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MATRIX METALLOPROTEINASES IN HUMAN HEALTH AND DISEASE: A MULTISYSTEM APPROACH TO UNDERSTANDING THEIR MOLECULAR FUNCTION AND CLINICAL RELEVANCE IN REPRODUCTION, MULTIPLE SCLEROSIS, AND FIBROSIS

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To my pillar of strength,

whose steadfast support never wavered

SUMMARY

ABSTRACT

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases involved in the remodelling of the extracellular matrix (ECM). Among MMPs, gelatinase A (MMP-2) and gelatinase B (MMP-9) are important contributors to physiological and pathological processes, such as wound healing and reproduction. On the other hand, MMP dysregulation may be involved in numerous diseases, including cancer, neuroinflammation and fibrosis. Throughout this PhD thesis, the pleiotropic and multifaceted role of MMPs was investigated using a multisystem approach in three diverse pathophysiological conditions: human reproduction, multiple sclerosis (MS), and Dupuytren's disease (DD).

In the reproductive context, MMPs are implicated in ECM remodelling during follicle growth, ovulation, corpus luteum formation, angiogenesis and embryo implantation. The composition and function of follicular fluid (FF) were analysed in n=126 women undergoing assisted reproductive techniques (ART). The study characterised the gelatinolytic activity of MMP-2 and MMP-9, both in the FF and FF-derived extracellular vesicles (FF-EVs) isolated by ultracentrifugation. Zymographic analysis showed a higher activity of proMMP-2 and less variability among patients, while proMMP-9 showed a lower activity and was more variable, suggesting distinct regulatory mechanisms. In FF-EVs, the active form of MMP-2 was also detected in 70% of the analysed samples, indicating a specific compartmentalisation of active enzyme. A significant inverse correlation emerged between patient age and MMP activity levels. Furthermore, the positive correlation between MMP-9 and C-reactive protein (CRP) suggested a link between gelatinase and the inflammation status of the patients. *In vitro* functional studies showed that FF from young women stimulates cell migration and reduces E-cadherin expression, indicating a possible effect on endometrial receptivity mediated by mechanisms similar to the epithelial-mesenchymal transition (EMT). These results propose MMPs as potential biomarkers predictive of oocyte quality and ART success.

In the neurological context, a multisystemic analysis was conducted on biological fluids from multiple sclerosis patients. MS is a chronic neuroinflammatory disease with blood-brain barrier (BBB) impairment, demyelination and neurodegeneration. Cerebrospinal fluid (CSF), serum, and plasma samples were obtained from n=62 patients with MS and compared with n=31 patients with other neurological diseases (OND), further classified as inflammatory and non-inflammatory diseases. High-resolution proteomic CSF profiling (LC-MS/MS) identified n=892 proteins, with upregulated and downregulated proteins involved in different aspects of

MS aetiology and progression. A special focus was deserved for the concomitant IGFBP-2 upregulation and IGF-2 downregulation in MS patients, also because IGFBP-2 was the leading discriminating marker by machine learning classification analysis. In parallel, zymographic analysis was conducted on MS biological fluids. In CSFs, MMP-2 and MMP-9 were mainly detected as proenzymatic forms, with higher expression of proMMP-2. Gelatinase activity was surprisingly lower overall in MS patients than in OND controls. Interestingly, there was a prominent decrease in active MMP-2 during relapse *vs.* remission. Correlation analysis also revealed a positive association between proMMP-2 and CRP cerebrospinal fluid levels, thereby connecting matrix remodelling to subclinical inflammatory events. To analyse the peripheral compartments, gelatinolytic activity was also examined in the plasma and serum of MS patients compared to controls. Serum zymography detected higher expression of proMMP-9, as well as its dimer and proMMP-9/TIMP-1 complex. Generally, also in serum the gelatinase activity was also considerably lower in MS patients. Serum CRP levels were also considered, and correlation analysis demonstrated a negative correlation with proMMP-2, suggesting a differential association between gelatinase activity and systemic inflammation across central and peripheral compartments. These findings highlight the dual role of MMPs in MS and remark on their potential as biomarkers of disease stage and therapeutic response. The elevated levels of proMMP-9 in serum compared with plasma are most likely due to leukocyte degranulation during clotting and highlight the need for additional work to clarify the clinical relevance of matrix-specific MMP activity in peripheral compartments.

Finally, in Dupuytren's disease, a fibrotic disorder of the palmar fascia, MMP-14 (MT1-MMP) is highly involved in extracellular matrix remodelling and fibroblast contractility. Primary fibroblasts from genotyped patients were analysed to assess the impact of a functional *MMP14* SNP. Conversely to the expectations, homozygous AA cells showed higher proMMP-2 activity, with minimal active MMP-2 in other genotypes. Anti-MMP-14 antibody treatment unexpectedly increased proMMP-2 activity across all genotypes, without affecting total MMP-2 levels. However, MMP-14 protein expression was reduced after the treatment, confirming antibody efficacy. These results suggest a genotype-dependent modulation of gelatinase activity and a functional dissociation between expression and activation. Future proteomic analysis of fibroblast media will lead to the identification of downstream targets involved in fibrosis, in order to evaluate the therapeutic potential of MMP-14 inhibition.

In conclusion, this PhD thesis illustrates MMPs as central molecular actors of tissue remodelling in numerous biological settings. Their expression, activation, and extracellular vesicle-mediated delivery are strictly controlled in response to physiological demands and

pathological insults. Through the synergy of biochemical, molecular, proteomic, and functional techniques, this thesis provides an integrative view of MMPs as candidate biomarkers and therapeutic targets in human reproduction, neuroinflammation, and fibrotic disorders.

INTRODUCTION

1 Matrix Metalloproteinases in Health and Disease

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases with several roles in extracellular matrix (ECM) degradation and tissue remodelling. Owing to this primary function, MMPs are implicated in a plethora of biological processes, both under physiological and pathological conditions (Cabral-Pacheco et al., 2020).

In addition to extracellular functions, several MMPs have also been shown to localise intracellularly (in the cytosol, mitochondria, and nucleus) where they are involved in the regulation of apoptosis, DNA repair, and transcriptional processes. Thus, these enzymes are involved in several cellular processes, including cell proliferation, migration, differentiation, and apoptosis, by activating precursors of growth factors and other bioactive molecules (Hadler-Olsen et al., 2011). Under physiological conditions, MMPs maintain tissue homeostasis by regulating ECM turnover. Indeed, they are involved in processes such as wound healing, angiogenesis, bone repair, immune response, ageing, but also embryogenesis and reproduction (Cabral-Pacheco et al., 2020; Serra, 2024). Notably, it has been shown that MMP-2 might play a positive role in the attainment of longevity (Cancemi et al., 2020). This functional versatility is made possible by the tight and fine tuning of MMP expression and activity, which occurs at different levels, including gene transcription, proenzyme activation, inhibition by tissue inhibitors of metalloproteinases (TIMPs) (Hadler-Olsen et al., 2011), cellular localization or compartmentalisation. Thus, dysregulation of this regulatory balance can contribute to the pathogenesis of several diseases, such as cancer, cardiovascular disease, chronic inflammation, arthritis, fibrosis, and neurodegeneration (Cabral-Pacheco et al., 2020; Serra, 2024). For instance, MMP-2 and MMP-9 are implicated in breast and colon cancer (Buttacavoli et al., 2020; Di Cara et al., 2018).

Over the years, studies have also highlighted the potential of MMPs as diagnostic and prognostic indicators, emphasising how their expression and regulation can vary significantly across different biological systems. This variability reflects the specificity of tissue context, cellular microenvironment, and pathological state.

1.1 MMP in Extracellular Matrix Remodelling

The extracellular matrix is a complex and dynamic network that surrounds and supports the cells of all tissues. Far more than its structural role, the ECM is a biologically active environment essential for tissue development, repair, and homeostasis. Within this microenvironment, cells share biochemical and mechanical signals that regulate basic processes such as proliferation, differentiation, migration, and survival.

The ECM is mainly composed of protein and non-protein components. Among proteins, collagens are the most abundant constituents of the ECM. Further fibrous ECM proteins are elastin, fibronectin, laminins, and tenascin. Additionally, ECM contains glycoproteins, growth factors, proteoglycans, glycosaminoglycans, water, and ions (Karamanos et al., 2021).

