processes of astrocytes (**Figure 22**).



**Figure 22. Constitution of the Blood Brain Barrier.**

One of the mechanisms that mediate the opening of the BBB is the digestion of the extracellular matrix that separates the capillary endothelium from the basement membrane by MMPs, released by inflammatory cells. This event increases the capillary permeability contributing to the formation of cerebral edema (Gasche Y et al., 1999; Rosenberg G et al., 1997). A growing body of evidence has led the researchers to investigate the role of these enzymes in contributing to the brain damage after ischemia and after trauma (Cunningham LA et al., 2005; Gasche Y et al., 2006), but MMPs are essential for neurogenesis processes, for the formation of the myelin sheath and for the axonal growth (Cunningham LA et al., 2005). Thus, the treatment of patients affected by intraparenchymal hematoma with inhibitors of these enzymes could be more harmful than helpful.

Studies on animal models have shown that the intracerebral injection of MMP-2 produced the opening of the BBB leading to bleeding, whereas the co-administration of TIMP-2 reduced this phenomenon (Rosenberg G et al., 1992). Other evidence of the role of MMPs were obtained for MMP-9 in knockout mice, which showed a small infarct volume and less damage to BBB after focal ischemia (Rosenberg G et al., 1997; Tejima E et al., 2007). Similar results were obtained in experiments on primates which showed an early opening of the BBB after stroke associated to increased expression of MMP-2, whereas the hemorrhagic transformation was related to increased MMP-9 expression (Heo JH et al., 1999; Chang DI et al., 2003).

The role of MMPs in cerebral ischemia have been studied extensively *in vitro* and using animal models; thus, there is little evidence on their role in cerebral haemorrhage in humans.

Few studies have been conducted to determine the concentration of gelatinases and their inhibitors, TIMP-1 and TIMP-2 in plasma of ICH affected patients (Alvarez-Sabin J et al., 2004; Ramos-Fernandez M et al., 2011). In particular, it has been observed that MMP-9 and TIMP-1 tended to be overexpressed in the acute phase of the ICH, with higher levels of these molecules after 24 hours from symptoms onset (Alvarez-Sabin J et al., 2004). On the contrary, MMP-2 expression seems to follow a biphasic behaviour: in fact, it has been observed a peak in MMP-2 levels immediately after the onset of ICH and another after one week from the onset of ICH (Alvarez-Sabin J et al., 2004).

Also, a relationship between the overexpression of MMP-9 and TIMP-1 and different stages of the ICH evolution was observed. In particular, it has been shown a positive correlation between MMP-9/TIMP-1 ratio,



considered a marker to monitor inflammation, and the volume of the edema in the peri-hematoma area (Alvarez-Sabin J et al., 2004).

Moreover, in another study Abilleira and co-workers found higher plasma levels of MMP-9 in patients with ICH, with a relation between relative peri-hematoma edema volume and MMP-9 concentration in patients with deep ICH (Abilleira S et al., 2003). Indeed, the plasma concentrations of TIMP-1 increased regardless of the hematoma topography.

In *in vitro* studies on primary culture of astrocytes from cerebral cortex of mice subjected to hemoglobin-mediated oxidative stress to mimic an haemorrhage, showed that MMP-9 concentration increased within one hour after the oxidative stress exposure in a dose-dependent manner, and the concentration was maintained up to 24 hours (Tejima E et al., 2007). Instead, no substantial changes of MMP-2 levels were observed.

To date, no quantitative data on serum levels of active form of MMP-9 and MMP-2 in patients with spontaneous cerebral hematoma exist.

### **Neutrophils and changes in environmental pH**

There is a growing body of evidence that acid-base abnormalities are capable to induce significant alterations on the immune response. In fact, it has been observed that changes in the extracellular pH can affect the synthesis and the release by cells of the immune system of pro-inflammatory mediators, such as tumor necrosis factor (TNF) and nitric oxide (NO) (Kellum JA et al., 2004).

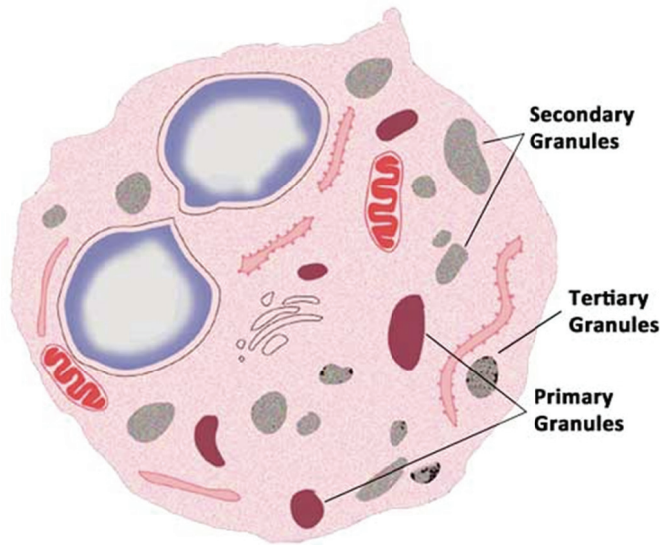
Furthermore, it has been shown that different acids (HCl [hydrochloric acid], lactic acid and lactate solutions) may produce different effects (increase or decrease in the levels of the mediators) despite the same extracellular pH. In particular, it appears that the HCl act as pro-

inflammatory agent, while the lactic acid appears to be an anti-inflammatory agent (Kellum JA et al., 2004).

Extracellular acidosis can also affect other aspects of the immune response, such as extreme acidity (pH between 6.0 and 5.5) causes dysfunction in the chemotactic process of leukocytes (Nahas GG. et al., 1971).

Among all leukocytes, neutrophils are the most abundant type of white blood cells in mammals and form an essential part of the innate immune system. Although they play an important role in host defense against infectious agents, they may also cause tissue damage in a wide range of diseases (Weiss SJ, 1989), where the tissue-damaging effects of neutrophils are completely dependent on the activation of degranulation (Cundall M et al., 2003; Lacy P et al., 2008).

Degranulation is defined as the secretion by receptor-mediated exocytosis of granule-derived substances. Granules of neutrophils may be classified from a functional point of view into peroxidase-positive (or azurophilic or primary) granules, defined by their content of Myeloperoxidase (MPO), and peroxidase-negative granules. The latter can be further subdivided into specific (or secondary) granules, defined by their content of lactoferrin, and gelatinase (or tertiary) granule, defined by their high concentration of gelatinase B (MMP-9) (**Figure 23**) (Borregard N et al., 1997).



**Figure 23. Granules of the human neutrophils.**  
*Adapted from King AE et al., 2003*

MMP-9 is essential for the neutrophil migration through the basal membrane of the endothelium and interstitial tissues, mainly composed by type IV and type V collagens (Murphy G et al., 1982). When overexpressed, MMP-9 may also have detrimental effects as occurs in pathologies such as multiple sclerosis, brain ischemia and Alzheimer disease (Lacraz S et al., 1995).

Studies on human neutrophils reported an activation of the respiratory burst and increased production of reactive oxygen species, phagocytosis and apoptosis upon environmental pH variation (Trevani AS et al., 1999; Martinez D et al., 2006).

In particular, it was observed that neutrophils can be activated by extracellular acidosis (Martinez D et al., 2006), a feature commonly associated with inflammatory reactions against pathogens and other diseases such as cancer, autoimmune diseases and asthma, where acidic microenvironment may occur (Schornack PA et al., 2003).

Such microenvironments may develop as a result of several factors (Martinez D et al., 2006):

- ❖ lowering of the oxygen partial pressure in the inflamed area (hypoxia) and the intense metabolic activity of inflammatory cells that switch from aerobic to anaerobic metabolism (glycolysis), leading to the accumulation of lactate;
- ❖ massive infiltration of neutrophils and macrophages and the production of protons during the respiratory burst activation;
- ❖ the accumulation of short-chain fatty acids produced by bacteria.

Extracellular acidification induces several changes in neutrophils, both functional and structural. It was observed that the levels of intracellular calcium increased significantly in neutrophils stimulated in acidic pH (6.5) compared to physiological pH (7.4) (Trevani AS et al., 1999). In addition, neutrophils change their shape in response to extracellular acidification. Two mechanisms have been proposed to explain such modification:

1. a reorganization of the cytoskeleton leading to disassembling of actin polymers in monomers;
2. activation of the  $\text{Na}^+/\text{H}^+$  antiport causing the cell swelling due to the movement of ions and the entry of water into the cell. It has been shown that the activation of this antiport is necessary for the efficient migration of neutrophils (Rosengren S et al., 1994).

Another effect of acidic pH is the up-regulation of receptors which promote the adhesion of neutrophils to the endothelium, such as the  $\beta 2$  integrin (CD18) (Trevani AS et al., 1999).

Considering that the changes in neutrophils shape and adhesive interactions with endothelial cells are essential steps for the migration to the sites of inflammation, this suggests that acidic extracellular pH can

promote the recruitment of neutrophils in inflammatory areas (Trevani AS et al., 1999).

Moreover, low extracellular pH can trigger the production of H<sub>2</sub>O<sub>2</sub> and the release of myeloperoxidase (marker of degranulation and oxygen-dependent cytotoxic response) from neutrophils stimulated with different agonists (Trevani AS et al., 1999).

However, other studies have shown conflicting results about the effect of extracellular acidosis on neutrophil responses. In particular, it has been postulated that extracellular acidosis can have a negative effect on neutrophils bactericidal activity. In fact, extracellular acidosis can activate the G protein-coupled receptor TDAG8 (T cell death-associated gene 8) leading to an increase in cAMP concentration that induce the inhibition of superoxide anion (O<sub>2</sub><sup>-</sup>) production (Murata N et al., 2009).

Two mechanisms for the action of pH on neutrophils have been proposed: the change in intracellular pH and the stimulation of proton-sensing receptors.

Acidification of the cytoplasm could be due to the diffusion of CO<sub>2</sub> into the cell causing an increase in intracellular protons. Therefore, the extracellular pH decrease can act as a second messenger in the activation of neutrophils. In particular, it has been observed that initial stages of chemotaxis are accompanied by transient cytosolic acidification (Yuli I. et al., 1987).

Another hypothesis is that extracellular acidosis can influence neutrophils through the activation of specific proton-sensing receptors expressed on the surface membrane surface.

Recent studies have shown that protons can be recognized by a subfamily of G protein-coupled receptors, namely GPR4. This subfamily consists of four receptors with high sequence homology: GPR4 (G protein-coupled

receptor 4), OGR1 (ovarian cancer G protein-coupled receptor 1), TDAG8 (T cell death-associated gene 8) and G2A (for G2 accumulation).

Extracellular pH changes are able to activate different pathways depending on the receptor involved: the activation of OGR1 and G2A leads to the accumulation of inositol phosphate, whereas the activation of GPR4 and TDAG8 leads to the formation of cyclic AMP (cAMP) (Martinez D et al., 2006).

In vitro experiments on HL-60 neutrophil-like cells and human neutrophils have shown that the G protein-coupled receptor mainly expressed is the TDAG8, followed by OGR1 and G2A, whereas the GPR4 is not expressed (Murata N et al., 2009).

Although the real mechanism responsible for the recognition of extracellular protons remains to be elucidated, it was shown that extracellular acidosis activates the signal transduction pathways that involve the phosphoinositide 3-kinase (PI3K)/Akt and ERK (Martinez D et al., 2006). Such signaling pathways play an important role in several neutrophils functions: in fact, both PI3K and ERK are required for the activation of chemotaxis, phagocytosis, respiratory burst and are also implicated in the control of survival (Andrews S et al., 2007).

### **Neurofilament subunits and N-Acetyl aspartic Acid (NAA) as markers of axonal damage in progressive multiple sclerosis**

As previously reported, different clinical subtypes of MS are defined (Lublin FD et al., 1996), for which Primary Progressive (PP) MS accounts for 10-15% of all the MS clinical subtypes (Miller DH et al., 2007).

The classification of the disease subtype is highly relevant because disease-modifying treatments are effective only in predominantly relapsing MS (Miller DH et al., 2007). In fact, focal inflammatory lesions are the main features of relapse-onset MS (RRMS), whereas the progressive form of the disease (PPMS) is characterised by a global inflammatory process, with diffuse axonal injury in white matter and cortical demyelination (Kutzelnigg A et al., 2005). This is confirmed by the observation of extensive cortical demyelination and diffuse damage with microglial activation and axonal injury in the healthy-looking white matter of patients with PPMS (Kutzelnigg A et al., 2005). Moreover, Revesz and colleagues (Revesz T et al., 1994) reported few inflammatory cells in lesions of PPMS patients: this finding, combined with early MRI studies that showed fewer focal white-matter lesions in PPMS patients (Thompson AJ et al., 1990), led to the notion that pathological features additional to classic inflammatory, demyelinating, white-matter lesions might be important for the disability in PPMS.

There is a strong evidence that axonal loss underlies irreversible and progressive disability, which has led to the search for potential biomarkers of axonal damage for use in in-vivo studies.

CSF N-acetylaspartate (NAA) as well as Neurofilaments (Nf) light and heavy subunit levels were recently identified as promising markers of axonal damage in MS patients (Teunissen CE et al., 2009).

### **Neurofilaments**

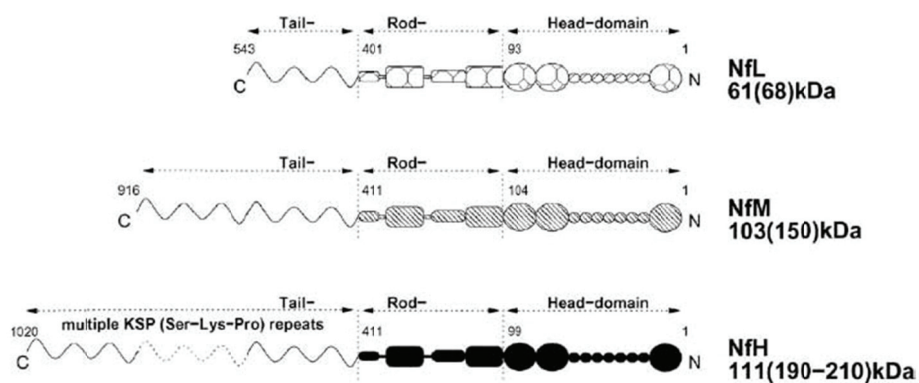
Neurofilaments (Nf) are neuron-specific proteins that have emerged as promising biomarkers for neurodegeneration, especially the Nf light and Nf heavy subunits (Rosengren LE et al., 1996; Norgren N et al., 2003;

Petzold A et al., 2005a). These subunits, together with the Nf medium, compose the cytoskeleton of the axon.

The Nf light consists of 543 amino acids with a calculated molecular mass of 61 kDa, but due to the phosphorylation and glycosylation, migration in SDS PAGE is slow with an apparent molecular mass of 68 kDa (Petzold A, 2005b).