Collagen is the most abundant structural protein in mammals. There are 28 different types of collagens, classified into fibrillar (types I, II, III, and V) and non-fibrillar (e.g., basement membrane collagen type IV) ones. In general, collagen is constituted by a triple-helix structure, composed of three α-chains. The triple-helix is rich in glycine, proline, and hydroxyproline, which confer flexibility and stability to the structure. This conformation allows high mechanical resistance to the molecule. For this reason, collagen primarily provides structural support and strength to tissues. Collagen is also important in tissue turnover and wound healing processes: indeed, it is subject to continuous remodelling within the ECM, regulated by proteases able to cleave the triple-helix conformation, particularly matrix metalloproteinases. Among MMPs, collagenases (MMP-1, MMP-8, MMP-13) and gelatinases (MMP-2 and MMP-9) are capable of degrading collagen fibres. Type I, II and III collagens can be recognized and cleaved by MMPs due to a specific proteolytic sequence localized at the *N*-terminal region, generating two fragments (¾ *N*-terminal and ¼ *C*-terminal) of denatured collagen, known as gelatine. Gelatine can be further degraded by gelatinases (Ricard-Blum, 2011) (Figure 1).

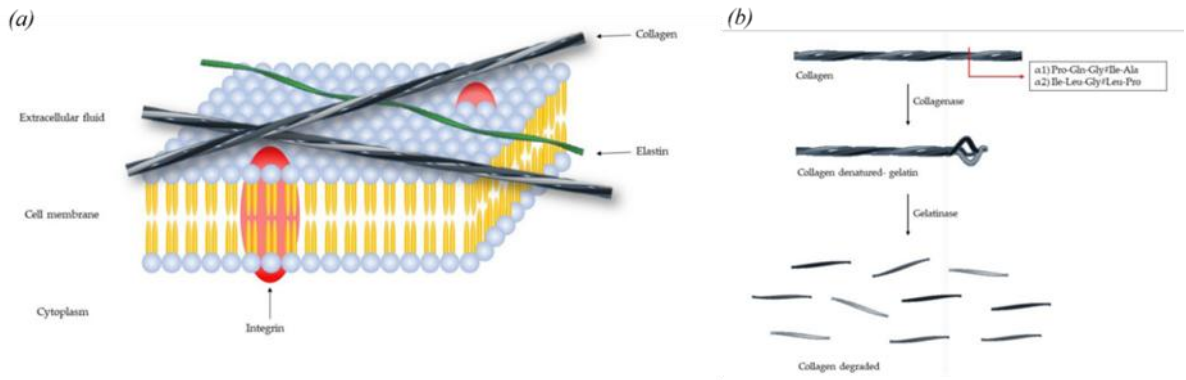

Figure 1 **Extracellular matrix (ECM) and collagen degradation.** (a) Schematic representation of the cell membrane interacting with ECM components (e.g., collagen and elastin). (b) Focus on collagen degradation by MMPs: collagenases cleave the triple helix in two fragments (¾ *N*-terminal and ¼ *C*-terminal) at a specific proteolytic sequence. Denatured collagen (or gelatine) can be in turn degraded by gelatinases (adapted from Laronha & Caldeira, 2020).

The ECM directly participates in the regulation of cellular processes, since the extracellular microenvironment regulates biochemical and mechanical stimuli that result in the activation of specific signalling pathways. Indeed, cells respond to stimuli from changes in the extracellular environment and matrix, specifically by the activation of surface receptors such as integrins, activating intracellular signalling pathways such as MAPK and AKT. Consequently, changes in the ECM can influence biological processes such as proliferation, differentiation, apoptosis, and cell migration. Moreover, the ECM's structure and stiffness determine cell differentiation: for example, collagen-rich culture supports osteogenic differentiation, while laminin and a more compliant matrix support neuronal differentiation. ECM induces apoptosis if cells are released from adhesion. MMPs modulate the ECM dynamically to facilitate migration and the delivery of growth factors. In short, the ECM is not merely a mechanical scaffold, but also a dynamic regulator of cell function in both physiologic and pathologic conditions (Karamanos et al., 2021).

1.2 MMP Classification

The human MMP family consists of 23 different members, listed in Table 1. The current nomenclature of MMP follows a sequential number system, although the names of some of them, designated over time by their discoverers, are still in use today (i.e., gelatinases). MMPs can be classified into two major subgroups according to their molecular structure: secreted-type MMPs and membrane-type MMPs (MT-MMPs). MMP-4, -5 and -6 are not listed because, after their discovery, they were verified to be identical to other already known MMPs. MMP-18 and 22 are also missing in this report because they are assigned to *Xenopus* (collagenase-4) and chicken (Okada, 2017).

The most widely supported classification of MMPs involves their subdivision into different subgroups based on their specificity for a substrate. Secreted-type MMPs can be classified into collagenases, gelatinases, stromelysins, matrilysins, and other MMPs with specific functions. MT-MMPs can be subdivided into transmembrane-type MMPs (types II and II) and

glycosylphosphatidylinositol (GPI)-linked MMPs (Okada, 2017; Visse & Nagase, 2003). Although MMPs are primarily classified based on their specificity for ECM substrates, it has become increasingly evident over time that they can also interact with non-ECM proteins, involved in numerous biological functions (Dufour & Overall, 2015). Below is a brief overview of each subgroup within the MMP family, detailing their specific substrates.

Collegenases. MMP-1 (interstitial collagenase or collagenase-1), MMP-8 (neutrophil collagenase or collagenase-2), and MMP-13 (collagenase-3) recognise fibrillar collagens (types I, II, and III). MMP-1, -8, and -13 can also degrade many other ECM macromolecules, such as gelatin, aggrecan, perlecan, and tenascin. Interestingly, among non-ECM substrates insulin-like growth factor binding proteins (IGFBP-2 and -3) are noted (Fowlkes et al., 1999; Okada, 2017).

Gelatinases. MMP-2 (gelatinase A) and MMP-9 (gelatinase B) can degrade the main component of the basal membranes, such as gelatin (denatured form of collagen), but also type IV and V collagen. Other ECM substrates are elastin and aggrecan. MMP-2, differently that MMP-9, can also degrade type XI collagen, fibronectin, and laminin; only MMP-9 can digest type III collagen (Okada et al., 1990, 1992). Among non-ECM substrates, gelatinases can also specifically regulate IGFBP family members (IGFBP-5 and -3, respectively recognised by MMP-2 and -9) (Fowlkes et al., 1999).

Stromelysin. This group encloses MMP-3 (stromelysin-1), MMP-10 (stromelysin-2), and MMP-11 (stromelysin-3). Their common ECM substrates are aggrecan, fibronectin, laminin, and collagen type IV (Chin et al., 1985). In addition to ECM substrates, MMP-3 can also regulate IGFBP-3 (Fowlkes et al., 2004), but also E-cadherin (Zheng et al., 2009). MMP-3 and MMP-10 also regulate the activation of several zymogenic forms of MMPs (Murphy et al., 1987; Nakamura et al., 1998).

Matrilysins. Matrilysin-1 and -2, also known as MMP-7 and MMP-26, respectively, are the smallest MMPs, as they consist only of the catalytic domain and the pro-peptide, lacking the hemopexin-like domain. MMP-7 efficiently degrades various ECM components, including collagens (III, IV, V, IX, X, XI), gelatin, elastin, fibronectin, and laminin, as well as non-ECM substrates like E-cadherin (Im et al., 2022; Lynch et al., 2010) and N-cadherin (Williams et al., 2010). MMP-26 targets gelatin, type IV collagen, fibronectin, and fibrinogen, but its substrate profile remains less characterized (Okada, 2017).

Other Secreted-type MMPs. Secreted MMPs include MMP-28 (epilysin), which is intracellularly activated by furin. MMP-28 degrades casein, but its natural substrates remain unknown. MMP-12, MMP-19, MMP-20. MMP-21 and MMP-27 share structural similarities with collagenase and stromelysin, but their classification is uncertain due to incomplete characterisation. MMP-12 degrades elastin, fibronectin, and type V collagen, while MMP-19 acts on collagen IV, laminin, and aggrecan. MMP-20 degrades amelogenin and cartilage matrix proteins. The substrates of MMP-21 and MMP-27 are still unknown (Okada, 2017).

Membrane-anchored MMPs (MT-MMPs). This group includes MMP-14, MMP-15, MMP-16, and MMP-24. MMP-14 can activate proMMP-2 and degrade collagen and other ECM proteins. MMP-17 and MMP-25, anchored via GPI, degrade gelatinase and fibrin. MMP-23, a type II MMP, is expressed in the reproductive organs and degrades gelatinase, but its precise function remains unknown (Okada, 2017).