The Nf heavy consists of 1020 amino acids with a molecular mass of the amino acids corresponding to 111 kDa. However, most authors refer to the molecular mass derived from SDS PAGE that ranges from 190 to 210 kDa, due to the phosphorylation of this subunit.

Each subunit is composed of a conserved  $\alpha$ -helical core region, flanked by the head domain (N-terminus) and the hypervariable tail (C-terminus) (**Figure 24**).



**Figure 24. Neurofilaments subunits.**

*NfL*: neurofilament light; *NfM*: neurofilament medium; *NfH*: neurofilament heavy.

The  $\alpha$ -helical domain is mainly relevant for Nf assembly, while the N- and C- terminal domains are responsible for protein-protein interactions.

We can distinguish two main pools of Nf subunits in the axoskeleton: a “static pool”, which is highly phosphorylated and constituted by 80% of

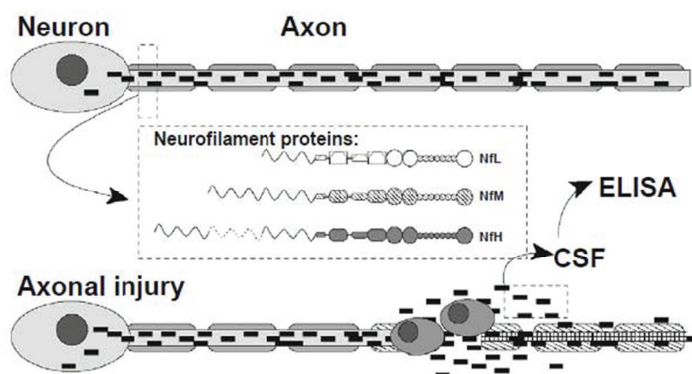


axonal neurofilaments; a “dynamic pool”, less extensively phosphorylated and composed by the remaining 20 % of the neurofilaments (Petzold A, 2005b).

It was proposed that one function of phosphorylation is to increase the charge-based repulsion of neighbouring filaments, increasing the axonal calibre (Waxman SG et al., 1995). However, it has been observed that radial axonal growth is not dependent on phosphorylation alone, and the original hypothesis has been modified in that Nf medium is the main regulator of radial growth (Elder GA et al., 1998).

Another possible function of phosphorylation might be the protection from protease digestion: in fact, it has been shown that susceptibility of Nf to protease degradation decreases increasing the phosphorylation (Goldstein ME et al., 1987).

The measurement of neurofilament subunits in the CSF of MS patients has been employed to assess the possible usage of these subunits as markers of axonal damage: in fact, neurofilaments are normally contained into the axoplasm and are released in the extracellular fluid during axonal disintegration following axonal injury (Petzold A, 2005b) (Figure 25).



**Figure 25. Neurofilaments release in the extracellular fluid following axonal injury.**  
*Adapted from Petzold A, 2005b.*

A growing body of evidence points to Neurofilament light and heavy subunits as surrogate markers for axonal injury in MS.

The light subunit has been related to early phases of MS and even as a prognostic marker in early relapsing-remitting MS (Salzer et al., 2010). In particular, Salzer and colleagues determined in a long-term follow-up study the Nf light levels in CSF of RRMS patients. Their findings associated high CSF Nf light levels with unfavourable prognosis, suggesting that the levels of this subunit could be used as a prognostic marker in early RRMS. Moreover, neurofilament light has been recently used as a marker to monitor the neuroprotective effect of Natalizumab, a disease-modifying treatment (Gunnarsson M et al., 2010). In their study Gunnarsson et al found a 3-fold reduction of Nf light levels after the treatment with the drug, suggesting that Natalizumab can reduce the accumulation of nerve injury in relapsing form of MS (Gunnarsson M et al., 2010). Also, the neurofilament heavy subunit has been shown to be related to disease progression (Petzold A et al., 2005a). In fact in their follow-up study, Petzold and colleagues found that patients with Secondary Progressive (SP)/PPMS experienced an increase in Nf heavy levels between baseline and follow-up compared with those with RRMS. Moreover, Nf heavy levels were higher in SP/PPMS patients than RRMS both at baseline and follow-up. Interestingly, they also found a positive correlation between neurofilament high levels and the EDSS (Expanded Disability Status Scale). All their results suggest that cumulative axonal loss is responsible for sustained disability and that high Nf heavy levels are a poor prognostic sign of MS progression (Petzold A et al., 2005a). Collectively taken, these results point to neurofilament light and heavy subunits as markers of axonal damage in MS.

## **NAA**

A proposed in vivo marker for axonal pathology in MS is N-Acetyl Aspartic Acid (NAA).

NAA is an amino acid present in the vertebrate brain, where its concentration is one of the highest of all free amino acids and it constitutes about 3–4 % of total brain osmolarity (Baslow MH, 2000). NAA is synthesized primarily in neurons being present in amounts up to 20 mM and exhibiting a high intracellular-extracellular gradient. Further, neuronal NAA is dynamic in that it turns over about 1.4 times per day (Moreno A et al., 2001) by virtue of its continuous efflux in a regulated intercompartmental cycling via extracellular fluids between neurons and a second compartment in oligodendrocytes (Baslow MH, 2002).

Post-mortem investigation of spinal cords from MS patients has shown that lower tissue concentrations of NAA are related to lower axonal volume. Furthermore, the average NAA concentration per axonal volume may decrease with increasing demyelination and functional impairment of neurons (Bjartmar C et al., 2000).

MR-spectroscopy (MRS) is widely used to determine parenchymal NAA concentrations in vivo. Clinical studies employing MRS (Bruhn H et al., 1992; Leary SM et al., 1999) universally show decreased NAA levels associated with the progression of the disease. Reduced amounts of NAA in MR-visible lesions and in normal appearing white matter are readily documented (Leary SM et al., 1999): these MRS observations are explained as axonal injury (Criste GA and Trapp BD, 2006). Axonal injury begins early in multiple sclerosis (De Stefano N et al., 2002), and cumulative axon loss results in progressive disability.

MRS studies show that NAA levels can be associated with neuronal dysfunction, as well as neuronal death, because levels have been shown

to recover when MRI visible plaques resolve (Arnold DL et al., 1990). Partial recovery of NAA levels has also been reported after treatment of patients with disease-modifying treatments (Narayan S et al., 2001) suggesting that NAA levels reflect not only neuronal and axonal integrity, but also may reflect improvements in neuronal energetics and possibly remyelination (Moffet JR et al., 2007).

Moreover, recent studies showed that CSF NAA levels are related to disease progression and brain atrophy (Jasperse B et al., 2007; Teunissen CE et al., 2009). In particular, it has been observed that SPMS patients had a significantly lower NAA concentration than RRMS patients (Jasperse B et al., 2007), whereas the levels were not different from patients without neurologic disease in early stages of disease, though decreased as the disease progressed (Teunissen CE et al., 2009). Furthermore, NAA concentrations correlated with EDSS, normalised brain volume and MRI measures of disease burden. Therefore, these studies suggest that NAA may be an important neuron specific marker of disease severity and possibly progression.

Recently, the combination of CSF N-acetylaspartate (NAA) and neurofilament light and heavy subunit levels has increased the potential to identify patients with axonal damage (Teunissen CE et al., 2009). Up to now, little is known about the relevance of Nf isoforms and NAA as markers of disease in the PPMS subtype. In fact, while Nfs have been extensively studied in the RRMS and SPMS (Teunissen CE et al., 2005), a lack of reliable data still remains for the PPMS subtype where small cohorts were studied and often pooled together with SPMS patients (Semra YK et al., 2002; Norgren N et al., 2003). Similarly, different levels of CSF NAA were found only in RRMS and SPMS patients but not in PPMS subtype compared to non-diseased controls, but also here

the studies included small cohorts of primary progressive patients (n=6-8) (Jasperse B et al., 2007; Teunissen CE et al., 2009).

## **AIMS OF THE THESIS**

Actually, the role of gelatinases in inflammation seems to be opposite.

MMP-2 has been related to both tissue repair and damage, although its role is not yet clear; on the contrary, MMP-9 which can be produced also by neutrophils, has been associated to inflammation in several pathologies. Also, the environmental conditions such as acidosis and alkalosis might influence the release of MMP-9 from neutrophils.

Furthermore, the possible presence in body fluids of another active form of MMP-9, which might not be modulated by its inhibitor TIMP-1, could open new insights on the use of active MMP-9 as biomarker in inflammation.

In the present thesis, I aimed to evaluate the role of active MMP-9 and MMP-2 as biomarkers in inflammatory conditions such as Multiple Sclerosis and in Spontaneous Intra Cerebral Hemorrhage; also, I aimed to investigate the presence in serum of the 66 kDa MMP-9, and pH effect on the neutrophils response since low tissue pH has been associated to inflammation.

Secondary, my purpose was to study the role of Nf light and heavy subunits, NAA and their combination as biomarkers of axonal damage in a relatively large cohort of PPMS, since the combination of neurofilaments and NAA were identified as promising markers of axonal degeneration in MS patients.

The final target is to verify if the use of different biomarkers, which reflect various aspects of pathological processes, and their combination could be useful in order to predict the evolution of a disease.

## **MATERIAL AND METHODS**

### **Active MMP-2 and TIMP-2 in Multiple Sclerosis Patients**

#### **Patient selection**

A total of 67 consecutive patients (45 women and 22 men; mean age =  $37.3 \pm 10.2$  years) meeting the criteria for definite MS according to the classification of McDonald et al. (McDonald WI et al., 2001) and admitted to the MS Center of the Department of Neurology, University of Ferrara during the period from October 2004 to January 2008 were prospectively recruited in the study. All patients had relapsing–remitting (RR) course in agreement with the criteria of Lublin and Reingold (Lublin and Reingold, 1996). Evidence of a relapse at admission, as defined by Poser’s criteria (Poser CM et al., 1983), was considered clinical disease activity. Accordingly, at entry 44 patients were clinically active and 23 were clinically stable. Disease severity was scored in all MS patients at the time of sample collection using Kurtzke’s Expanded Disability Status Scale (EDSS) (Kurtzke JF, 1983) (mean at entry =  $2.1 \pm 1.2$ ; range from 0 to 5.5). The duration of the disease was expressed in months (mean at entry =  $36.8 \pm 58.4$ ; range from 0.5 to 300). At entry, none of the patients had fever or other symptoms or signs of acute infections. Moreover, at the time of inclusion in the study none of the patients had received any potential disease-modifying therapies (e.g., azathioprine or methylprednisolone, interferon-beta or glatiramer acetate) during the six months before the study. In all, 64 patients with other inflammatory neurological disorders (OIND) and 65 subjects with other non-inflammatory neurological disorders (NIND) were selected as neurological controls. The OIND group (33 women and 31 men, mean

age =  $52.5 \pm 17.5$  years) included 13 patients with viral encephalomyelitis, 12 with chronic inflammatory demyelinating polyneuropathy, 10 with acute inflammatory demyelinating polyneuropathy, 8 with bacterial meningitis, 6 with HIV encephalopathy, 5 with intracerebral abscess, 4 with neurolupus, 3 with acute demyelinating encephalomyelitis, 2 with neuroSjogren, and 1 with Neurobehcet. The NIND group (28 women and 40 men, mean age =  $54 \pm 15$  years) consisted of 10 patients with headache, 9 with migraine, 7 with transient ischemic attack, 7 with epilepsy, 6 with cervical spondylosis, 5 with vascular dementia, 5 with mild cognitive impairment, 4 with Alzheimer's disease, 4 with hereditary ataxia, 2 with amyotrophic lateral sclerosis, 2 with compression neuropathy, 2 with paresthesias, 1 with confusion, and 1 with vascular myelopathy. Patients with OIND and NIND were free of immunosuppressant drugs, including steroids, at the time of sample collection.

### **Cerebrospinal fluid and serum sampling**

Cerebrospinal fluid and serum samples were collected under sterile conditions and stored in aliquots at  $-70^{\circ}\text{C}$  until assay. "Cell-free" CSF samples were obtained after centrifugation at room temperature of specimens taken by atraumatic lumbar puncture performed for purposes of diagnosis in the absence of contraindications. Serum samples derived from centrifugation of blood specimens withdrawn by puncture of an antero-cubital vein at the same time of CSF extraction. Paired CSF and serum samples from RRMS, OIND, and NIND patients were stored and measured under exactly the same conditions. All CSF and serum analysis were performed within 2 weeks of the onset of clinical symptoms in relapsing RRMS patients and at least 2 months after the end of clinical



exacerbation in clinically stable RRMS patients. Informed consent was given by all patients before inclusion, and the study design was approved by the Regional Committee for Medical Ethics in Research. Cerebrospinal fluid and serum albumin levels were measured by immunochemical nephelometry with the Beckman Array Protein System (Beckman Instruments, Inc. Fullerton, California, USA) according to the procedure in the study by Salden et al. (Salden HJM et al., 1988). Blood-CSF-barrier (B-CSF-B) dysfunction was determined by CSF/serum albumin quotient (QA<sub>lb</sub>) (Tibbling G et al., 1977).

### **Magnetic resonance imaging examination**

Brain magnetic resonance imaging (MRI) scans were performed at entry using a standard head coil in all patients with a 1-tesla MRI unit (GE Signa Horizon, General Electric Medical Systems, Milwaukee, Wisconsin, USA) within 48 h after CSF sampling. Routinely used T1-weighted axial spin echo images were obtained approximately 10 min after intravenous injection of 0.1mmol/kg of gadolinium (Gd)-DTPA in each patient. Lesions showing Gd- enhancement on T1-weighted scans were defined as indicative of MRI activity. Accordingly, 33 MS patients (26 with clinically active and 7 with clinically stable disease) were considered MRI active and 34 (19 with clinically active and 15 with clinically stable disease) were considered MRI inactive. All brain MRI scans were evaluated by one investigator (EF) blinded to clinical and sample data.

### **MMP-2 activity assay**

Cerebrospinal fluid and serum concentrations of active MMP-2 were measured by a commercially available Activity Assay System kit (Activity Assay System, Biotrak, Amersham Biosciences, Little Chalfont, UK, Cat. N. RPN 2631) according to the manufacturer's instructions. All reagents and standards were included in the kits. Briefly, undiluted CSF or serum diluted 1:100 were analysed in duplicate into 96-microwell microtiter plates pre-coated with anti-MMP-2 antibody. Six serial dilutions of standard were dispensed on each plate in duplicate with a range of 0.19–6 ng/mL. After an overnight incubation at 2–8°C and four washing cycles, only the enzyme had bound to the wells. The standard, a human pro-MMP-2, was activated by adding 50 µL/well of p-aminophenylmercuric acetate (APMA). In parallel, to detect endogenous levels of active MMP-2, 50 µL of dilution buffer was dispensed into each well instead of APMA. At the same time, 50 µL of the detection reagent was pipetted into each well. The amount of active MMP-2 was determined by interpolation from the standard curve. Cerebrospinal fluid and serum samples with concentrations outside the range of determination were re-assayed at greater dilutions to obtain values falling into the standard curve. The lower limit of quantification of the assay was 0.19 ng/mL, the range of intra-assay coefficient of variations (CV) was 4.4–7.0%, whereas the range of inter-assay CV was 16.9–18.5%.

### **TIMP-2 ELISA assay**

Cerebrospinal fluid and serum levels of TIMP-2 were measured by using a commercially available “sandwich” ELISA kit (Biotrak, Amersham Bio- sciences, Little Chalfont, UK, Cat. N. RPN 2618) according to the manufacturer’s instructions. Both CSF and corresponding serum samples were diluted at 1:4. The limit of sensitivity of the assay was 3 ng/mL.

### **Determination of total and active MMP-2 and TIMP-2 Indices**

According to the IgG index (Tibbling G et al., 1977), intrathecal production of active MMP-2 and TIMP-2 was calculated using the following formula:

$$\text{MMP-2 Index} = \text{CSF/serum MMP-2} : \text{CSF/serum albumin}$$

$$\text{TIMP-2 Index} = \text{CSF/serum TIMP-2} : \text{CSF/serum albumin}$$

where the ratio between CSF and serum albumin concentrations represents the status of B-CSF-B. Intrathecal synthesis was assumed for values greater than 3 SD above the mean of NIND.

### **Statistical analysis**

The normality of each variable was checked by using the Kolmogorov–Smirnov test. When normality of data distribution was found in all variables, statistical analysis was performed by a parametric approach. Conversely, when normality of data distribution was rejected in several variables, a non-parametric analysis was applied. Accordingly, serum mean concentrations of active MMP-2 and CSF mean levels of TIMP-2 were compared by using (a) ANOVA, followed by Scheffè test, among RRMS, OIND, and NIND and (b) t-test between RRMS patients grouped

according to clinical and MRI activities. In contrast, CSF mean levels of active MMP-2, serum mean concentrations of TIMP- 2, and CSF and serum mean values of active MMP-2/TIMP-2 ratio were compared by means of (a) Kruskal–Wallis test, followed by Mann–Whitney U-test with Bonferroni correction for multiple comparisons, among RRMS, OIND, and NIND and (b) Mann–Whitney U-test between RRMS patients classified according to clinical and MRI activities. Chi-square test was used to compare the patient group percentages of intrathecal synthesis of MMP-2 and TIMP-2, as indicated by the presence of specific Index abnormal values. In cases of multiple comparisons, a Bonferroni post hoc was applied. As normality of data distribution was not detected in several MS variables, possible relationships among them were assessed by using the Spearman’s rank correlation coefficient test. A value of  $p < 0.05$  was considered statistically significant.

## **Active gelatinases and TIMPs in SICH**

### **Patient selection**

Twenty-eight patients (18 males and 10 females; mean age  $\pm$  SD = 71.4  $\pm$  10.8 years) with diagnosis of acute supratentorial SICH proven by admission non-enhanced computed tomography (CT) scan carried out within 24 hours of onset were prospectively included in the study. Time of onset was considered as the last time the patient was known to be neurologically normal. Exclusion criteria at the time of enrollment were: 1) occurrence of infratentorial hemorrhage; 2) hematoma related to tumor, trauma, coagulopathy, aneurysms, vascular malformations; 3) hemorrhagic transformation of brain infarction; 4) intraventricular extension of hemorrhage; 5) surgical hematoma evacuation performed after the first non-contrast CT scans; 6) age < 20 years; 7) evidence of pregnancy. Disease severity was scored in all patients using the National Institutes of Health Stroke Scale (NIHSS) [3] at 24 hours (admission), 48 hours and 7 days (follow-up) from onset.

### **CT examination**

All CT scans were performed by using a single-section CT scanner (CT HiSpeed ZX/i; GE Medical System, Milwaukee, Wis) at the same time points as NIHSS calculation: 24 hours (baseline), 48 hours and 7 days (follow-up) after bleeding. Based on their location, hematomas were classified as basal ganglia and lobar. Accordingly, SICH was located within basal ganglia regions in 18 patients and within lobar regions in the remaining 10 patients. Hematoma volume was calculated at non-enhanced initial CT scan using the formula  $A \times B \times C / 2$  where A, B and C represent the dimensions of CT hyperdensity in 3 axes perpendicular to

each other (Kothari RU et al., 1996). The volume of hematoma plus that of perihematoma low density area was determined with the same method. Perihematoma edema volume was measured by subtracting the hematoma volume from the combined hematoma and perihematoma low density area volumes. Hematoma expansion was defined as a volume increase > 33% between the baseline CT scan obtained at 24 hours and the follow-up CT scan performed at 48 hours (Brott T et al., 1997). Informed consent was obtained from each patient or from his close relatives before the CT was performed.

### **Serum sampling**

Serum samples were obtained from centrifugation at room temperature of blood specimens previously withdrawn by puncture of an antecubital vein collected under sterile conditions at the same time points as NIHSS calculation and CT scan performance: 24 hours (baseline), 48 hours and 7 days (follow-up) after SICH. Serum specimens were then stored in aliquots at  $-70^{\circ}\text{C}$  until assay.

### **MMPs activity assays and TIMPs assays**

As reported previously (Fainardi E et al., 2006), serum concentrations of active MMP-9 and MMP-2 were measured by using commercially available Activity Assay System kits (Activity Assay System, Biotrak, Amersham Biosciences, UK, MMP-9 Cat. N. RPN 2634; MMP-2 Cat. N. RPN 2631) according to the manufacturer's instructions, with a sample dilution of 1:20 for MMP-9 and 1:100 for MMP-2 determinations. The lower limit of quantification was 0.125 ng/ml for MMP-9 and 0.19 ng/ml for MMP-2.

Serum levels of TIMP-1 and TIMP-2 were measured by using commercially available “sandwich” enzyme-linked immunosorbent assay (ELISA) kits (Biotrak, Amersham Biosciences, UK; TIMP-1 Cat. N. RPN 2611; TIMP-2 Cat. N. RPN 2618) according to the manufacturer’s instructions. Serum samples were diluted at 1:40 for TIMP-1 and at 1:4 for TIMP-2 determinations. The limit of sensitivity of the assay was 3 ng/ml for both TIMP-1 and TIMP-2.

### **Statistical analysis.**

After checking data for normality by using the Kolmogorov-Smirnov test, repeated measures ANOVA followed by post-hoc Scheffè test, Friedman ANOVA followed by post-hoc Mann-Whitney test, Spearman rank correlation coefficient test and linear regression analysis were used when appropriate. A value of  $p < 0.05$  was considered as statistically significant.

## **Detection of 66 kDa active MMP-9 in Serum**

### **Blood specimen collection and storage**

Serum samples from 28 healthy volunteers (12 female and 16 male; mean age  $\pm$  SD = 32.4  $\pm$  8.2 years) were collected in Vacutainer<sup>TM</sup> tubes and centrifuged within 30-60 minutes after venipuncture at 3000 rpm for 12 minutes at room temperature. After removal of the supernatants, serum samples were aliquoted and stored at  $-80^{\circ}\text{C}$  until assay. Each sample was analysed in duplicate in the same session. Serum specimens were chosen since active MMP-9 concentrations are not altered by clotting phenomena that take place in collector tubes (Fainardi E et al., 2007; Castellazzi M et al., 2007). Informed consent was given by all volunteers before inclusion and the study design was approved by the Local Committee for Medical Ethics in Research.

### **Enzymes, antibodies and reagents**

In all experiments, recombinant human MMP-2 and MMP-9 proenzymes used as standards as well as APMA and buffers, were included in commercially available Activity Assay System kits (Activity Assay System, Biotrak, Amersham Biosciences, UK, Cat. N. RPN 2631 and Cat. N. RPN 2634). In preliminary experiments, MMP-9 and MMP-2 proenzymes were tested for the presence of MMP-2 and MMP-9, respectively, with MMP-2 and MMP-9 activity assays. No contamination was found in either experiments. TIMP-1 was purchased from Sigma-Aldrich (Sigma-Aldrich, Milan, Italy, Cat. N. T8947). Concanavalin A (ConA) Sepharose and Protein G Sepharose were purchased from Sigma-Aldrich (Sigma-Aldrich, Milan, Italy, Cat. N. C9017 and Cat. N. P3296 respectively). Tris Base was purchased from Merck (VWR International,



Milan, Italy,); Sodium Chloride, Calcium Chloride, Zinc Chloride, BRIJ™ 35 and Coomassie Blue-R were purchased from Sigma-Aldrich (Sigma-Aldrich, Milan, Italy). The Assay Buffer used for immunoprecipitation and activation of MMP-9 was composed as follows: 50 mM Tris-HCl buffer pH 7.6 containing 1.5 mM Sodium Chloride, 0.5 mM Calcium Chloride, 1  $\mu$ M Zinc Chloride and 0.01% (v/v) BRIJ™ 35.

### **Gelatin Zymography**

Zymographic patterns of gelatinases were evaluated by using the method described by Heussen and Dowdle (Heussen and Dowdle, 1980) on gelatin-copolymerized gel. Briefly, samples were applied without reduction and boiling to a 10% polyacrylamide gel containing 0,1% gelatin. Gel electrophoresis was performed with these settings: 30 mA, 35W and 450 V/h for about 45 minutes. Then electrophoresis gels were incubated with 2.5% (v/v) Triton for 20 minutes three times and then in 50 mM Tris, pH 7.6, containing 150 mM NaCl, 5 mM CaCl<sub>2</sub> and 0.02% (v/v) Brij 35 for 22 hours. The gels were then stained with 0.3% Coomassie Blue-R.

### **Densitometric analysis of the zymographic patterns**

Gel was scanned using a Canon scanner (CanoScan D660U) interfaced with a Windows PC and acquired with Adobe Photoshop (Adobe System Inc., Mountain View, CA, USA) in grey scale mode at 600 dpi. The corresponding images were saved to disk in TIFF format and then analysed using Quantity One® software (Bio-Rad Laboratories). The bands were manually selected using the volume tool within the program

and their densitometric values were adjusted for the background, giving an integrated density reported in volume units of pixel intensity X mm<sup>2</sup>.

### **MMP-9 activity assay**

Active forms of MMP-9 were measured using a commercially available activity assay system (Activity Assay System, Biotrak, Amersham Biosciences, UK, Cat. N. RPN 2634) following the manufacturer's instructions. Briefly, each sample was analysed in duplicate into 96 microwell microtiter plates precoated with anti-MMP-9 antibodies. Six serial dilutions of standard were dispensed on each plate in duplicate in the range of 0.125 - 4 ng/ml. After an overnight incubation at 2–8 °C and four washing cycles, only the enzymes had bound to the wells. The standards, human pro-MMP-9, were activated by adding 50 µl/well of APMA. In this way, total amounts of MMP-9 were measured. Conversely, to detect only endogenous levels of active MMP-9, 50 µl/well of dilution buffer, instead of APMA, was dispensed into each well. Fifty µl of detection reagent, was pipetted into each well. The amount of active MMP-9 in all samples was determined by interpolation from the standard curve. As reported in the manufacturer's instructions, the lower limit of quantification was assumed at 0.125 ng/ml, the range of intra-assay CV was 3.4–4.3%, whereas the range of inter-assay CV was 20.2–21.7%. The MMP-9 activity assay was not able to distinguish between the two active MMP-9 forms, but only detected both MMP-9 activities.

### **MMP-9 inhibition by TIMP-1**

Inhibition of MMP-9 by TIMP-1 was measured by slight modification of the above mentioned activity assay. Briefly, after an overnight incubation of the samples in microtiter plates precoated with anti-MMP-9 antibodies, 100 µl/well of TIMP-1 (100 ng/ml) were added to all samples in order to inhibit the 82 kDa MMP-9 active form. After 90 minutes of incubation at 37°C and three cycles of washing the detection reagent was applied, and the residual MMP-9 activity was assigned to 66 kDa active MMP-9. The optimal concentration of TIMP-1 used for these determinations was tested in preliminary experiments which showed that higher concentrations of TIMP-1 did not cause a further decrease in MMP-9 activity (data not shown).

### **MMP-2 activity assay**

Active forms of MMP-2 were measured using a commercially available activity assay system (Activity Assay System, Biotrak, Amersham Biosciences, UK, Cat. N. RPN 2631) as previously reported. According to the manufacturer's instructions, the lower limit of quantification of the assay was 0.19 ng/ml, the range of intra-assay coefficient of variations (CV) was 4.4–7.0%, whereas the range of inter-assay CV was 16.9–18.5%.

### **Activation of pro-MMP-9**

A sample of recombinant human MMP-9 (8 ng/ml) was incubated with 1 mM APMA (diluted in Assay Buffer) at 37 °C up to six hours in order to activate the enzyme. Samples were withdrawn from the incubation mixture at baseline, two, four and six hours. Then, the samples were incubated overnight at 4 °C in microtiter plates precoated with anti-

MMP-9 antibodies to test by activity assay with and without TIMP-1, or kept at 4° C overnight and assayed by gelatin zymography.

### **Immunoprecipitation**

To identify the 66 kDa MMP-9 from a serum sample, a two-step immunoprecipitation procedure was conducted by using monoclonal antibody against MMP-9 (R&D Systems, Minneapolis, MN, USA, Cat. N. MAB936). As indicated by the manufacturer's instructions, this antibody is able to bind pro (92 kDa) and active (82 kDa) forms of recombinant human MMP-9, but it does not react with the C-terminal truncated form (66 kDa) of MMP-9 and does not show cross-reactivity with recombinant human MMP-2. Diluted serum sample (1:20 in assay buffer) was incubated overnight at 4°C on a tube rotator with 4 µg/ml of anti-MMP-9 antibody and 1 µl of 2.5% Triton. After this first step, Protein G Sepharose fast flow was added to the sample in a ratio of 1:3 and incubated for 3 hours at 4°C on a tube rotator. After centrifugation, supernatant was removed and immunoprecipitation was repeated with the same antibody. The sample was then treated with Protein G Sepharose fast flow, as previously indicated, and finally centrifuged to obtain supernatant. The untreated serum and supernatant obtained after the two-step immunoprecipitation were then tested for MMP-9 activities.

### **Gelatinases separation by Concanavalin-A chromatography**

Serum MMP-2 and MMP-9 were separated by using a concanavalin-A-Sepharose affinity chromatography following the method described earlier by Rantala-Ryhanen (Rantala-Ryhanen S et al., 1983) and adapted by us. Briefly, a microcolumn with 500 µl of concanavalin-A-Sepharose was equilibrated with PBS. After serum sample application the column

was washed with a 0.3 M NaCl buffer and then with a 0.3 M NaCl and 50 mM methyl  $\alpha$ -D-mannopyranoside buffer. Fractions of the flow through and wash were kept. Finally, the column was eluted with a buffer containing 0.5 M methyl  $\alpha$ -D-mannopyranoside. The fractions were analyzed with gelatin zymography, MMP-2 and MMP-9 activity assays and MMP-9 inhibition by TIMP-1.