Table 1 **Substrates of Human Matrix Metalloproteinases**

Enzyme	MMP	Substrates/Functions
Collagenases		
Interstitial Collagenase; Collagenase I	MMP-1	Fibrillar collagens (Types I, II, III)
Neutrophil Collagenase; Collagenase II	MMP-8	Fibrillar collagens (Types I, III), Elastin, Proteoglycans
Collagenase-3	MMP-13	Fibrillar collagens (Types I, II, III), Cartilage
Gelatinases		
Gelatinase A; Type IV Collagenase	MMP-2	Gelatin, Type IV collagen, Elastin, Fibronectin
Gelatinase B; Type IV Collagenase	MMP-9	Gelatin, Type IV collagen, Elastin, Aggrecan
Stromelysins		
Stromelysin-1	MMP-3	Proteoglycans, Laminin, Fibronectin
Stromelysin-2	MMP-10	Proteoglycans, Fibronectin, Gelatin, Elastin
Stromelysin-3	MMP-11	Precursor proteins, Cytokine modulators
Matrilysins		
Matrilysin-1	MMP-7	Elastin, Fibronectin, Laminin, Proteoglycans, E-cadherin
Matrilysin-2	MMP-26	ECM components, ProMMP-9
Membrane-Type MMPs		
Transmembrane type		
MT1-MMP	MMP-14	Fibrillar collagens (Types I–III), Gelatin, ProMMP-2
MT2-MMP	MMP-15	Collagens, Gelatin, ProMMPs
MT3-MMP	MMP-16	Collagens, ECM components, ProMMP-2
MT5-MMP	MMP-24	ECM components, Neural remodeling
GPI-anchored		
MT4-MMP	MMP-17	ECM degradation, Cell-surface shedding
MT6-MMP	MMP-25	ECM proteins, Immune cell migration
Others		
Macrophage elastase	MMP-12	Elastin, Fibronectin, Basal membrane
-	MMP-19	Type IV collagen, Laminin, Basal membrane
Enamelysin	MMP-20	Enamel proteins (Amelogenin, Enamelin)

-	MMP-21	Undefined substrates, Development, Reproduction
CA-MMP	MMP-23	Ion channel regulators, IGF-binding proteins
-	MMP-27	Undefined substrates, Tissue homeostasis, Ovarian functions
Epilysin	MMP-28	ECM components, Tissue repair, Inflammation, Nerve regeneration

1.3 MMP Structure and Domains

The MMPs have a modular structure composed of various functional domains, related to their proteolytic activity and substrate specificity, but also the different regulatory mechanisms they undergo. However, all MMP members contain the archetypal domain, which consists of:

i. an *N*-terminal signal sequence (pre-peptide) of variable length;
ii. a pro-domain of ~80 amino acids (a.a.) that maintains the enzyme in an inactive state and is removed by proteolysis;
iii. a catalytic domain (~160 a.a.) with the Zn^{2+}-binding motif and three Calcium (Ca^{2+}) ions, composed of five β-sheets and three α-helixes;
iv. a linker domain or "hinge region" (between 14 and 69 a.a.), which connects the catalytic domain to the hemopexin-like (Hpx) domain;
v. a highly conserved hemopexin-like domain (~210 a.a.), composed of four β-propellers (Cui et al., 2017; De Almeida et al., 2022; Tallant et al., 2010).

Furthermore, many MMPs have additional domains that modulate their activity and substrate specificity. For example, gelatinases A and B (MMP-2 and MMP-9) contain three tandem fibronectin type-II modules essential for collagen binding (Shipley et al., 1996). Moreover, MMP-9 uniquely comprises a central O-glycosylated (OG) domain, which acts as a linker sequence between the active site and the Hpx domain (Wilhelm et al., 1989). Some MMPs can be activated by convertases (like furin), as they contain a furin-cleavage site (RKRR sequence) within their pro-peptide region. Since the MT-MMPs are localized on the plasma membrane, they present a transmembrane segment or a glycosylphosphatidylinositol (GPI) anchor domain. MMP-23 differs from other MMPs because, in addition to its shared basic structure, it is the only one with a cysteine array (CA) and an immunoglobulin-like (Ig-like) domain (Galea et al., 2014) (Figure 2).

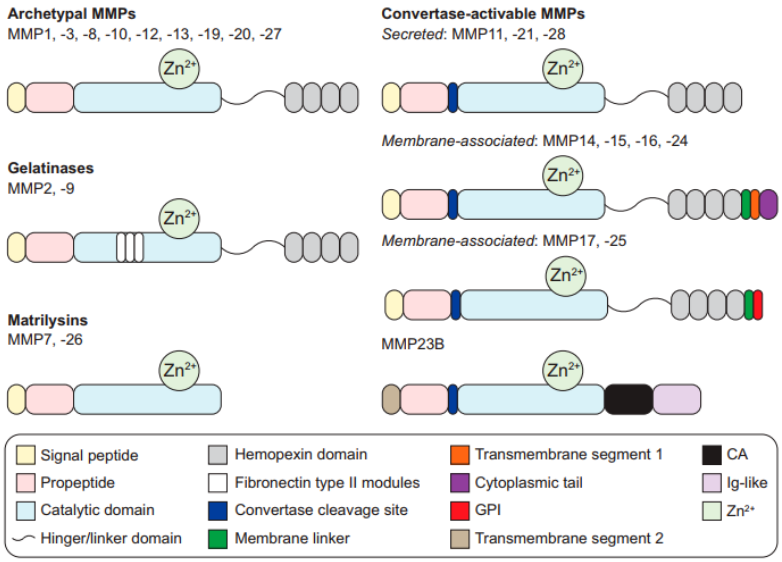

Figure 2 **Structure of Human MMP Domains.** Schematic representation of the 23 human modular structure of MMP domains. The archetypal MMP domain is composed of signal peptide, pro-peptide, catalytic domain (Zn^{2+} binding), linker or "hinge domain", and hemopexin domain. Gelatinases (MMP-2, MMP-9) contain an additional fibronectin type-II module, while matrilysins (MMP-7, MMP-26) lack the hemopexin domain. Convertase-activable MMPs have a furin-cleavable peptide. Membrane-associated MMPs include a transmembrane segment or a glycosylphosphatidylinositol (GPI) anchor domain. MMP-23 presents a cysteine array (CA) and an immunoglobulin-like (Ig-like) domain.

All MMPs are synthesized as pre-pro-enzymes. The pre-peptide signal domain is in the N-terminal region. It guides the transport of the protein to the endoplasmic reticulum (ER), where the signal sequence is removed to obtain the zymogenic form of MMP (pro-MMP) (Laronha & Caldeira, 2020).

The pro-peptide domain contains three α-helixes (α1-, α2-, and α3-helix) and is connected by flexible loops susceptible to proteolysis. Within these loops, there are specific regions where proteolytic cleavage by other proteases can occur. This site is commonly referred to as the "bait region": in proMMP-2, it contains the target sequence (SCN*LF) for proteolytic cleavage and protease activation (Figure 3). Similarly, pro-MMP-1 also contains a bait region (with an EKRRN sequence) subject to proteolytic cutting (Maskos, 2005). The primary function of the pro-domain is to regulate enzymatic activity by keeping the enzyme in its inactive state, preventing premature activation. In the pro-domain, a conserved "cysteine switch" motif (with a specific sequence, PRCGXPD) (Figure 3) binds to the active site containing the functional zinc ion, needed for the enzymatic reaction, keeping the zymogen inactive. Going into details,

a sulfhydryl (SH) group in the Cys residue coordinates with the Zn^{2+} of the catalytic domain (Nagase et al., 2006). The cysteine switch is bent due to an Arg-Asp salt bridge within the PRCGXPD motif, contributing to maintaining the stability of the inactive form and forming a tetrahedral coordination sphere. Moreover, the presence in the pro-domain of amino acid residues of tyrosine and phenylalanine helps create a hydrophobic environment (Maskos, 2005). This way, the pro-domain displaces the water molecule, preventing salt bridge disruption, and thereby keeping the inactive state.

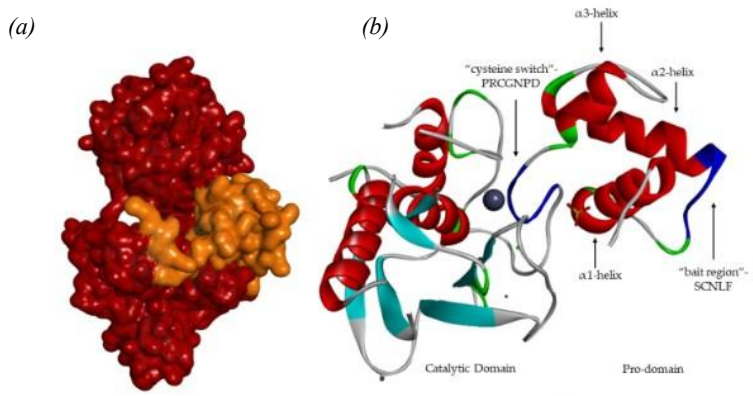

Figure 3 **Structure of MMP-2 with its pro-domain.** (a) Surface representation of MMP-2, with the pro-domain highlighted in orange. (b) Three-dimensional structural model of MMP-2. Indicated the "bait region" (SCNLF) and the "cysteine switch" (PRCGNPD), both highlighted in blue. The catalytic domain, composed of three α-helixes (α1-, α2-, and α3-helix) and five highly twisted β-sheets, is represented (Laronha & Caldeira, 2020).