## **MMP-9 released from neutrophils**

### **Separation of Neutrophils from leukocytes-enriched Buffy Coats**

Used solutions:

- ❑ Ficoll-Paque Plus (GE Healthcare);
- ❑ Dextran 6% (dissolved in PBS);
- ❑ Sodium Citrate (170 mM) / Citric Acid (76 mM) (anticoagulant);
- ❑ Lysis Buffer: 30 mM NaCl, 10 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.4;
- ❑ Restoring Buffer: 270 mM NaCl, 10 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.4.

Neutrophils were isolated from buffy coats of healthy donors obtained from the blood bank, using density gradient centrifugation with Ficoll-Paque PLUS (GE Healthcare) and sedimentation with dextran. The procedure was performed under a laminar flow hood to avoid any contamination.

Briefly, the buffy coat was layered on the Ficoll in a sterile tube (buffy coat : Ficoll ratio 2:1). Then, the tubes were centrifuged at 2200 rpm for 15 minutes at room temperature.

After centrifugation, the supernatant (containing Ficoll, the PBMCs and PBS) was discarded from each tube and the remaining pellets, containing erythrocytes and granulocytes, were collected in a sterile tube adding anticoagulant and 6% dextran. The formed layers were mixed gently by inversion then left to settle at room temperature one hour in a vertical position.

At the end of the incubation time, the upper layer containing the granulocytes was collected and centrifuged at 1100 rpm for 10 minutes. After centrifugation the supernatant was removed and the contaminating

RBCs were eliminated by hypo-osmotic lysis with Lysis Buffer, then, to restore the ionic strength Restoring Buffer was added. If necessary, the lysis procedure was repeated until neutrophils were no longer contaminated by RBCs.

Finally, the cells were resuspended in 1 ml of 155 mM NaCl not buffered and 10  $\mu$ l of the suspension were collected for the cell count.

#### **Stimulation of neutrophils with LPS**

$4 \times 10^6$  cells were resuspended in 1 ml of RPMI 1640 medium previously adjusted to the necessary pH (6.6, 7.0, 7.4, 7.8, 8.2) and subjected to stimulation with 10  $\mu$ g/ml of LPS for 30 minutes at 37 °C. At the end of stimulation, the samples were centrifuged 10 minutes at 1100 rpm and the conditioned media were collected and then stored at -80 °C until MMP-9 assay.

#### **Stimulation of neutrophils with IL-8**

$4 \times 10^6$  cells were resuspended in 1 ml of RPMI 1640 medium previously adjusted to the necessary pH (6.6, 7.0, 7.4, 7.8, 8.2) and subjected to stimulation with 20 ng/ml of IL-8 for 30 minutes at 37 °C. At the end of stimulation, the samples were centrifuged 10 minutes at 1100 rpm and the conditioned media were collected and then stored at -80 °C until MMP-9 assay.

#### **Total MMP-9 Assay (ELISA)**

The concentration of total MMP-9 in culture media was determined using a commercial kit from GE HealthCare (RPN 2614); all reagents useful for the determination (wells coated with specific antibodies, standard, buffers and detection reagent) were included in the kit.

Briefly, 100 µl/well of diluted medium were placed in a 96 well microtiter plate, together with a serial dilution (in duplicate) of MMP-9 standard in a range 1-16 ng/ml and using dilution buffer as blank. After 1 hour of incubation at room temperature and four wash cycles, the MMP-9 present in the sample was bound to the wells of the plate.

Afterwards, 100 µl of anti-MMP-9 HRP-conjugated antibody were dispensed in all wells and the plate was incubated for 2 hours at room temperature. At the end of incubation, four wash cycles were performed with wash buffer to remove the excess of secondary antibody. Then, 100 µl of detection reagent (ready to use TMB/H<sub>2</sub>O<sub>2</sub> mix) were dispensed in all wells and the plate was incubated 20 minutes at room temperature with slow shaking.

The reaction was stopped with 100 µl of 1M H<sub>2</sub>SO<sub>4</sub> and the absorbance read with a microplate reader at a wavelength of 450 nm. The amount of MMP-9 present in each sample was determined by interpolation with the standard curve.

### **Statistics**

Comparisons of pHs with the control group 7.4 were performed by One Way ANOVA using the Dunnett's post-hoc test. The comparisons between the acid and basic pHs were performed by One Way ANOVA, using the Bonferroni post-hoc test excluding the control group 7.4.



## **Neurofilament subunits and NAA in progressive multiple sclerosis**

### **Patients and controls**

We included PP (n = 28, according to Lublin and Reingold) (Lublin and Reingold, 1996) and transitional progressive (n = 10, according to Thompson et al.) (Thompson AJ et al., 1997) MS patients. Transitional progressive MS are patients with a progressive course without relapses, which are characterized by a single relapse before or after the onset of disease progression (Stevenson et al., 1999). Because of their similarities, PPMS and TPMS patients were considered as a unique patient group, defined as progressive hereafter. Of these, a subgroup of 21 patients participated in a phase II randomized, double-blind, placebo-controlled trial of interferon-beta (IFN $\beta$ )-1b (Montalban et al., 2009), where 10 patients (8 PPMS, 3 transitional progressive patients) were treated with subcutaneous IFN $\beta$ -1b and 11 patients (7 PPMS, 3 transitional progressive patients) received placebo. As for patients who were not part of the clinical trial, 16 patients were untreated and 1 patient was receiving treatment with azathioprine at the time of CSF evaluation. The local ethical committee approved the study and informed consent was obtained from all participants.

The control group was composed of patients with non-inflammatory neurological diseases (OND, n = 15): headache (n = 7), vertigo (n = 3), hydrocephalus secondary to tumor (n = 1), trigeminal neuropathy (n = 1), polyradiculopathy (n = 1), vestibular syndrome (n = 1), epilepsy (n = 1).

## MR methods

Patients included in the clinical trial underwent MR examinations at baseline and at months 12 and 24. Brain T2 lesion load (T2LL), brain T1 lesion load (T1LL), and brain parenchymal fraction (BPF) were calculated as described elsewhere (Montalban et al., 2009). The median time (interquartile range, IQR) between CSF collection and MR examinations was 2.5 months (0.1–3.1 months). No MR scans were available for the patients who were not part of the clinical trial (n=17).

Demographics and baseline clinical and radiological characteristics of MS patients and controls are summarized in **table 4**. No differences in age, gender distribution, EDSS scores or disease duration were observed between the different groups.

**Table 4. Demographic and baseline clinical characteristics of progressive MS patients and OND controls.**

Characteristics	Progressive MS (whole Group)	Placebo <sup>1</sup>	IFN $\beta$ <sup>1</sup>	OND
n	38	11	10	15
Age (years)	50.4 (46.1 – 54.6)	51.1 (48.8 – 59.6)	52.1 (48.1 – 56.7)	38.9 (31.5 – 51.0)
Females/Males (% females)	19/19 (50.0%)	6/5 (54.5%)	6/4 (60%)	7/8 (46.7%)
Disease duration (years)	10.0 (5.3 – 13.0)	11.0 (8.0 – 13.5)	12.0 (10.0 – 16.5)	-
EDSS	5.5 (4.0 – 6.0)	6.0 (5.3 – 6.5)	6.0 (4.5 – 6.0)	-
Progression/Not Progression (% progressed)	-	2/8 (20 %)	4/7 (36%)	-
T2LL (mm <sup>3</sup> ) <sup>2</sup>	14594.1 (10551.5 – 28252.6)	15100.5 (10291.1 – 26414.4)	13319.5 (10551.5 – 52539.8)	-
T1LL (mm <sup>3</sup> ) <sup>2</sup>	5184.2 (3347.4 – 14534.0)	3696.4 (3144.3 – 12791.6)	5269.0 (3773.7 – 17809.9)	-
BPF <sup>2</sup>	72.8 (70.4 – 74.6)	72.2 (71.2 – 73.5)	73.5 (69.2 – 75.9)	-

Data are expressed as median (interquartile range). BPF is expressed as percentage of total brain content.

<sup>1</sup>Includes only patients that participated in the clinical trial. EDSS: Expanded Disability Status Scale.

<sup>2</sup>It refers to MR scans performed at baseline of the clinical trial.

IFN $\beta$ : subcutaneous interferon beta-1b. T2LL: T2-weighted lesion load. T1LL: T1-weighted lesion load. BPF: Brain parenchymal fraction. OND: controls with non-inflammatory neurological diseases.

### **Preparation of cell-free CSF**

CSF samples were collected by lumbar puncture and centrifuged for 5 min at 1500 rpm to remove cells. The cell-free CSF supernatants were aliquoted and stored at  $-80^{\circ}\text{C}$ . In patients that participated in the clinical trial, the median time (interquartile range) between the study onset and CSF collection was 11.8 months (11.6–14.9 months), and was similar between placebo and IFN $\beta$  treated patients [12.0 months (11.7–14.7 months) vs. 11.8 months (11.6–15.0 months) respectively].

### **CSF NAA measurements**

NAA was measured using a modified stable isotope dilution gas chromatography-mass spectrometry method, as previously reported (Jakobs et al., 1991; Teunissen et al., 2009). The intra-assay and inter-assay CV were 2% (n = 8) and 2% (n = 6). All samples were analyzed within the linear range of the standard curve (0-0.5 nmol).

### **CSF Neurofilament measurements**

#### **Nf light ELISA**

Nf light was determined essentially as described before (Norgren et al., 2004), however the first antibody was replaced by the in-house produced anti-Nf light monoclonal antibody, clone 4F8. For Nf light the inter-assay CV was 27.5% and intra-assay CV was 9.5%.

#### **Nf heavy Luminex assay**

Nf heavy was measured in a novel home-made developed multiplex assay. Activated beads from Qiagen (USA) were covalently immobilized with an anti-Nf heavy monoclonal antibody (9C9 generously provided by Carsten Korth, Germany). After blocking the Durapore<sup>TM</sup> filter plates

(HTS screening plates, Millipore) with PBS/2%BSA/0.05% Tween-20; 50  $\mu$ L of standard (Nf heavy, Cat. 62010, Progen), controls, CSF samples, and blanks were incubated with a suspension of coupled beads (2500 beads/well) for 14-18 hours at 4°C on an orbital plate shaker (600 rpm). CSF samples were 5 times diluted in PBS/1% BSA/ 6 mM EDTA. After washing with PBS/1%BSA/0.05% Tween-20, the wells were incubated with 25  $\mu$ L of a 1:1000 diluted detector antibody (PAb- $\alpha$ -Nf heavy, Cat. N4142, Sigma, The Netherlands) for 1 hour at room temperature under continuously shaking (600 rpm). After washing, wells were incubated with 50  $\mu$ L of a 1:200 diluted phycoerythrin labelled donkey-anti-rabbit-polyclonal antibody (Cat. 711-116-152, Jackson ImmunoResearch) for 1 hour at room temperature under continuously shaking (600 rpm). The plate was then washed again and 100  $\mu$ L reading solution (Bio-plex Sheath fluid) was applied. The plate was read on a Bio-Plex™ 200 System (Bio-Rad) (50-100 beads/well). The mean fluorescence intensity data were analyzed using a 5-parameter logistic curve-fitting method and sample concentrations were calculated. All analyses were performed in duplicate and normalized to quality controls. Inter-assay CV was 18.4% and intra-assay CV was 5.4%.

### **Statistics**

Normality of distributions was tested with the Shapiro-Wilk test. Since normality could not be assumed for the majority of variables, CSF data for axonal damage are represented as medians and multiple comparison were performed using a Kruskal-Wallis test. Two-group comparisons were performed using a Mann-Whitney U test. Possible relationships among all the variables and clinical data were assessed using the Spearman's rank correlation coefficient test. Frequency distributions

were examined using the Fisher's exact test. To control for possible confounding factors, such as age or therapy usage, analysis of variance was performed on the ranks of the biomarker levels. For all the tests, a  $P < 0.05$  was considered significant.

## RESULTS

### Active MMP-2 and TIMP-2 in Multiple Sclerosis Patients

#### Cerebrospinal fluid and serum levels and intrathecal synthesis of active MMP-2 and TIMP-2 in MS patients and controls

Cerebrospinal fluid and serum concentrations of active MMP-2 and TIMP-2 were detectable in 100% of cases in RRMS, OIND, and NIND. As reported in the **table 5**, CSF and serum mean levels of active MMP-2 were statistically different among the groups of patients examined ( $p < 0.001$  and  $p < 0.02$ , respectively). Specifically, CSF and serum mean concentrations of active MMP-2 were significantly higher in RRMS ( $p < 0.001$  and  $p < 0.05$ , respectively) and in OIND ( $p < 0.01$  and  $p < 0.05$ , respectively) than in NIND. On the other hand, while CSF mean values of TIMP-2 were statistically different among the groups evaluated ( $p < 0.01$ ), serum mean levels of TIMP-2 did not statistically differ among RRMS, OIND, and NIND. In particular, CSF mean concentrations of TIMP-2 were significantly more increased in OIND ( $p < 0.01$ ) than in RRMS and in NIND, without statistical differences for these values between RRMS and NIND. In addition, CSF mean values of active MMP-2/TIMP-2 ratio were statistically different among the groups analysed ( $p < 0.01$ ), whereas only a trend toward a statistically difference ( $P = 0.0602$ ) was found for serum mean values of active MMP-2/TIMP-2 ratio among RRMS, OIND, and NIND. More precisely, CSF and serum mean values of active MMP-2/TIMP-2 ratio were significantly greater in RRMS than in NIND ( $p < 0.001$  and  $p < 0.05$ , respectively), without any

statistical difference between RRMS and OIND and between OIND and NIND. Finally, an intrathecal synthesis of active MMP-2, as reflected by specific index values above mean + 3 SD of NIND, was significantly more frequent in RRMS than in OIND ( $p < 0.01$ ) and NIND ( $p < 0.0001$ ) and in OIND than in NIND ( $p < 0.05$ ). In contrast, abnormal values of TIMP-2 Index, suggesting a local production within the CNS, were similar among RRMS, OIND, and NIND.

**Table 5. CSF and serum levels of active MMP-2, TIMP-2, MMP-2/TIMP-2 ratios and intrathecal synthesis in patients with MS, OIND and NIND controls.**

	RR MS (n = 67) median, IQR, mean $\pm$ SD	OIND (n = 64) median, IQR, mean $\pm$ SD	NIND (n = 65) median, IQR, mean $\pm$ SD	ANOVA or Kruskal-Wallis
CSF active MMP-2 levels (ng/ml)	2.1, 0.9 – 3.8, 2.9 $\pm$ 3.6 <sup>a</sup>	2, 0.9 – 2.9, 2.2 $\pm$ 1.5 <sup>b</sup>	0.8, 0.7 – 1.9, 1.7 $\pm$ 1.9	$p < 0.001$ (Kruskal-Wallis)
Serum active MMP-2 levels (ng/ml)	273, 182.4 – 387.3, 288.9 $\pm$ 147.5 <sup>c</sup>	227.8, 165.4 – 412.7, 291.7 $\pm$ 168.9 <sup>d</sup>	203.5, 126.2 – 287.2, 222.6 $\pm$ 122.5	$P < 0.02$ (ANOVA)
CSF TIMP-2 levels (ng/ml)	58.4, 45.7 – 80.9, 64.4 $\pm$ 23.9	80, 45.3 – 117.9, 83.5 $\pm$ 45.2 <sup>e,f</sup>	59.8, 39.3 – 79.9, 63.7 $\pm$ 32	$P < 0.01$ (ANOVA)
Serum TIMP-2 levels (ng/ml)	44.4, 20.1 – 55.1, 46.3 $\pm$ 40.2	53.3, 20.6 – 70.2, 50.4 $\pm$ 36.2	40, 21.5 – 69.7, 50.9 $\pm$ 35.6	$P = 0.2176$ (Kruskal-Wallis)
CSF active MMP-2/TIMP-2 Ratio ( $\times 10^{-2}$ )	2.8, 1.9 – 5.7, 6.2 $\pm$ 11.8 <sup>g</sup>	2.3, 1.4 – 4.1, 3.7 $\pm$ 5.6	1.8, 1 – 2.9, 2.6 $\pm$ 3.7	$P < 0.001$ (Kruskal-Wallis)
Serum active MMP-2/TIMP-2 Ratio	8.7, 3.5 – 12.5, 14 $\pm$ 22.9 <sup>h</sup>	6.2, 3.4 – 15.2, 12.1 $\pm$ 15	4.4, 2.1 – 10, 7.2 $\pm$ 7	$P = 0.0602$ (Kruskal-Wallis)
Active MMP-2 ITS (%)	26/67 (38.8%) <sup>l</sup>	8/64 (12.5%) <sup>m</sup>	1/65 (1.5%)	
TIMP-2 ITS (%)	5/67 (7.5%)	2/64 (3.1%)	1/65 (1.5%)	

*IQR* = interquartile range; *SD* = standard deviation; *RR* = relapsing remitting; *ITS* = intrathecal synthesis.

CSF active MMP-2 levels (Mann-Whitney): <sup>a</sup>RR MS vs. NIND ( $P < 0.001$ ); <sup>b</sup>OIND vs. NIND ( $p < 0.01$ ).

Serum active MMP-2 levels (Scheffè test): <sup>c</sup>RR MS vs. NIND ( $P < 0.05$ ); <sup>d</sup>OIND vs. NIND ( $p < 0.05$ ).

CSF TIMP-2 levels (Scheffè test): <sup>e</sup>OIND vs. RR MS ( $P < 0.01$ ); <sup>f</sup>OIND vs. NIND ( $p < 0.01$ ).

CSF active MMP-2/TIMP-2 Ratio (Mann-Whitney): <sup>g</sup>RR MS vs. NIND ( $p < 0.001$ ).

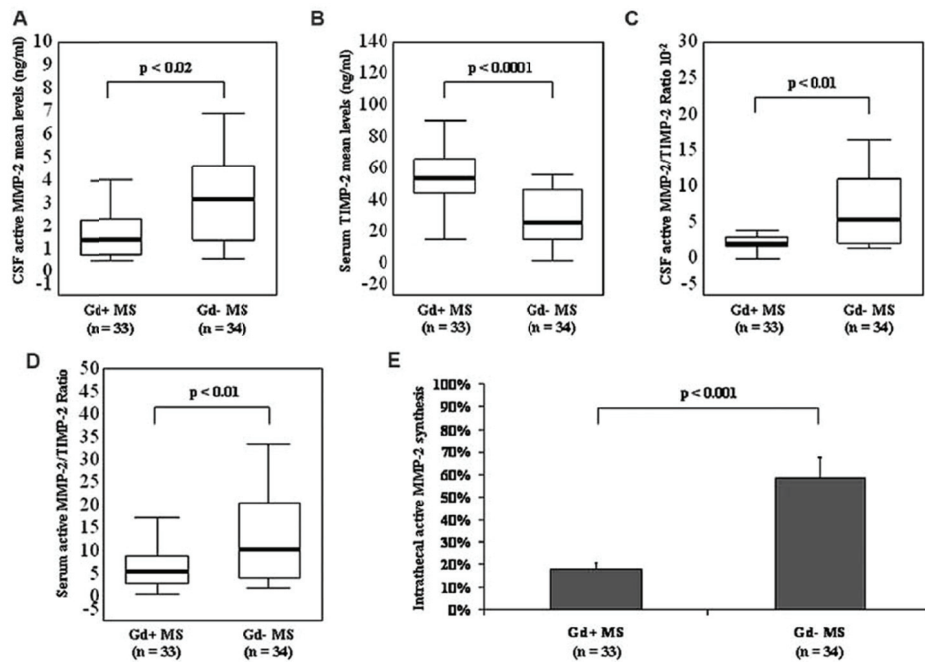
Serum active MMP-2/TIMP-2 Ratio (Mann-Whitney): <sup>h</sup>RR MS vs. NIND ( $p < 0.05$ ).

Active MMP-2 ITS (Chi-square): <sup>i</sup>RR MS vs. OIND ( $p < 0.01$ ); <sup>l</sup>RR MS vs. NIND ( $p < 0.0001$ ); <sup>m</sup>OIND vs. NIND ( $p < 0.05$ ).

### **Cerebrospinal fluid and serum levels and intrathecal synthesis of active MMP-2 and TIMP-2 in MS patients categorized according to clinical and MRI activity**

When MS patients were grouped according to clinical activity, there were no statistical differences between RRMS patients with and without clinical evidence of disease activity for CSF and serum mean concentrations of active MMP-2 and TIMP-2, for CSF and serum mean values of active MMP-2/TIMP-2 ratio, and for intrathecal production of active MMP-2 and TIMP-2 (data not shown). On the other hand, when MS patients were stratified according to MRI activity, CSF mean levels of active MMP-2 significantly increased more ( $p < 0.02$ ) in RRMS patients with MRI inactive ( $4.0 \pm 4.6$  ng/mL) than in those with MRI active disease ( $1.9 \pm 1.7$  ng/mL) (**figure 26, panel A**), whereas serum mean concentrations of TIMP-2 were significantly more pronounced ( $p < 0.0001$ ) in MRI active ( $63.7 \pm 49.2$  ng/mL) than in MRI stable ( $29.3 \pm 16.7$  ng/mL) RRMS patients (**figure 26, panel B**). Furthermore, CSF and serum mean values of active MMP-2/TIMP-2 ratio were significantly higher ( $p < 0.01$ ) in RRMS patients with MRI stable ( $8.2 \pm 14.4 \times 10^{-2}$  ng/mL and  $18 \pm 25.7$  ng/mL, respectively) than in those with MRI active disease ( $4.1 \pm 8.2 \times 10^{-2}$  ng/mL and  $10 \pm 19.2$  ng/mL, respectively) (**figure 26, panel C and D**). Next, an intrathecal synthesis of active MMP-2 was significantly more frequent ( $p < 0.001$ ) in RRMS patients without MRI appearance of disease activity (58.8%) than in those with disease activity as measured by MRI scans (18.2%) (**figure 26, panel E**). We did not observe other significant differences between RRMS patients with and without Gd- enhancing lesions for serum active MMP-2 mean levels, for CSF TIMP-2 mean concentrations, and for intrathecal synthesis of TIMP-2 (data not shown).





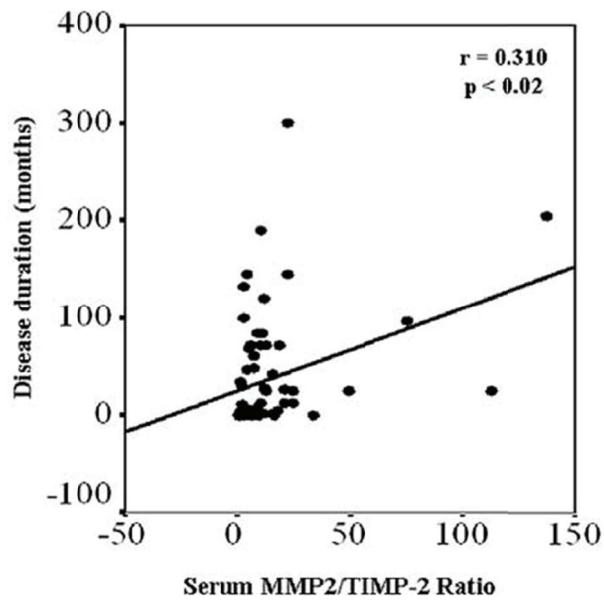
**Figure 26.** CSF active MMP-2 levels, serum TIMP-2 levels, and CSF and serum values of active MMP-2/TIMP-2 ratio, and percentages of intrathecal MMP-2 synthesis in MRI active (Gd+) and MRI inactive (Gd-) RRMS patients.

Panel A: CSF active MMP-2 levels; Panel B: Serum concentrations of TIMP-2; Panel C: CSF values of active MMP-2/TIMP-2 ratio; Panel D: serum values of active MMP-2/TIMP-2 ratio; Panel E: Intrathecal production of active MMP-2.

The boundaries of the box represent the 25th–75th quartile. The line within the box indicates the median. The whiskers above and below the box correspond to the highest and lowest values, excluding outliers.

### Correlation between CSF and serum levels and intrathecal synthesis of active MMP-2 and TIMP-2 and MS clinical features

In RRMS patients, a trend toward a positive correlation ( $p < 0.02$ ) was observed between serum mean values of active MMP-2/TIMP-2 ratio and the duration of the disease (**figure 27**). No further definite relationships were detected between disease duration and severity of the disease, as expressed by EDSS and CSF and serum parameters assessed in this study (data not shown).

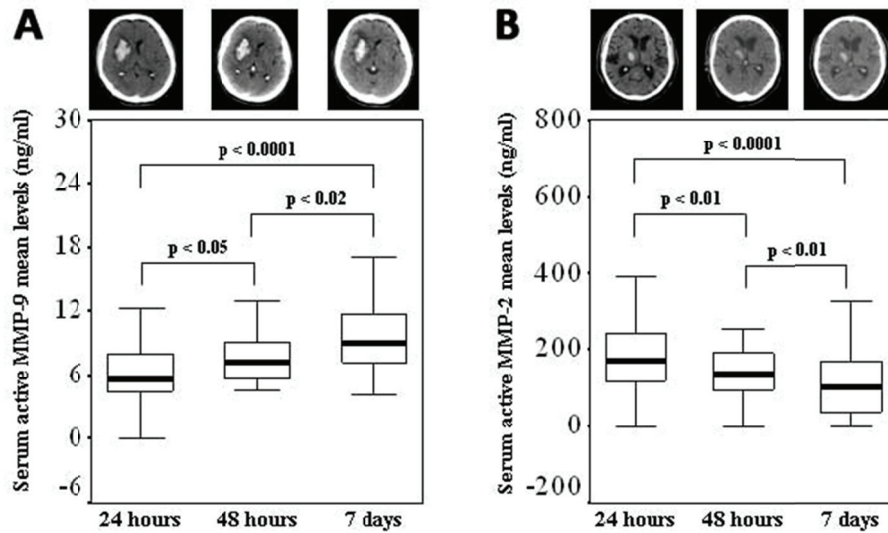


*Figure 27. Correlation between serum mean values of MMP-2/TIMP-2 ratio and disease duration. r and p Spearman correlation coefficients are indicated.*

## Active gelatinases and TIMPs in SICH

### MMPs and TIMPs time course

As illustrated in **figure 28**, serum mean levels of active MMP-9 and MMP-2 were statistically different over time ( $p < 0.0001$ ). Specifically, in comparison to baseline mean values observed at 24 hours ( $6.1 \pm 3.7$  ng/ml), serum active MMP-9 mean concentrations (**panel A**) were significantly higher at 48 hours ( $7.5 \pm 4.0$  ng/ml;  $p < 0.05$ ) and at 7 days ( $9.5 \pm 4.1$  ng/ml;  $p < 0.0001$ ). In addition, serum active MMP-9 mean levels were significantly more elevated at 7 days than at 48 hours ( $p < 0.02$ ). On the other hand, serum mean titers of active MMP-2 (**panel B**) were significantly lower at 48 hours ( $154.5 \pm 110.9$  ng/ml;  $p < 0.01$ ) and at 7 days ( $117.8 \pm 93.7$  ng/ml;  $p < 0.0001$ ) than at baseline ( $194.1 \pm 122.7$  ng/ml). Moreover, serum active MMP-2 mean levels were significantly decreased at 7 days as compared to 48 hours ( $p < 0.01$ ). No statistical differences were found among the time points examined for serum mean levels of TIMP-1 and TIMP-2 (data not shown).

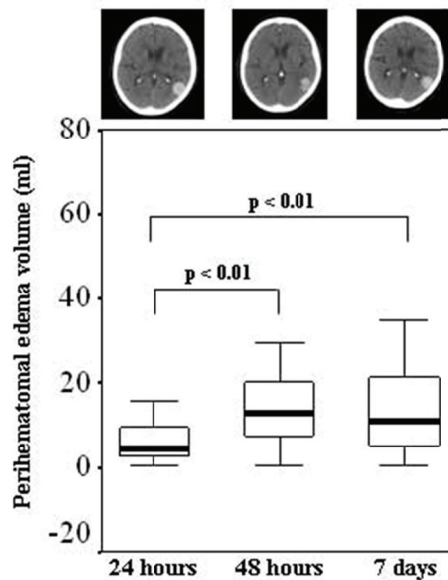


**Figure 28. Temporal profile of serum active MMP-9 and MMP-2 levels in SICH patients.**

Panel A: Serum mean concentrations of active MMP-9; Panel B: Serum mean levels of active MMP-2. Values are represented as median and interquartile range (IQR). The boundaries of the box represent the 25th-75th quartile. The line within the box indicates the median. The whiskers above and below the box correspond to the highest and lowest values, excluding outliers. On the top, sequential CT scans coming from three illustrative cases which show the time course of hematoma and surrounding perihematoma edema in basal ganglia.