The catalytic domain typically consists of three α-helixes (α1-, α2-, and α3-helix) and five highly twisted β-sheets, connected by eight loops. Moreover, two subdomains can generally be distinguished: an *N*-terminal larger upper subdomain and a smaller lower one at the *C*-terminus. Between the two subdomains lies the *S1'* pocket, which is the hydrophobic active site where the enzymatic reaction resides. This pocket determines the substrate specificity (Maskos, 2005) (Figure 4b). In this active site, the catalytic Zn^{2+} is coordinated by three histidine residues in the conserved domain sequence HEXXHXXGXXH. Near the C-terminal subdomain, the highly conserved methionine (Met) residue is located in a tight turn (the so-called "met-turn", sequence XBMX) that helps stabilise the structure. To improve the conformational and structural stability of the domain, an additional zinc ion is present in the catalytic side, along with three calcium ions. Finally, between the α1- and α2-helices lies the Ω-loop, which determines the specificity of each MMP; its amino acid composition is highly variable (Laronha & Caldeira, 2020; Maskos, 2005) (Figure 4).

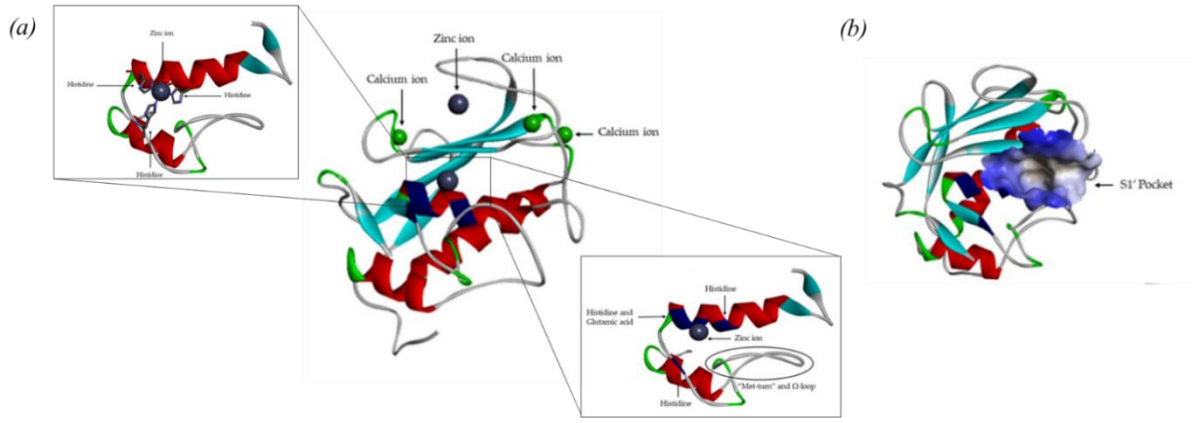

Figure 4 **Three-dimensional structure of the MMP-2 catalytic domain.** (a) Structural representation of the catalytic domain with the functional zinc-binding site (Zn^{2+}) and the structural Zn^{2+} coordinated to the three calcium ions, which contribute to MMP-2 stability. Top left: detail of Zn^{2+} catalytic site coordinated by three histidine residues in a tetrahedral coordination sphere; Bottom right: zinc-binding site and its interactions with histidine and glutamic acid, along with the "Met-turn" and Ω-loop. (b) Three-dimensional structural model of *S1'* pocket binding a substrate (adapted from Laronha & Caldeira, 2020).

The hinge region is a flexible linker whose length varies depending on each specific MMP. It is generally rich in prolines and contributes both to protein stability and the degradation of substrates. Indeed, this linker region is implicated in collagenase activity, promoting the proper disposition and coordination between the catalytic domain and the hemopexin-like one (Figure 5). This coordination is essential for achieving the correct three-dimensional arrangement of the protein, which allows the substrate hydrolysis. Moreover, studies have demonstrated that the hinge region can directly interact with the substrate, participating in collagen binding and degradation (Maskos, 2005; Tam et al., 2004).

The hemopexin-like domain consists of a β-propeller, composed of four β-sheets arranged rather symmetrically around a central channel. Some Hpx-like domain contains calcium, chloride, and sodium in the central channel (Figure 5). Its function is related to substrate recognition and specificity (Chung et al., 2004).

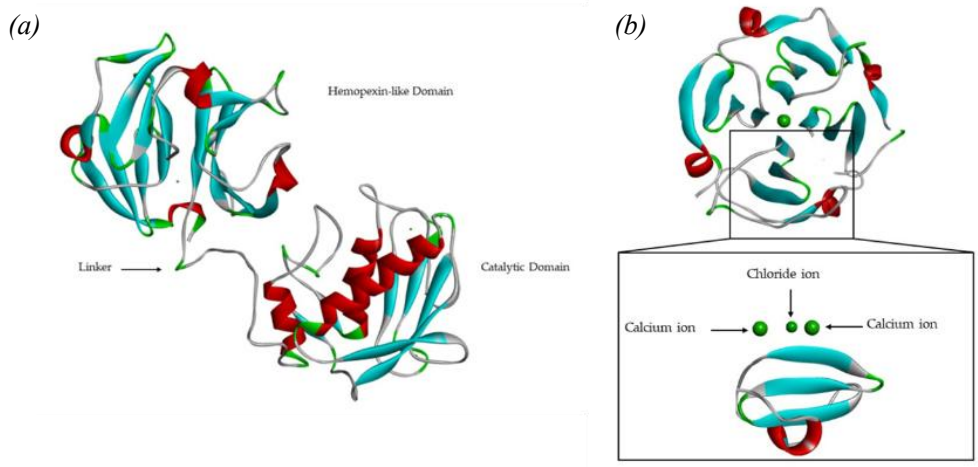

Figure 5 **Structure of catalytic domain and hemopexin-like domain.** (a) MMP-1 catalytic domain linked by the "hinge domain" (linker) to the hemopexin-like (Hpx) domain. (b) Structure of MMP-1 Hpx domain, composed of 4 β-propellers (4 β-sheets and 1 α-helix) around a central channel containing calcium, chloride, and sodium (adapted from Laronha & Caldeira, 2020).

1.4 MMP Regulation

Under normal physiological conditions, the biological activity of MMPs is tightly regulated at multiple levels, including gene transcription, modulation of mRNA stability, regulation of MMP secretion, activation of zymogenic forms, inhibition through physiological endogenous inhibitors (TIMPs), and specific compartmentalisation.

1.4.1 Transcriptional Regulation and Gene Expression

MMP gene expression is finely regulated at transcriptional and post-transcriptional levels to ensure a balance between matrix degradation and synthesis, which is essential for physiological cell processes such as proliferation, differentiation, migration, and apoptosis, as well as for tissue homeostasis and remodelling during wound healing, embryogenesis, and neuroplasticity.

Many *MMP* genes share highly similar promoters, which contain several *cis*-regulatory elements. At the transcriptional level, the promoters can be linked by various transcriptional factors or gene regulatory complexes to control *MMP* expression in response to different cellular signals. *Trans*-activators include AP-1 (Activator Protein-1), PEA-3 (Polyoma Enhancer Activator 3), Sp-1 (Specificity Protein 1), and NF-kB (Nuclear Factor-kappa B).

Based on the composition of *cis*-elements, *MMP* promoters can be distinguished into three categories. The first one consists of a TATA box sequence and an AP-I binding site. AP-1 complex is composed of the Jun and Fos proteins generally activated in response to inflammatory and mitogenic stimuli. In numerous *MMP* promoters with proximal AP-1 sites, a PEA3-binding consensus sequence is often located upstream. The majority of *MMP* genes possess promoters with this structural configuration, including *MMP9*. The second group includes promoters that also contain a TATA box but lack the site for AP-1. The third group, on the other hand, does not have either of the previous two *cis*-elements. These genes are therefore regulated by other transcriptional factors, such as Sp-1 and NF-kB. *MMP2* and *MMP14* belong to this category (Clark et al., 2008).

Gelatinase MMP-2 and MMP-9 gene expression. The activation of the *MMP2* and *MMP9* genes occurs through a multi-level regulation. However, while the gene regulatory pathways of gelatinase B are relatively well characterised, those involving the *MMP2* gene are still poorly understood and require further investigation. The *MMP9* promoter contains one NF-κB and two AP-1 binding sites, whereas *MMP2* is regulated by TNF-α and the p38-MAPK pathway via NF-κB, but independently of AP-1 (Figure 6).