### Hematoma and edema volumes time course

Hematoma volume did not statistically differ among the time points selected since similar mean values were detected at baseline ( $15.8 \pm 21.4$  ml), at 48 hours ( $18.4 \pm 26.2$  ml) and at 7 days ( $6.4 \pm 21.6$  ml). In this setting, a hematoma enlargement was present in 5/28 (17.9 %) SICH patients. In contrast, as shown in **figure 29**, perihematoma edema volume was statistically different over time ( $p < 0.0001$ ). In particular, its mean values were significantly increased at 48 hours ( $18.4 \pm 26.2$  ml;  $p < 0.01$ ) and at 7 days ( $18.77 \pm 27.1$  ml;  $p < 0.01$ ) compared to those found at 24 hours ( $8.9 \pm 13.3$  ml).



**Figure 29. Perihematomal edema volume in SICH patients.** Values are represented as median and interquartile range (IQR). The boundaries of the box represent the 25th-75th quartile. The line within the box indicates the median. The whiskers above and below the box correspond to the highest and lowest values, excluding outliers. On the top, sequential CT scans coming from three illustrative cases which show the time course of lobar SICH.

### **Relationships between MMPs and TIMPs and CT and clinical findings**

As reported in **table 6**, hematoma volume was positively associated to serum mean concentrations of active MMP-9 and MMP-2 at 24 hours ( $p < 0.05$ ) and to serum mean levels of active MMP-9 at 48 hours ( $p < 0.02$ ), whereas it was negatively correlated to serum active MMP-2 mean levels at 7 days ( $p < 0.05$ ). Likewise, perihematomal edema volume was positively correlated with serum active MMP-9 and MMP-2 mean titers at 24 hours ( $p < 0.02$  and  $p < 0.05$ , respectively) and with serum mean levels of active MMP-9 at 48 hours ( $p < 0.05$ ), but it was inversely correlated with serum active MMP-2 mean concentrations at 7 days ( $p < 0.02$ ). No further definite associations were identified between

temporal profile of serum MMP-9, MMP-2, TIMP-1 and TIMP-2 mean levels and hematoma and perihematoma edema volumes (data not shown). Serum MMPs and TIMPs measurements were not significantly correlated to NIHSS in the different time points analysed.

**Table 6. Significant correlations between serum mean levels of MMP-9 and MMP-2 and hematoma and perihematoma edema volumes in 28 patients with spontaneous intracerebral hemorrhage (SICH).**

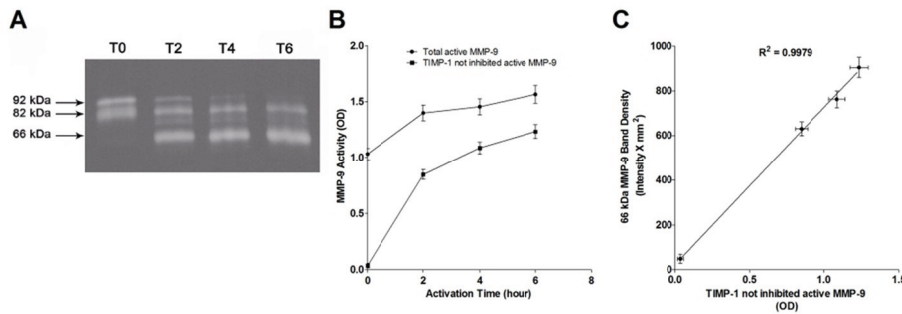
<b>Spearman's correlation</b>	<b>Correlation coefficient</b>	<b>Significance</b>
MMP-9 vs. hematoma volume at 24 hours from onset	r = 0.394	p < 0.05
MMP-9 vs. hematoma volume at 48 hours from onset	r = 0.447	p < 0.02
MMP-2 vs. hematoma volume at 7 days from onset	r = - 0.405	p < 0.05
MMP-9 vs. edema volume at 24 hours from onset	r = 0.460	p < 0.02
MMP-9 vs. edema volume at 48 hours from onset	r = 0.422	p < 0.05
MMP-2 vs. edema volume at 24 hours from onset	r = 0.385	p < 0.05
MMP-2 vs. edema volume at 7 days from onset	r = - 0.471	p < 0.02

## Detection of 66 kDa active MMP-9 in Serum

### Inhibition of MMP-9 by TIMP-1

To verify whether inhibition by TIMP-1 is useful to distinguish between the 82 kDa and 66 kDa active forms of MMP-9, we performed a time-dependent activation of MMP-9. As shown in **figure 30 (panel A)** an increasing incubation time with APMA corresponded to a decreasing intensity of the gelatinolytic bands at high molecular weights (pro-MMP-9 and 82 kDa MMP-9, respectively) and an associated increasing intensity of the lower molecular weight band (66 kDa MMP-9). It must be noted that there was a band between 82 kDa and 66 kDa forms, most likely representing an intermediate form in the process of activation (Okada Y et al., 1992). These findings confirmed that in the presence of APMA, pro-MMP-9 was converted into the 82 kDa form and then into the 66 kDa form.

The same samples were assayed also for the MMP-9 activities. As indicated in **figure 30 (panel B)**, the total MMP-9 activity (circles) increased with the incubation time, due to the progressive conversion of the inactive 92 kDa form to the active 82 kDa and 66 kDa forms. When TIMP-1 was added to the assay mixture the uninhibited activity (**figure 30, panel B**, filled squares), which was very low at time zero, showed a remarkable time-dependent increase indicating that under the assay conditions only a part of the active enzyme was inhibited. Furthermore, the uninhibited activity increased in parallel with the 66 kDa band intensity, showing a strong positive correlation with the zymographic data (**figure 30, panel C**) ( $p < 0.01$ ; Pearson correlation  $r = 0.9987$ ). This suggested that the assay performed in presence of TIMP-1 allowed a quantitative evaluation of the 66 kDa form.



**Figure 30. Time course activation of proMMP-9.**

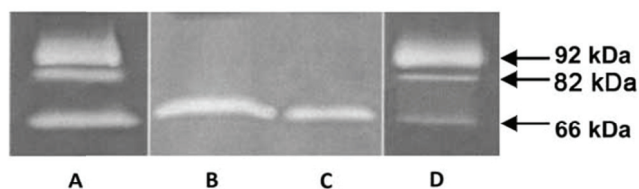
Panel A: gelatin zymography time course activation of proMMP-9 with APMA.; Panel B: total active MMP-9 (circles) and TIMP-1 not inhibited active MMP-9 (squares) values were measured at the same time points with the different activity assays. Experiments were repeated three times; values are indicated as Mean  $\pm$  standard deviation; Panel C: Correlation between temporal changes of TIMP-1 not inhibited active MMP-9 and 66 kDa MMP-9 band density expressed as mean value  $\pm$  standard of optical density and intensity  $\times$  mm<sup>2</sup>, respectively.

### Identification of 66 kDa MMP-9 form in serum

A fresh serum sample was subjected to an affinity chromatography on ConA-Sepharose in order to verify whether the 66 kDa MMP-9 form was present in biological fluids or whether it was an “in vitro” artefact. The obtained chromatographic fractions were analyzed by gelatin zymography, MMP-2 activity assay and MMP-9 activity assays. Gelatin zymography (**figure 31**) showed the presence of a single 66 kDa band in the flow through (Lane B) and wash (Lane C) samples corresponding to the unbound MMP-2. This was supported by the MMP-2 activity assay data, which showed the presence of the enzyme only in the flow through and wash fractions (**figure 31**, table). In the eluate, three bands were present (Lane D) which migrated at 92 kDa, 82 kDa and 66 kDa in accordance with the three forms of MMP-9. In fact, MMP-2 activity assay did not show the presence of the enzyme in the eluate, ruling out the possibility that the 66 kDa band in the eluate was residual MMP-2.



Additionally, when MMP-9 activity was assayed in the eluate, the addition of TIMP-1 only partially inhibited its activity (**figure 31**, table). This indicated that both MMP-9 active forms, 82 kDa and 66 kDa, were present in the serum sample. To further demonstrate the presence of the 66 kDa MMP-9 form in biological fluids, we performed a two-step immunoprecipitation of a serum sample with an anti-MMP-9 antibody which recognizes only the 92 kDa and 82 kDa forms of the enzyme; thus, only the 66 kDa form was expected to be present in the supernatant. However, gelatin zymography (not shown) demonstrated that even a second treatment with the antibody did not remove all 92 kDa and 82 kDa forms of MMP-9.

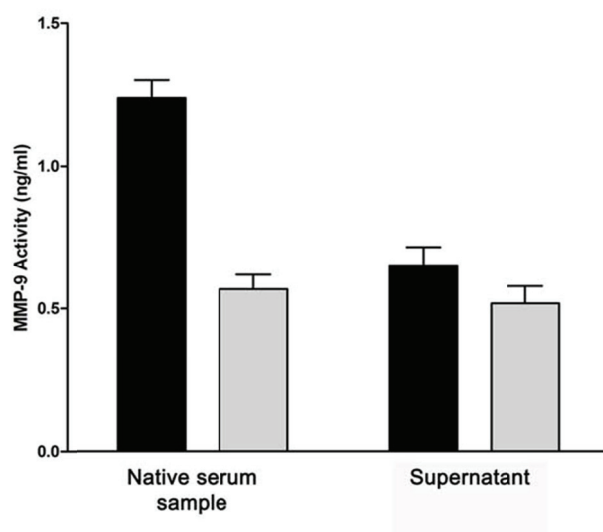


	Total active MMP-9 (OD)	TIMP-1 not inhibited active MP-9 (OD)	Active MMP-2 (OD)
Flow through (B)	0	0	1.324
Wash (C)	0	0	1.019
Elution (D)	0.439	0.288	0

**Figure 31. MMP-9 and MMP-2 activity before and after concanavalin-A-sepharose chromatography.**

Gelatin zymography profiles of native serum (A), flow through (B), wash (C) and elution (D) obtained after concanavalin-A-sepharose chromatography. Table reported below describes absorbance values in flow through, wash and elution samples of MMP-2 and MMP-9 activity assay in presence or absence of TIMP-1.

Nevertheless, as shown in **figure 32** when TIMP-1 was added to the MMP-9 assay of a native serum sample, an inhibition in MMP-9 activity of about 54% was observed. Conversely, only a slight inhibition was found in the supernatant obtained with the two-step immunoprecipitation after the addition of TIMP-1. These data indicate that an active form of MMP-9, not inhibited by TIMP-1 and not recognized by antibodies specific for the 92 kDa and 82 kDa forms of the protease, occurs in serum. On the basis of the results obtained also by ConA chromatography, this MMP-9 should be the 66 kDa form.

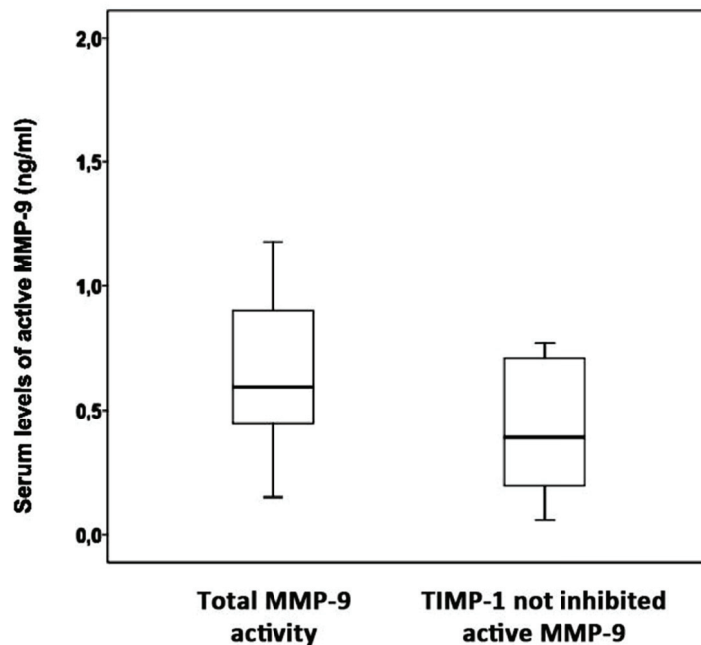


**Figure 32.** *MMP-9 activity before and after two-step immunoprecipitation. Total MMP-9 activity (black) and TIMP-1 not inhibited MMP-9 activity (grey) in native serum sample and supernatant obtained after two-step immunoprecipitation.*

### **Quantification of 66 kDa MMP-9 form in serum of healthy donors**

In order to measure the levels of 66 kDa form in biological samples, we assayed the MMP-9 activity and TIMP-1 inhibition on the sera of 28 healthy donors. **Figure 33** depicts serum levels of total active (82 kDa

and 66 kDa) and 66 kDa active MMP-9. Serum mean levels of total active MMP-9 were  $1.5 \pm 2.2$  ng/mL, whereas serum mean concentrations of only 66 kDa MMP-9 were  $1.1 \pm 1.7$  ng/mL. In addition, comparison between the results obtained by MMP-9 activity assay and MMP-9 inhibition by TIMP-1 in each serum sample showed that 66 kDa MMP-9 values, expressed as a percentage of corresponding total active MMP-9 levels, fluctuated from 10.6% to 92.8 %. Therefore, serum levels of 66 kDa and total active MMP-9 demonstrated a high variability.



**Figure 33. Total active MMP-9 and TIMP-1 not inhibited active MMP-9 in sera of healthy donors.**

Median and interquartile range (IQR) of total active and TIMP-1 not inhibited active MMP-9 serum levels in 28 healthy donors as measured with MMP-9 activity assay in absence or presence of TIMP-1, respectively. The boundaries of the box represent the 25th-75th quartile. The line within the box indicates the median. The whiskers above and below the box correspond to the highest and lowest values, excluding outliers.

## MMP-9 released from neutrophils

### Levels of MMP-9 released by not stimulated neutrophils

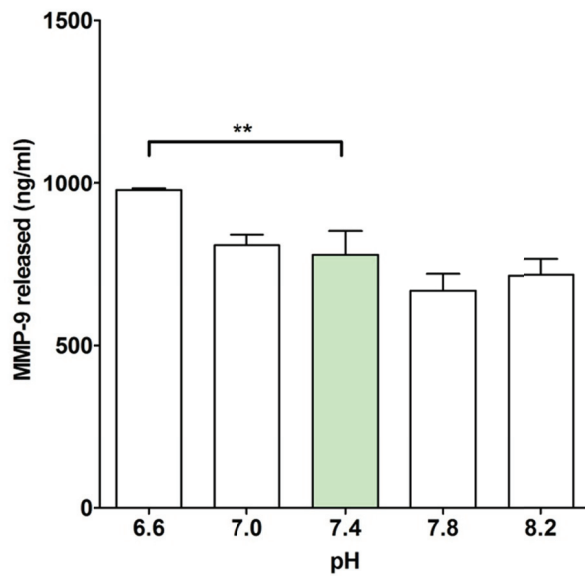
**Table 7** shows the mean MMP-9 levels in absence of stimuli, at different pHs.

Comparing the levels of MMP-9 released at different pH with the control group 7.4 (physiological pH) we found significant higher levels of MMP-9 at more acidic pH (6.6) (**figure 34**; One way ANOVA, Dunnett's post-hoc test vs. 7.4;  $p < 0.01$ ). Moreover, the levels of MMP-9 were higher than all the other pHs (**table 10**), whereas comparable levels were found between pH 7.0, 7.8 and 8.2.

**Table 7. Levels of total MMP-9 (ng/ml) released at different pH by not stimulated neutrophils.**

Values are represented as mean  $\pm$  SD of 5 experiments. To compare the levels of MMP-9 a one way ANOVA followed by a Dunnett's post-hoc test using pH 7.4 as control was performed. ns = not significant ( $p > 0.05$ ).

pH	MMP-9 released (ng/ml) mean $\pm$ SD	Significance (pH vs. 7.4)
6.6	978,00 $\pm$ 5,51	$p < 0.01$
7.0	810,48 $\pm$ 32,63	ns
<b>7.4</b>	<b>780,89 <math>\pm</math> 73,08</b>	-
7.8	668,73 $\pm$ 54,35	ns
8.2	717,33 $\pm$ 48,94	ns



**Figure 34. Mean levels of MMP-9 released by not stimulated neutrophils in medium at different pH.**

The bars above the histograms represent standard deviations. Significant comparisons between pHs and pH 7.4 are marked with star (\*); \*\* =  $p < 0.01$

### **Levels of MMP-9 released by neutrophils stimulated with LPS**

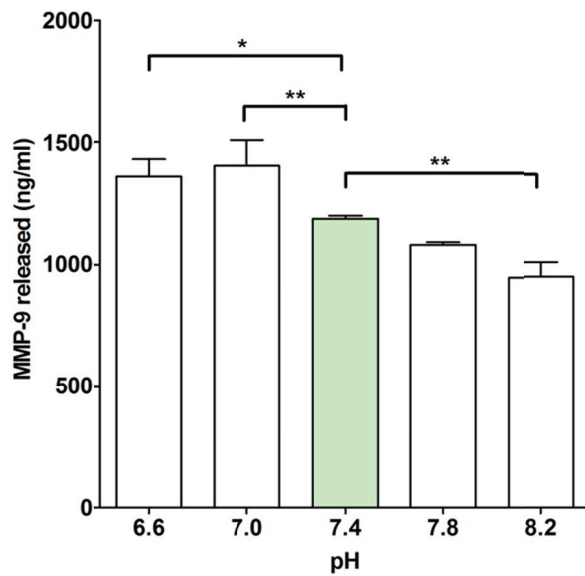
We investigated the release of MMP-9 at different pHs after stimulation of neutrophils with lipopolysaccharide (LPS), in order to mimic an infective pro-inflammatory stimulus. The different levels of total MMP-9 measured are reported in **table 8** and represented as mean  $\pm$  SD of 5 experiments.

**Table 8. Levels of total MMP-9 (ng/ml) released by neutrophils stimulated with LPS.** Values are represented as mean  $\pm$  SD of 5 experiments. To compare the levels of MMP-9 a one way ANOVA followed by a Dunnett's post-hoc test using pH 7.4 as control was performed. ns = not significant ( $p > 0.05$ ).

pH	MMP-9 released (ng/ml)	Significance (pH vs. 7.4)
	mean $\pm$ SD	
6.6	1358.67 $\pm$ 70.43	p<0.05
7.0	1403.09 $\pm$ 103.73	p<0.01
<b>7.4</b>	<b>1187.33 <math>\pm</math> 13.26</b>	-
7.8	1081.40 $\pm$ 10.46	ns
8.2	947.27 $\pm$ 60.83	p<0.01

**Figure 35** shows the course of MMP-9 levels measured at different pHs. We observed a pH-dependent release of MMP-9 when neutrophils were stimulated with LPS: there was an increase at low pH values (6.6 and 7.0) and a decrease at higher pH (8.2), respect the 7.4 (**table 8**). However, the amount of MMP-9 released at pH 7.8 and 7.4 did not differ.

Moreover, significant higher levels of MMP-9 released at acid pHs than the basic pHs were found (**table 10**), whereas no differences were observed between the two acid pH (6.6 vs.7.0), or the two basic pH (7.8 vs. 8.2) (**table 10**).



**Figure 35. Mean levels of MMP-9 released by LPS stimulated neutrophils in medium at different pH.**

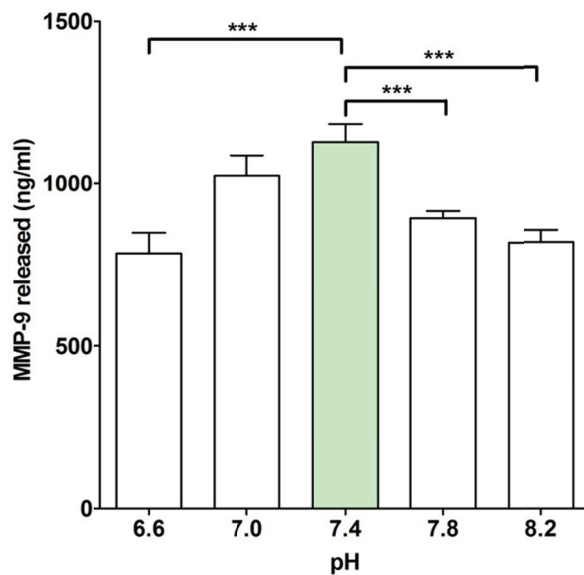
The bars above the histograms represent standard deviations. Significant comparisons between pHs and pH 7.4 are marked with star (\*) \* =  $p < 0.05$ ; \*\* =  $p < 0.01$

### **Levels of MMP-9 released by neutrophils stimulated with IL-8**

The release of MMP-9 at different pHs after stimulation of neutrophils with IL-8, in order to mimic an endogenous pro-inflammatory stimulus, was investigated. The measured levels, reported as mean of 5 experiments, of total MMP-9 are reported in **table 9**.

**Table 9. Levels of total MMP-9 (ng/ml) released by neutrophils stimulated with IL-8.** Values are represented as mean  $\pm$  SD of 5 experiments. To compare the levels of MMP-9 a one way ANOVA followed by a Dunnett's post-hoc test using pH 7.4 as control was performed. ns = not significant ( $p > 0.05$ ).

pH	MMP-9 released (ng/ml)	Significance (pH vs. 7.4)
	mean $\pm$ SD	
6.6	785,36 $\pm$ 63,95	p<0.001
7.0	1023,28 $\pm$ 62,67	ns
<b>7.4</b>	<b>1126,78 <math>\pm</math> 124,93</b>	-
7.8	894,02 $\pm$ 21,57	p<0.001
8.2	818,60 $\pm$ 37,41	p<0.001



**Figure 36. Mean levels of MMP-9 released by IL-8 stimulated neutrophils in medium at different pH.**

The bars above the histograms represent standard deviations. Significant comparisons between pHs and pH 7.4 are marked with star (\*); \*\*\* =  $p < 0.001$

**Figure 36** depicts the course of MMP-9 released in function of the pH. The maximum release of MMP-9 from human neutrophils was found at



physiological pH (7.4), with significant lower levels at more acid pH (6.6) and both basic pHs (7.8 and 8.2) (**table 9**).

**Table 10. One way ANOVA followed by Bonferroni post-hoc test.**  
*The comparison with the control group 7.4 were excluded. ns = not significant (p>0.05).*

<b>Comparison</b>	<b>Significance</b>		
	<b>not stimulated</b>	<b>LPS</b>	<b>IL-8</b>
<b>6.6 vs 7.0</b>	p<0.05	ns	p<0.01
<b>6.6 vs 7.8</b>	p<0.001	p<0.01	ns
<b>6.6 vs 8.2</b>	p<0.001	p<0.001	ns
<b>7.0 vs 7.8</b>	ns	p<0.001	ns
<b>7.0 vs 8.2</b>	ns	p<0.001	p<0.01
<b>7.8 vs 8.2</b>	ns	ns	ns

## Neurofilament subunits and NAA in progressive multiple sclerosis

### CSF NAA, Nf light and Nf heavy levels in patients with progressive MS and controls with OND

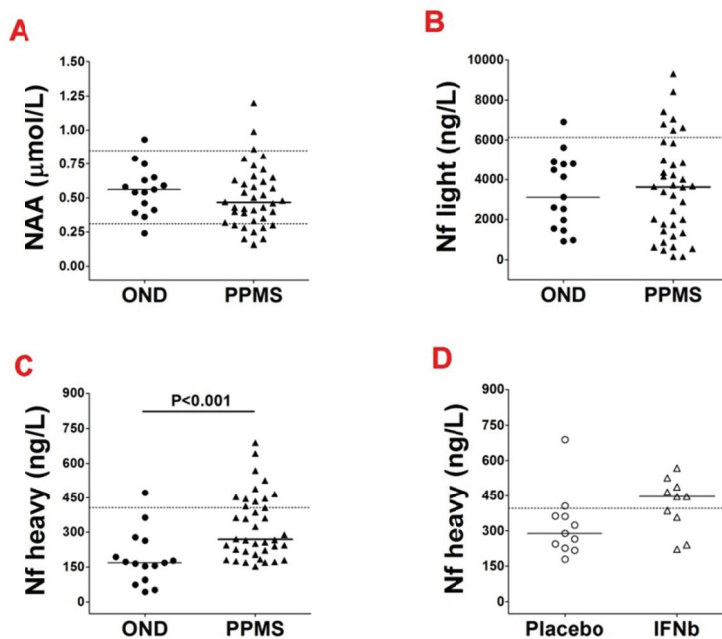
The levels of NAA, Nf light and Nf heavy were detectable in 100% of MS patients and OND (**table 11**). We first compared CSF levels of NAA, Nf light and Nf heavy between the whole MS group and controls with OND. As shown in **figure 37 (panel A and B)**, NAA and Nf light levels were comparable between the two groups. However, Nf heavy levels were two fold higher in MS patients than OND controls ( $P < 0.001$ ; **Figure 37, panel C**). Of note, NAA, Nf light and Nf heavy levels were comparable between transitional progressive ( $n = 10$ ) and primary progressive ( $n = 28$ ) MS patients ( $P = 0.260$ , data not shown).

Since part of the patients in the progressive MS group were receiving treatment with IFN $\beta$ -1b as part of the clinical trial, we evaluated whether IFN $\beta$ -1b could affect CSF Nf light, Nf heavy and NAA levels by stratifying patients into treated ( $n=10$ ) and untreated ( $n=11$ ) groups. While NAA and Nf light were very similar between the two groups ( $P < 0.05$ ; data not shown), we found only a trend towards a statistical significant difference in the Nf heavy levels (**figure 37, panel D**;  $P = 0.067$ ). Of note, CSF Nf heavy levels remained significantly higher in untreated MS patients compared to controls after correction for IFN $\beta$ -1b usage ( $P=0.004$ ; data not shown). No effect of age was observed (data not shown).

**Table 11. CSF levels of NAA, Nf light and Nf heavy in progressive MS patients and OND controls.**

Data are expressed as median (interquartile range).

	Progressive MS patients (n=38)	Controls with OND (n=15)	P value (Z)
NAA ( $\mu\text{mol/L}$ )	0.46 (0.32 – 0.63)	0.56 (0.41 – 0.65)	P = 0.264 (-1.116)
Nf light (ng/L)	3618 (1394 – 5191)	3113 (1541 – 4789)	P = 0.984 (-0.020)
Nf heavy (ng/L)	268 (213 – 437)	167 (95 – 263)	P < 0.001 (-3.337)

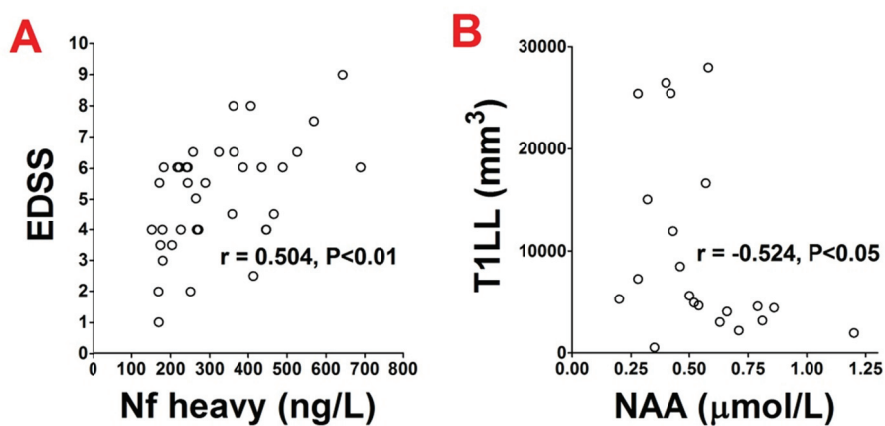


**Figure 37. Concentrations of the analyzed biomarkers in CSF of controls and MS patients.**

OND (filled circle), progressive MS patients (filled triangle), untreated MS patients (open circle), IFN $\beta$  treated patients (open triangle). Filled horizontal lines indicate median values; dashed horizontal lines indicate the 90% cutoff values in the OND group (NAA, 0.31 and 0.85  $\mu\text{mol/L}$ ; Nf light, 6138 ng/L; Nf heavy, 406 ng/L). A: N-acetylaspartate levels in controls and MS patients. B: Nf light levels in controls and MS patients. C: Nf heavy levels in controls and MS patients. CSF Nf heavy concentrations were higher in MS patients than OND ( $P < 0.001$ ). D: Nf heavy levels in IFN $\beta$  untreated (Placebo) and treated (IFN $\beta$ ) MS patients.

## Correlation between CSF biomarkers for axonal damage and clinical and radiological parameters

We investigated possible linear associations between biomarkers and disease progression outcomes. A moderate positive correlation was found between EDSS scores at the time of CSF collection and Nf heavy levels (**Figure 38, panel A**; Spearman  $r = 0.504$ ,  $P < 0.01$ ). No association was found between NAA and Nf heavy or between the two Nf isoforms. Concerning association between biomarkers and radiological parameters in IFN $\beta$  treated and untreated patients, no correlation was found between Nf light or Nf heavy and T1LL, T2LL and BPF. A significant negative correlation was found between NAA levels and T1LL (**Figure 38, panel B**; Spearman  $r = -0.524$ ,  $P < 0.05$  [ $P = 0.015$ ]). None of the biomarkers correlated with the change in radiological markers over 24 months.



**Figure 38. Correlation between biomarkers and clinical and radiological parameters.** The graphs represent the raw data. The Spearman's correlation coefficients and p-values are indicated on top of each graph. A: Nf heavy in relation to EDSS. B: NAA in relation to T1weighted Lesion Load (T1LL) in IFN $\beta$  treated and untreated progressive MS patients.