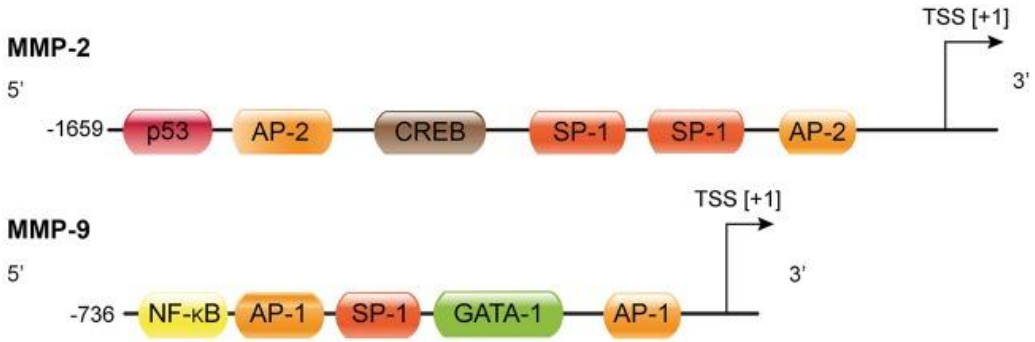

Figure 6 **Schematic representation of the MMP-2 and MMP-9 promoter regions highlighting transcription factor binding sites.** Each box indicates the location of a specific transcription factor binding motif. Abbreviations: TSS, transcription start site; AP-1, activator protein 1; AP-2, activator protein 2; GATA-1, erythroid-specific GATA-binding transcription factor; SP-1, specificity protein 1; NF-κB, nuclear factor kappa B; CREB, cAMP response element-binding protein; p53, tumour suppressor protein p53.

Extracellular signals (involved in proliferation, angiogenesis, migration and immune response) activate several intracellular signalling pathways that converge on the recruitment of transcription factors that bind the gene promoter. Pro-inflammatory cytokines (TNF-α, IL-8 and IL-1β) and growth factors (TGF-β, PDGF and bFGF) can bind to their receptors and activate

downstream signalling cascades involved in the activation of transcription factors (NF-κB, SP1 and AP-1), which can then bind to specific sequences of the *MMP9* gene promoter to trigger transcription (Augoff et al., 2022). MEK-1/ERK and NF-kB signalling pathways are shown to be essential to induce *MMP9* expression, after treatment with phorbol 12-myristate 13-acetate (PMA) (Ma et al., 2004). The activity of *MMP9* transcription factors also depends on coactivators and epigenetic modifications, required to promote chromatin remodelling and transcriptional process. The recruitment of coactivators (such as CBP, p300, and CARM1), chromatin remodelling, and histone modifications (acetylation of H3/H4 and methylation of H3-R17, H3-K4) are necessary for nucleosome opening to allow promoter binding sites (Ma et al., 2004).

1.4.2 Post-Translational Modifications

Post-translational modifications (PTM) consist of the addition of functional groups or other macromolecules to amino acid residues of proteins, during or after their synthesis and maturation. These modifications can alter the structure of the protein through conformational changes, regulate its function by activating or inhibiting it, and influence its localisation by guiding it to specific cellular compartments. This way, PTMs contribute to a complex regulatory network involved in numerous cellular processes, such as cell cycle, migration, apoptosis and immune response. Modifications involving the addition of functional groups include phosphorylation, oxidation, methylation and acetylation. The binding of more complex groups regards instead glycosylation, ubiquitination, SUMOylation, acylation and prenylation. Also, extended structures as GAGs can be linked (Ramazi & Zahiri, 2021).

To perform their pleiotropic activities, MMPs also undergo various and highly specific PTMs that can occur at the level of multiple functional domains. These modifications can influence MMPs in various ways: activating or inhibiting them, triggering proteolytic cutting of the substrate or modifying their specificity. In addition, modifications to the substrate or in other complexed proteins can influence the cutting site (Madzharova et al., 2019). Glycosylation and phosphorylation are the modifications that most commonly occur on MMPs and have been most studied over the years (Madzharova et al., 2019). In MMP-9, glycosylation can occur in two *N*-linked sites, in the pro-domain (Asn^{38}) and the catalytic domain (Asn^{120}), and it is generally associated with MMP-9 secretion, since required for the interaction with calreticulin (Duellman et al., 2015). Studies also show that *N*-glycosylation is related to proMMP-9 activation (S. Kumar & Cieplak, 2018), in particular by promoting the proteolytic cleavage of the pro-domain

by MMP-3 (Boon et al., 2019). Furthermore, MMP-9 can be *O*-glycosylated at the proline-rich linker sequence between the hemopexin domain and the catalytic site (Van Den Steen et al., 2006). Regarding MMP-2, it presents two potential *N*-glycosylation sites in the Hpx domain, although their function is not yet well understood (Madzharova et al., 2019). On the other hand, it is known that *O*-linked glycosylation of MMP-14 is important for the formation of the complex MMP-14/TIMP-2/proMMP-2, necessary for proMMP-2 cell-surface activation (Wu et al., 2004). Also, phosphorylation influences MMP activity: in MMP-2, of the 29 possible phosphorylation sites, five are recognised as regulators of its activity; in particular, the greater the degree of phosphorylation of MMP-2, the lower its proteolytic activity; conversely, dephosphorylation increases MMP-2 degradation capacity (Jacob-Ferreira et al., 2013).

1.4.3 MMP Compartmentalisation

One of the most important features in MMP activity control is their compartmentalisation at specific intracellular compartments, in the pericellular space or at the cell surface. Indeed, their localisation is tightly regulated and well correlated with the microenvironment wherein they are supposed to act. This precise spatiotemporal distribution is necessary to ensure their effective proteolytic activity on target substrates. Thus, MMP compartmentalisation is not only complementary but also determining of their action efficiency (Ra & Parks, 2007).

As previously mentioned, MMPs are synthesised as zymogens (pre-pro-peptide form) in the endoplasmic reticulum (ER) and subsequently undergo maturation in the Golgi apparatus. Within the Golgi, MMP trafficking is tightly regulated (Hey et al., 2022). For instance, the transport of MMP-2 and MMP-14 is mediated by nucleobindin-1 (NUCB1) in MDA-MB-231 breast cancer cells (Pacheco-Fernandez et al., 2020). After maturation, MMPs can be transported within vesicles along microtubules via motor proteins such as kinesins and dyneins (Hey et al., 2022).

Exocytosis of MMPs often occurs at specific sites in the plasma membrane – i.e. MMPs are often found accumulated at focal adhesions or in invadopodes, such as in macrophages, where their action facilitates matrix degradation during migration and infiltration through endothelial vessels (Wiesner et al., 2013). Soluble MMPs are secreted into the extracellular space, whereas membrane-type MMPs can undergo ectodomain shedding, thereby releasing their extracellular portions (Elkington et al., 2009). Moreover, both soluble and MT-MMPs can be included as cargo in extracellular vesicles (EVs) (Shimoda & Khokha, 2017).

EVs are small membrane-bound vesicles released by almost all cells in normal and disease states. They are considered intercellular messengers transporting proteins, lipids, mRNA and miRNA. EVs play a significant role in cell-cell communication, tissue remodelling, immune response, and in pathologies such as inflammatory and neurodegenerative diseases or cancer. EVs were canonically distinguished based on dimension in exosomes (30-150 nm) and microvesicles (about 1 μm). The two types of vesicles also differ in origin: exosomes are formed through the endosomal pathway as intraluminal vesicles (ILVs) that are released externally through the fusion of multivesicular bodies (MVBs) with the plasma membrane; on the contrary, microvesicles originate directly from the cell surface through membrane budding. Another category of EVs are the apoptotic bodies (1-5 μm), generated during the programme death process (Doyle & Wang, 2019; Van Niel et al., 2018). Being included as cargo in EVs, MMPs may act as long-range mediators in modulating the ECM microenvironment, facilitating physiological processes such as angiogenesis, tissue repair and embryonic development, but also contributing to the formation of pre-metastatic niches or promoting lymphocyte infiltration during inflammatory responses (Thuault et al., 2022). Some studies demonstrated that MMPs, including MMP-2 and MMP-9, but also MMP-14, can be incorporated into EVs, modulating the ECM microenvironment over distance (Shimoda & Khokha, 2017). However, the MMP-cargo function in EVs as a long-distance mediator requires further investigation. This is relevant, considering that MMPs have not only functions in ECM remodelling, but they also modulate a number of other functional proteins in the cell. Thus, their presence within EVs could be significant in both physiological and pathological conditions, offering new insights into the understanding of biological mechanisms and potentially paving the way for application or prognostic developments in the field of personalised medicine.

1.4.4 Activation Mechanisms

MMPs' activity is tightly controlled by a well-regulated process. The main mechanisms of MMP activation are outlined here, ranging from the classical activation mechanism (based on cysteine switch) to the stepwise model of proteolytic cleavage, focusing then on individual activation processes, such as cell surface-mediated activation of proMMP-2 by MT1-MMP. Both biochemical and cellular control pathways participate in the complex regulation of MMP activation, reflecting their importance in physiological and pathological processes.

According to the classical model, the activation of MMPs essentially occurs with the release of

the catalytic site. The thiol group (SH) in the PRCGXPD motif interact with the zinc ion of the catalytic domain. The removal of this interaction makes the protein active, which can then bind its specific substrate. In 1990. Van Wart and Birkedal-Hansen defined this mechanism as the "cysteine switch", a theory that is still widely recognised today (Van Wart & Birkedal-Hansen, 1990). Removal of the SH-Zn^{2+} bond can occur through cleavage of the pro-domain by another protease, as mentioned above. An important aspect of this model is that proteolytic removal of the pro-domain is not essential for the enzyme to be activated. Chemical modifications or allosteric agents that change the protein conformation can also activate the catalytic site of MMPs. For example, MMPs can be activated by aminophenylmercury acetate (APMA), mercury chloride ($HgCl_2$), and N-ethylmaleimide (NEM) which interfere with the thiol group (SH) of the cysteine motif, releasing the catalytic site. Glutathione, on the other hand, induces oxidative stress and the production of reactive oxygen species (ROS) that also destabilise the cysteine switch. Moreover, chaotropic agents and sodium dodecyl sulphate (SDS) alter the structure of the protein, facilitating the removal of the pro-peptide domain from the catalytic site. This concept underlies the zymography method (described in the "Materials and Methods" section below), in which inactive forms can also be visualised because activated by SDS. Acidic pH and high temperatures can also induce enzyme activation (Visse & Nagase, 2003). Finally, interactions with non-substrate macromolecules or PTMs can also convert latent forms into active ones. However, *in vivo*, pro-domain detachment is the last essential step in MMP activation. (Ra & Parks, 2007).

According to the "stepwise activation mechanism", previously described by Nagase et al. (1990), the activation of MMPs results from a multistep process, rather than a simple punctual release event of the active site. As the term "stepwise" implies, this model predicts that activation occurs through a series of successive proteolytic steps. The removal of the pro-peptide is the first important step in the activation of MMPs. The presence of the "bait" region in the pro-peptide allows tissue and plasma proteinases to trigger MMP activity. The proteolytic cut of the pro-peptide can be carried out by specific proteinases, including other MMPs (He et al., 1989; Kollet et al., 2024). In general, the cleavage at the "bait region" induces the destabilisation of the pro-peptide, leading to a conformational change that is transmitted to the catalytic site (Laronha & Caldeira, 2020; Maskos, 2005; Nagase et al., 2006). The "stepwise activation" theory is well fitted to the explanation of MMP activation *in vivo*, where extracellular or pericellular space is tightly controlled and dynamic, and where protein activation usually originates from interaction between multiple factors and signals.

1.4.4.1 Cell Surface Activation of proMMP-2

The hemopexin domain is essential for MMP-2 functionality, since it plays an important role in proMMP-2 activation mediated by MT1-MMP (or MMP-14) via the intermediary binding of proMMP-2 and TIMP-2, in a quaternary tetrameric complex (Tam et al., 2004) (Figure 7). To activate proMMP-2, MMP-14 must first dimerise at the cell surface through interactions involving its hemopexin (Hpx) and transmembrane domains. After dimerisation, MMP-14 binds the *N*-terminal region of TIMP-2, which in turn is bound to proMMP-2 via its non-inhibitory *C*-terminal domain. ProMMP-2 /TIMP-2 complex does not exert an inhibitory effect but is crucial for the proteolytic activation of the inactive MMP-2.

The dimeric MMP-14 recognises the *N*-terminal region of TIMP-2, aligning the pro-peptide region of proMMP-2 with the catalytic site of the second, unbound MMP-14 molecule, thereby facilitating its activation, which in turn orients the pro-peptide portion of proMMP-2 towards the free dimeric MMP-14's proteolytic site. This precise arrangement allows the pro-peptide of proMMP-2 to be cleaved, thereby converting it into its active form. Once activated, MMP-2 is released into the extracellular space (Gifford & Itoh, 2019).

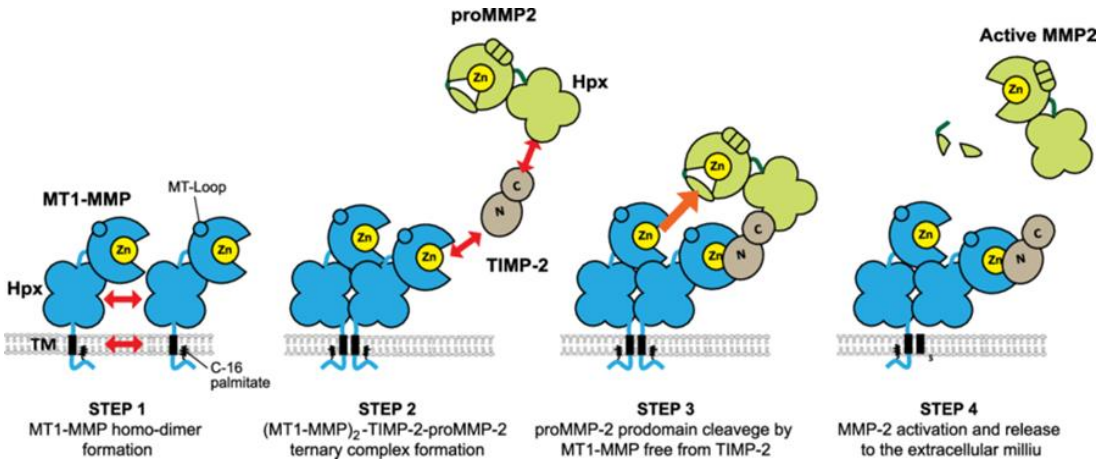

Figure 7 **Current model of cell surface activation of proMMP-2 by MT1-MMP.** Schematic representation of MT1-MMP (MMP14) forming a homo-dimeric complex through hemoplexin-like and transmembran (TM) domain (step 1); one MMP14 binds the N-terminus of TIMP-2, bound to proMMP-2 at the non-inhibitory domain (step 2); TIMP-2 facilitates proMMP-2 orientation to allow its pro-peptide cleavage by MT1-MMP free from TIMP-2 (step 3); after pro-peptide cleavage, MMP-2 active form is reliesed in the extracellular space (step 4) (Gifford & Itoh, 2019).

1.4.5 ProMMP-9 dimerization

Gelatinase B (MMP-9) exists in both monomeric and dimeric forms; recent *in silico* studies suggest a trimeric oligomerisation of MMP-9 (Charzewski et al., 2021). MMP-9 dimerisation is possible due to the presence of the hemopexin-like domain at the *C*-terminus of the protein. According to Cha et al. (2002), MMP-9 dimerises asymmetrically through interactions at the blade IV level of the Hpx domain, forming hydrophobic interactions instead of covalent ones. Hpx domain influences biochemical and functional properties of MMPs. Indeed, it can regulate enzyme activity, modulating the accessibility of the catalytic domain. Moreover, Hpx domain is important for substrate recognition, determining how MMP-9 interacts with collagen and other specific substrates (Vandooren et al., 2013). Finally, dimerisation could favour the interaction of MMPs with the cell surface or with specific receptors, influencing signalling pathways and cell communication. Other MMPs, including the transmembrane protein MMP-14, can dimerise. MT1-MMP dimerisation is a key step in proMMP-2 activation on the cell surface (see above).

1.4.6 Tissue Inhibitors of Metalloproteinases

Tissue inhibitors of metalloproteinases (TIMPs) are endogenous proteins that regulate MMPs by inhibiting their proteolytic activity. TIMPs are involved in maintaining ECM homeostasis, preventing excessive ECM degradation. There are four TIMPs (TIMP-1, TIMP-2, TIMP-3, and TIMP-4), each with distinct regulatory functions and tissue distribution. Beyond MMP inhibition, TIMPs influence cell proliferation, apoptosis, and angiogenesis, making them key modulators in physiological and pathological processes, including tissue remodelling, cancer, and fibrosis (Cabral-Pacheco et al., 2020).

In the human genome, there are four paralogous genes encoding TIMPs (TIMP-1 to -4). The four human TIMP sequences show a high degree of identity (40-50%). to the structure and function of the TIMPs, all of which are capable of inhibiting MMPs, although with different affinities. TIMP proteins have a size of approximately 21-28 kDa and are primarily composed of two domains: an *N*-terminal one with about 125 amino acids and a domain of 65 residues at the *C*-terminus (Okada, 2017).

The full-length structure of the protein has a distinctive conformation, termed “wedge-shaped” due to the shape of the *N*-terminal region, responsible for inhibiting metalloproteases. Indeed,

this domain is highly conserved in all TIMPs and is characterised by the presence of cysteine residues, whose thiol groups form disulfide bridges, essential for both protein stability and inhibitory function. Going into detail, the *N*-terminus contains five amino acid residues, including a highly conserved cysteine, forming a sort of edge. This edge fits into the active site of the metalloproteinase, placing the conserved cysteine directly in contact with the catalytic Zn^{2+} of the MMP. This interaction prevents the access of water molecules, necessary for the hydrolysis reaction, thus blocking the enzyme activity. Among the other residues, one (usually threonine or serine) binds to the S1' pocket, which determines the substrate specificity of MMP; the other four residues interact with different regions of the catalytic site. This combination of precise structural interactions allows TIMPs to inhibit metalloproteases (Brew & Nagase, 2010; Maskos, 2005; Okada, 2017).