### **Combination of CSF biomarkers for axonal damage improves discrimination between MS patients and controls**

Based on the 90% cut-off values of the levels of Nf light, Nf heavy and NAA in the OND group (**figure 37 A-D**, dashed horizontal line), we defined in all subjects whether Nf light or Nf heavy were increased and whether the NAA levels were increased or decreased. None of the patients had simultaneous increased Nfs and increased or decreased NAA, but 7 patients out of 38 (18.1 %) and 1 control out of 15 (6.7 %) showed simultaneous increase in both Nfs.

Abnormal axonal damage biomarkers were more frequent in progressive MS patients than controls (Fisher Exact test, 55% vs. 20%,  $P < 0.05$ ) and the combination of biomarkers allowed us to classify a higher number of patients with abnormal biomarker levels than single markers (**table 12**). No difference was found in the EDSS scores in patients with abnormal and normal axonal damage biomarkers, indicating that the combination of the three biomarker levels seemed not related to worse clinical condition within the progressive MS group, although Nf heavy only was, as shown before.

In addition, we determined the cut-off values for the biomarkers based on the first and third tertile in the whole OND and MS merged group and we analysed if high levels of Nf light, Nf heavy and low or high values of NAA could be predictive for progressive MS.

We found that Nf levels in the upper tertile were related to a Odds ratio of 7.2 to be in the group of the progressive MS patients (ODDS ratio 7.2; 95 % Confidence Interval 1.3 - 38.2;  $P < 0.025$ ). Indeed, Nf light or NAA did not improve the model.

**Table 12. Percentage of progressive MS patients and OND controls with abnormal biomarker values.**

*The percentages are defined by single markers or the combination of Nfs and NAA.*

<b>Percentage abnormal in each subgroup</b>		
	<b>Progressive MS</b>	<b>OND</b>
<b>NAA ↓</b>	21.1 % (8/38)	6.7 % (1/15)
<b>Nfs (Nf light ↑ AND Nf heavy ↑)</b>	18.4 % (7/38)	6.7 % (1/15)
<b>Nfs (Nf light ↑ OR Nf heavy ↑)</b>	42.1 % (16/38)	13.3 % (2/15)
<b>NAA or Nfs</b>	55.3 % (21/38)	20 % (3/15)

## DISCUSSION

A biomarker is defined as a quality objectively measured and evaluated as indicator of physiological and pathological processes or pharmacologic responses to a therapy (Biomarkers Definitions Working Group, 2001). Often, to monitor a disease only one biomarker that has proven to be specific for that pathology is used. The results found in the present study improve the concept of the use of different biomarkers related to pathologies. In fact, not only a single biomarker could be useful to monitor a disease, but especially the combination of biomarkers can give a more accurate sight of a disease state. A clear example is the combination of different biomarkers of axonal damage, such as Neurofilaments (Nf) light and heavy and N-acetylaspartic acid (NAA), which recently has been hypothesized as a good choice to monitor the different stages of axonal degeneration in Multiple Sclerosis (MS) (Teunissen CE et al., 2009). In particular, it has been shown that Nf light levels may be a biomarker for acute axonal damage caused by inflammation. On the other hand, the levels of Nf heavy may increase during irreversible late-stage axonal degeneration, whereas NAA probably increase during early stages of acute axonal damage, being increasingly excreted as a consequence of increased mitochondrial activity. However, decreased NAA levels likely reflect the axonal loss at the end stage of the process of axonal degeneration (Teunissen CE et al., 2009). The results we found in our study about biomarker levels in a relatively large cohort of essentially progressive MS patients, characterized by prominent axonal degeneration rather than inflammation (Kutzelnigg A et al., 2005), are in line with these assumptions. In fact, we did not find any difference on the Nf light levels between patients with a progressive course of disease (PPMS) and controls with other

neurological diseases (OND). This suggests that Nf light may be associated to relapse-onset or inflammation-induced axonal loss or injury, since it is increased during relapses and normalized during remission phases (Norgren N et al., 2004). Also, we can hypothesize that lower NAA levels may reflect the black hole formation, as we found a negative correlation between its levels and the lesion burden measured by T1 weighted Magnetic Resonance Imaging (MRI). However, the median NAA levels in progressive MS patients were similar to controls, arguing the possibility that NAA lowering is a common process which may be not-specific for MS. Furthermore, we can confirm that Nf heavy is associated to irreversible axonal damage, since we found significant high levels of this marker in PPMS patients. Therefore, our results suggest that Nf heavy can be considered as a surrogate marker of axonal damage in patients with essentially progressive MS course. Finally, using the combination of the biomarkers we were able to classify a higher number of patients with MS with abnormal axonal damage biomarker values than for single markers. This confirms that together these biomarkers may reflect different stages of axonal damage (Teunissen CE et al., 2009) and this can give a more accurate sight of the disease.

Different pathological processes can take place during MS: beside the axonal damage, which accumulates during the course of the disease and is responsible for the major disability experienced by patients, also inflammation has a key role (Hauser S et al., 2006). In fact, the triggered inflammatory response can lead to the recruitment of cells from the immune system which can in turn cause tissue damage as well as axonal degeneration (Martino GV et al., 2002; Hauser S et al., 2006). For this reason, we also studied the possible role of the gelatinases in pathologies with a neuroinflammatory component. The ability of MMP-9 and MMP-



2, the two gelatinases, to degrade components of the extracellular matrix and to regulate the activity of a number of soluble proteins confers an important role to various physiological and pathological processes (Van den Steen PE et al., 2002). A growing body of evidence has accumulated associating MMP-9 to inflammatory conditions, in particular to neuroinflammation (Rosenberg GA, 2002; Van den Steen PE et al., 2002; Tejima E et al., 2007). Indeed, MMP-9 can impair the Blood Brain Barrier (BBB), favoring the recruitment of inflammatory cells into the Central Nervous System (CNS) increasing the ongoing inflammation (Opdenakker G et al., 2003). Conversely, the significance of MMP-2 in inflammation seems controversial: in fact, especially in neuroinflammation MMP-2 has been associated to both protective and harmful role (Rosenberg GA, 2002; Yong VW, 2005; Yong VW et al., 2007). However, its real role is still unclear.

Previously, we identified in the active MMP-9 and the active MMP-9/TIMP-1 ratio good surrogate biomarkers for disease progression and activity in Relapsing-Remitting Multiple Sclerosis (MS) (Fainardi E et al., 2006). In particular, in such neuroinflammatory disease we found both increased active MMP-9 and MMP-9/TIMP-1 ratio in patients during active stage of disease than in those inactive. Moreover, the levels of MMP-9 were higher in patients with MS and other inflammatory neurological diseases (OIND) than the one with non-inflammatory neurological disease (NIND), confirming that MMP-9 is implicated in neuroinflammation (Fainardi E et al., 2006). The results we found in the present study about patients affected by SICH (Spontaneous Intracerebral Hemorrhage), another disease with a major inflammatory component, confirm the key role of MMP-9 in neuroinflammation. In fact, active MMP-9 levels rose over time moving from the acute to the subacute

phase compared to baseline levels. Also, a gradual increase in perihematomal edema volume was seen over time. More interestingly, MMP-9 levels were positive correlated with the perihematomal edema suggesting that MMP-9 is strictly related to neuroinflammation, as the edema formation is associated to a triggered inflammatory response. Thus, MMP-9 may exert a detrimental effect impairing the BBB and exacerbating the perihematomal edema formation (Abilleira S et al., 2003). On the contrary, our findings about active MMP-2 support the hypothesis of a protective function of this enzyme, favoring the resolution of neuroinflammation and tissue repair. In fact, we found a decrease in active MMP-2 levels over time in SICH patients and a negative correlation with perihematomal edema, suggesting an opposite role of MMP-2 than MMP-9 in neuroinflammation. Moreover, the results found in our study about MS support the repairing role of MMP-2. Higher active MMP-2 levels we found here in MS patients and in OIND than NIND, confirm that MMP-2 is implicated in the regulation of neuroinflammation (Yong VW, 2005). More attractive were the results coming from the analysis of MS patients that revealed the presence of more increased concentrations of active MMP-2 during Magnetic Resonance Imaging (MRI) inactive than MRI active stage of disease. Moreover, TIMP-2 levels were greater in MS patients with than in those without MRI evidence of disease activity. As MRI Gd-enhancement is widely accepted to be more powerful than clinical examination in measuring MS disease activity (Miller DH et al., 1993), this implies that a shift in MMP-2/TIMP-2 balance toward activity of MMP-2 could be involved in mechanisms leading to the resolution of neuroinflammation, which are associated with the remission of the disease. Therefore, our findings argue for a potential role of CSF and serum levels of active

MMP-2 as appropriate indicators of the termination of MS inflammatory disease activity. In particular, serum active MMP-2/TIMP-2 ratio may be useful as a surrogate biomarker for monitoring MS disease recovery since it is easily measurable.

Therefore, our results suggest a possible different implication of the two gelatinases in ongoing inflammatory response taking place in the CNS: MMP-9 may promote the disruption of the BBB increasing the inflammation, whereas MMP-2 might exert a protective function favouring the resolution of neuroinflammation. Thus, the combination of the active MMP-9 and MMP-2 might be useful to monitor the different stages of an inflammatory disease.

In addition, the involvement of MMP-9 in inflammation has been shown by *in vitro* experiments where we studied the release of MMP-9 from neutrophils subjected to environmental pH changes. In fact, it is well documented that an acidic pH is present in inflamed tissues and that this environmental condition can influence the immune response (Kellum JA et al., 2004; Trevani AS et al., 1999; Martinez D et al., 2006). Thus, the finding of more MMP-9 released during extracellular acidosis suggests that neutrophils can be activated by this condition, increasing their response. Therefore, a more prominent production of MMP-9 can be directly associated to inflammation. In addition, supporting this hypothesis we found that extracellular pH can also modulate the response of these cells to pro-inflammatory stimuli. In fact, we found a different behavior on the basis of the used stimulus: when neutrophils were subjected to LPS stimulation, there was an increase of MMP-9 release at acid pH, whereas when stimulated with IL-8 a decrease occurred, compared to physiological pH (7.4). A possible explanation of this non-conventional behavior could be the different role of the used stimuli. In

fact, while IL-8 is used to recruit neutrophils towards the site of inflammation from an environment with a physiological pH, LPS have to trigger a strong consistent response against pathogens. Conversely, a basic environment seems to affect negatively the release of MMP-9 with both stimuli, according to previous observation on diminished neutrophils functions during extracellular alkalosis (Kleinberg I. et al., 1968). Our observations are in line with studies that report a positive modulation of neutrophils functions by extracellular acidosis (Trevani AS et al., 1999; Martinez D et al., 2006), whereas are contrasting with other studies that report a negative modulation (Lardner A. et al., 2001; Murata N et al., 2009).

Finally, the finding of the 66 kDa active MMP-9 in serum of healthy subjects opens new avenues in understanding the implication of MMP-9 in inflammation and its role as marker. In fact, our results suggest that this particular form is normally present in serum in variable amounts in relation to total endogenous active MMP-9 concentration. This may indicate that the 66 kDa MMP-9 is not likely a background artifact due to autoproteolysis of the sample, as thought up to now (O'Connell JP et al., 1994). Therefore, this form of MMP-9 deserve more attention as it might not be inhibited by TIMP-1: the unbalance of the activation network towards a more prominent production of the 66 kDa form can lead to both increased tissue damage and inflammation. However, the main limitation of our study is the inability to establish the actual functional properties of this active MMP-9. In fact, it is not clear yet whether the balance between 82 kDa and 66 kDa MMP-9 has a physiological significance, although we can hypothesize that this might reflect the activity of the enzymes upstream the activation cascade of MMP-9. However, further studies including several inflammatory pathologies as

well as a large cohort of healthy subjects are necessary. Collectively taken, our results evidence the key role that MMP-9 might has in inflammation, since it can act both directly and indirectly increasing tissue damage and reinforcing the inflammation itself (Van den Steen PE et al., 2002).

From our study, we can conclude that the presence of more biomarkers which reflect different aspects of pathological processes and their combination can have a predicting value for the evolution of a disease, also giving useful information in therapies efficiency.

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## Personal publication related to the thesis

Some of the results presented here were published in the following journals:

1) Fainardi E, Castellazzi M, Tamborino C, **Trentini A**, Manfrinato MC, Baldi E, Tola MR, Dallochio F, Granieri E, Bellini T.: Potential relevance of cerebrospinal fluid and serum levels and intrathecal synthesis of active matrix metalloproteinase-2 (MMP-2) as markers of disease remission in patients with multiple sclerosis. *Mult Scler.* 2009; 15(5): 547-554

2) Castellazzi M, Tamborino C, De Santis G, Garofano F, Lupato A, Ramponi V, **Trentini A**, Casetta I, Bellini T, Fainardi E.: Timing of serum active MMP-9 and MMP-2 levels in acute and subacute phases after spontaneous intracerebral hemorrhage. *Acta Neurochir Suppl.* 2010; 106:137-140

3) **Trentini A**, Comabella M, Tintoré M, Koel-Simmelink MJA, Blom HJ, Rovira A, Korth C, Blankenstein MA, Bellini T, Montalban X, Teunissen CE.: N-acetylaspartate and Neurofilaments as Biomarkers of Axonal Damage in Progressive Multiple Sclerosis. *Archives of Neurology, Submitted for publication*

4) Bellini T, **Trentini A**, Manfrinato MC, Tamborino C, Di Foggia V, Fainardi E, Dallochio F, Castellazzi M.: Matrix metalloprotease-9 activity detected in body fluids is the result of two different enzyme forms. *Biochimie, Submitted for publication*

## Attendance to National and International Congresses during the

### PhD course:

- 1) Cervellati C, **Trentini A**, Manfrinato MC, Dallochio F, Bellini T, Castellazzi M, Fainardi E.: Gelatinases: enzymatic activity as potential biomarker in multiple sclerosis. *The FEBS Journal* 2008; 275 (Supplement 1): 215
  
- 2) **Trentini A**, Manfrinato MC, Castellazzi M, Fainardi E, Dallochio G, Volta CA, Bellini T.: Extracellular pH modulates the release of Metalloproteinases-9 (MMP-9) by stimulated neutrophils and its activity. *The FEBS Journal*. 2009; 276 (Supplement 1): 393-394
  
- 3) **Trentini A**, Manfrinato MC, Castellazzi M, Fainardi E, Dallochio F, Volta CA, Bellini T.: Matrix Metalloprotease-9 activity detected in body fluids is the result of two different enzyme forms. *Proteine 2010 (Parma)* 2010; P-85
  
- 4) Manfrinato MC, Bellini T, **Trentini A**, Chiavegato G, Farabegoli L, Alvisi R, Volta CA, Dallochio F.: The influence of different replacement solutions on neutrophils response. *55<sup>th</sup> National Meeting of the Italian Society of Biochemistry and Molecular Biology (SIB)*. 2010; IMMUNO-2