Several studies have shown that TIMPs can also interact with zymogenic forms of MMPs, but this only occurs in the case of gelatinases (proMMP-2 and -9). This interaction involves the *C*-terminal portion of TIMPs and the hemopexin-like domain of pro-gelatinases. Thus, TIMP-2, -3 and -4 can interact with proMMP-2, while TIMP-1 and -3 interact with proMMP-9 (Ogata et al., 1995; Ward et al., 1994). About the proMMP-2/TIMP-2 complex, the interaction occurs between the *C*-terminal end portion of TIMP-2 and the β-propeller structure of the Hpx domain of proMMP-2. This specific interaction, however, has no inhibitory action, but is the one that will then allow proMMP-2 to interact in turn with MMP-14 on the cell surface, according to the current accepted activation model described previously (Gifford & Itoh, 2019). TIMP-4 can also interact with proMMP-2 and MMP-14, forming a ternary complex; in this case, however, the effect of TIMP-4 is inhibitory on the activation of proMMP-2 by MMP-14 (Bigg et al., 2001). Similarly, TIMP-3 binds proMMP-2 to form an inactive complex, also exhibiting an inhibitory effect (English et al., 2006). Interestingly, the same interaction can have opposite effects on gelatinase regulation depending on the type of TIMP involved. These mechanisms highlight how the control of MMPs, particularly gelatinases, is very fine and necessary for precise control of their enzymatic activity.

Inhibition of MMP-9 is mainly regulated by TIMP-1, which interacts with the enzyme at several levels. One of the main mechanisms of regulation is binding to the hemopexin-like domain of MMP-9 through the *C*-terminus, inactivating both monomeric and oligomeric forms of MMP-9.

A further level of inhibition occurs via the catalytic domain: the *N*-terminus of TIMP-1 can

interact directly with this region, blocking the proteolytic activity of MMP-9 (Vandooren et al., 2013). In addition to enzyme inhibition, TIMP-1 has also been shown to influence cell migration. Roderfeld et al. (2007) analysed the expression and localisation of MMP-9 and TIMP-1 in HT-1080 cells after stimulation with phorbol 12-myristate 13-acetate (PMA), used as an inducer of MMPs expression. Immunofluorescence experiments revealed that cells co-expressing MMP-9 and TIMP-1 showed a distinct distribution of the two proteins in well-defined regions near the nucleus, suggesting a functional interaction that could influence cell migration. This suggests that TIMP-1 also participate in modulating cell behaviour. However, TIMP-1 activity is not always stable: certain proteases, such as neutrophil elastase (HNE), can degrade it when complexed with MMP-9, thus removing its inhibitory effect and reactivating gelatinase B (Jackson et al., 2010). Finally, the secretion of TIMP-1 and MMP-9 occurs through distinct mechanisms depending on the cellular context. In some cases, they are released as a pre-assembled complex, as in neutrophil granulocytes; to prevent premature activation of fibroblasts, TIMP-1 and MMP-9 are stored separately in distinct compartments, with proMMP-9 located in the Golgi and TIMP-1 in the endoplasmic reticulum. This allows an adaptation of the extracellular matrix, regulating processes such as inflammation and tumour metastasis (Roderfeld et al., 2007).

2 Reproduction Physiology: Focus on the Ovarian Follicle

Human reproduction is a tightly regulated physiological process that requires the coordination of endocrine signals, tissue remodelling and gamete competence. In females, reproductive success depends on the timely development and release of a mature oocyte, the creation of a receptive endometrium and the correct implantation of the embryo. These events are mainly orchestrated by the ovaries, which function as endocrine and gametogenic organs. Among the molecular factors involved in reproductive physiology, MMPs participate in the remodelling of the ECM. Their activity is essential during follicular development, ovulation and preparation of the endometrium for implantation, enabling structural changes in ovarian and uterine tissues. Dysregulation of MMP expression or activation can impair follicle maturation, oocyte release or embryo implantation, highlighting their importance in reproductive homeostasis.

2.1 Follicle Microenvironment

The ovary is the central organ of female reproductive function, since it is involved in both oocyte maturation and hormone production (mainly oestrogen and progesterone), necessary for fertilisation and embryonic development. Within the cortical zone, folliculogenesis is the process that leads to the maturation of primordial follicles. Folliculogenesis begins with the primordial follicle, containing an immature oocyte surrounded by flattened granulosa cells. Through the primary, secondary and antral stages, the follicle grows, is enriched with theca cells and develops the follicular antrum. The Graaf's follicle (mature follicle) releases the oocyte during ovulation. The remaining follicle transforms into corpus luteum, which produces progesterone, and in the absence of fertilisation, degenerates into corpus albicans (Rimon-Dahari et al., 2016) (Figure 8).

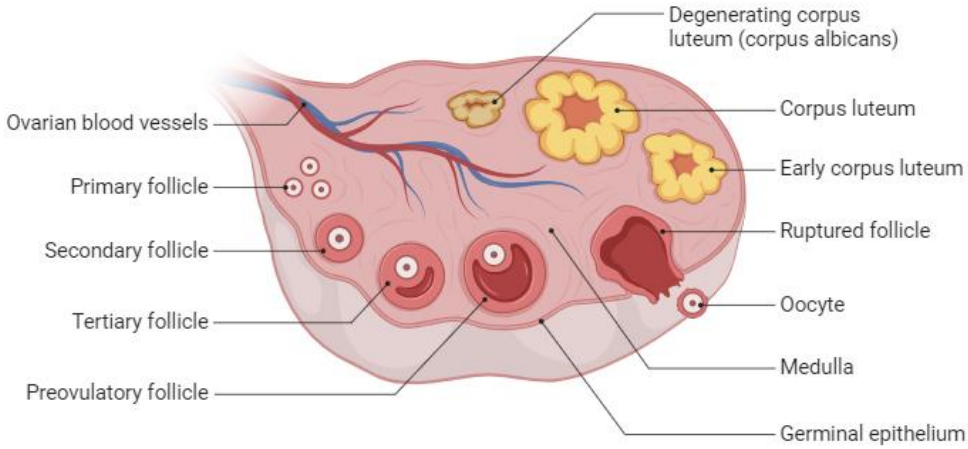

Figure 8 **Schematic representation of ovarian anatomy and main stages of folliculogenesis.** The figure shows the progression from the primary stage, with small follicles containing immature oocytes, to the secondary and tertiary stages. After maturation, the Graaf's follicle releases the oocyte during ovulation, as indicated by the ruptured follicle. After ovulation, the ruptured follicle transforms into the corpus luteum. In the absence of fertilisation, the corpus luteum degenerates into an albicans body. Created in https://BioRender.com.

The ovarian follicle is the functional unit of the ovary. It belongs to the cortical region of the ovary, composed of stroma and parenchyma. The stroma provides structural support through a network of collagen and reticular fibres, while the parenchyma represents the active cellular component. Here are found the oocyte and the specialised cells of the granulosa and theca, which together constitute the follicular structure. These cells are implicated in both oocyte development and the synthesis of female sex hormones, such as oestrogen and progesterone (Findlay, 1991).

In the ovarian follicle, the granulosa cells constitute the inner wall of the follicle, the theca cells form the outer boundary of the follicle, while the cumulus cells surround the oocyte, forming a cumulus-oocyte complex (COC). The follicle is enveloped by a basal lamina, an extracellular matrix that separates the granulosa cells from theca cells (Hennet & Combelles, 2012) (Figure 9).

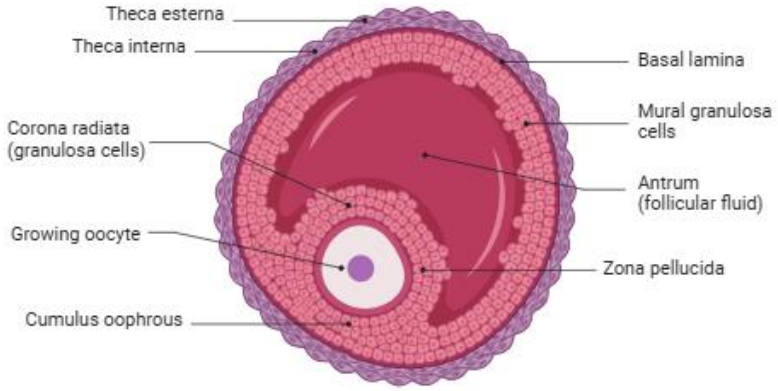

Figure 9 **Schematic section of an antral follicle.** The main structural components: growing oocyte, zona pellucida, oophorous cumulus, corona radiata, cells of the mural granulosa, follicular antrum, basal lamina and layers of the inner and outer theca. Created in https://BioRender.com.

Crosstalk between follicle cells is essential in the follicular microenvironment. Cell communication occurs via direct intercellular junctions, but also via paracrine and autocrine signals, mediated by hormones, cytokines and metabolites. This communication is highly regulated and significantly mediated by follicular fluid (FF). The FF reflects the functional state of the follicle and contributes to the regulation of follicular growth, oocyte maturation and hormone production.

2.1.1 The Follicular Fluid

The follicular fluid is contained within the follicle and is essential for optimal oocyte development and maturation. FF is mainly produced by theca and granulosa cells, but also includes exudates of blood serum from capillary diffusion. Its formation begins during the pre-antral phase, when secretions from the granulosa, theca cells and oocyte create an osmotic gradient exerted by high molecular weight molecules that induce the diffusion of plasma components through theca capillaries, with the accumulation of fluid in the follicle. The passage occurs mainly through aquaporins in the granulosa cells and requires remodelling of the cell junctions of the follicular stromal cells (Pan et al., 2024; Rodgers & Irving-Rodgers, 2010).

2.1.1.1 FF Composition and Functions

The composition of FF consists mainly of bioactive molecules (metabolites, lipids, amino acids, and steroid hormones) and a vast protein complexity (Pan et al., 2024). Changing levels of FF components influence oocyte quality and competence, endometrial receptivity, fertilisation, and perhaps even embryo development (Albeitawi et al., 2025; Kirillova et al., 2021; Revelli et al., 2009).

Since FF is partly composed of serum exudate, its composition partly reflects that of serum. However, the molecules capable of crossing the blood-follicular barrier are mainly negatively charged electrolytes and low molecular weight proteins. Among the highest molecular weight proteins are those released by the follicle cells, including matrix components such as hyaluronic acid and proteoglycans (Pan et al., 2024; Rodgers & Irving-Rodgers, 2010).

The protein composition of FF has been highly investigated over the years. Several proteins involved in ECM remodelling, immune response, angiogenesis, coagulation and oxidative stress have been highlighted in various proteomics studies. Ambekar et al. (2013) used mass spectrometry to analyse the FF of six regularly menstruating healthy women undergoing IVF and identified n=480 proteins, most of them were lytic enzymes (transferases, hydrolases, metalloproteases and serine proteases) and others involved in ECM remodelling (e.g. FN1, PTX3, TNFAIP6, VCAN), but also in oxidative stress, angiogenesis, coagulation and inflammatory response. Mass spectrometry analysis was also performed in FFs of n=10 patients undergoing successful ART outcome: high expression of proteins such as IGFBP1, transferrin, SERPIN1, and antithrombin III was found (Shen et al., 2017). Furthermore, the composition of follicular fluid changes concerning the phases of folliculogenesis, particularly during ovulation. One study investigated how the composition of follicular fluid changed in n=25 patients undergoing ART treatment after 32 and 36 hours from ovulation induction. A total of n=400 proteins were identified, with significant changes observed in the levels of 40 proteins across ovulation, including TIMP-1 and SERPINE1 (Poulsen et al., 2019).

Among the hormones released in the FF, FSH stimulates follicular growth, luteinizing hormone (LH) induces ovulation, oestradiol influences the selection of the dominant follicle, while progesterone induces luteinisation and ovulation. Many growth factors, such as IGF-2 (insulin-like growth factors 2) that stimulates cell proliferation and the angiogenic promoter VEGF (vascular endothelial growth factor), also influence oocyte maturation and embryo quality, in a synergic way together to the hormone signalling (Pan et al., 2024; Revelli et al., 2009).

Basically, the FF represents the environment in which communication between the cells of the follicle takes place, acting as a medium of exchange for extracellular signals, soluble factors and bioactive molecules, and constitutes an important vehicle for extracellular vesicles, which contribute to the paracrine and autocrine regulation of oocyte growth and maturation processes.

2.1.1.2 Intercellular Communication in the Ovarian Follicle

The intercellular communication within the ovarian follicle is essential for healthy follicle and oocyte growth and development. In particular, the bidirectional communication between oocyte and granulosa cells is fundamental and occurs through direct contact (i.e. via gap-junction) and in a paracrine, autocrine or endocrine manner. Many factors in a complex interaction network finely regulate primary follicle growth. Ovarian reserve is maintained by high expression of the PTEN protein that negatively regulates the PI3K/PDK1/Akt pathway. At puberty, primordial follicles emerge from a long dormant state, maintained by the transcription factor FOXO3. FOXO3 inactivation occurs via the PI3K/PDK1/Akt signalling cascade, activated by growth factors such as Kit ligand, which promotes follicle activation. The serine/threonine protein kinase (PKB/Akt) takes part in the pathways of direct follicle activation: indeed, Akt is activated through phosphorylation by PDK1 kinase, which, in turn, is activated by the PI3K/PIP2/PIP3 phosphorylation cascade triggered by different growth factors (such as IGF, KIT ligand, EGF) (Chen et al., 2020). Akt phosphorylates and inhibits FOXO3 transcriptional factor, activating latent primordial follicles. In parallel, Akt regulates the mTOR pathway, promoting oocyte growth and granulosa cell differentiation (Guo & Yu, 2019; Rehnitz et al., 2017). Follicular growth is also enabled by the activation of YAP/TAZ signalling and the inactivation of the Hippo pathway, an inhibitor of cell proliferation (Hsueh & Kawamura, 2020). In this context, the cross talk between oocyte and granulosa is essential for proliferation and differentiation of the latter: the oocyte in fact stimulates proliferation via TGF-β family markers (i.e. GDF-9 and BMP-15), but also activation of the Wnt/β-catenin pathway (H.-X. Wang et al., 2013). Furthermore, hormonal regulation is essential during folliculogenesis. In the antral phase, follicle-stimulating hormone (FSH) stimulates the cAMP/PKA pathway in the granulosa cells, leading to the production of oestrogen. Ovulation, on the other hand, is reached at the peak of LH, which activates MAPK pathways, promoting follicle rupture and release of the mature oocyte (Fan et al., 2009; Oduwole et al., 2021).

2.1.1.3 Extracellular Vesicles in FF

Extracellular vesicles mediate cell communication within the ovarian follicle and influence various physiological processes, such as follicular growth, oocyte maturation, fertilisation and embryonic development (Machtinger et al., 2015). Indeed, in the follicular microenvironment, EVs add a further level of intercommunication between cells, especially between oocyte and granulosa cells (Machtinger et al., 2015). The presence of EVs within human follicular fluid has been amply demonstrated, and recent research is focused on their characterisation (Neyroud et al., 2022; Shomali et al., 2020; Soares et al., 2023). However, current knowledge about the content of human FF-derived EVs and their function is yet to be expanded. The isolation of EVs from follicular fluid is challenging. Currently, several methods are employed for the isolation of EVs, but ultracentrifugation (UC) is the most adopted technique. This approach is based on the application of high centrifugal forces for prolonged times to sediment the EVs into a pellet. For small-volume samples, commercial kits for isolating EVs or chromatography columns are frequently used (Neyroud et al., 2022; Soares et al., 2023). Among the markers canonically used to identify EVs are the tetraspanins CD9, CD63 and/or CD81. Among these, CD9 is present on human granulosa cells, oocytes, as well as uterine cells (Jaslow et al., 2010).

Exosomes and microvesicles make significant contributions in carrying proteins and nucleic acids (e.g. microRNA) within the follicle. In a study, EVs from the follicular fluid of n=20 patients undergoing ART, subdivided by age (<35 and ≥35 years), were analysed and characterised. Younger women presented EVs with significantly higher levels of progesterone, lipids and proteins such as HSP70 and annexin A2, and proteins involved in oocyte maturation and sperm activation, such as clusterin and izumo1 (Sysoeva et al., 2024). Another proteomics study, on the other hand, revealed an increase in inflammatory response proteins (complement components, immunoglobulins and proteins associated with B-cell activation) in the group of older women undergoing ART, also highlighting the different expression of proteins associated with folliculogenesis and the PI3K/AKT signalling pathway (Z. Liu et al., 2025). These results indicate that follicular EVs reflect molecular changes related to ovarian age, as well as represent potential markers of follicular quality in ART.

Although interest in the contents of human FF-derived EVs is steadily growing, most of the studies conducted so far are limited to their characterisation, while the functional role that these vesicles may play in the process of folliculogenesis remains poorly understood. Considering that the extracellular matrix is essential in follicle development, an unexplored aspect concerns the possible interaction between EVs and the ECM, particularly through MMPs. MMPs have

been identified as potential cargoes of EVs (Shimoda & Khokha, 2017), but their functional role as long-range mediators in EVs has not yet been properly investigated. Since the presence of MMPs in follicular EVs could directly influence oocyte quality and, consequently, the success of treatments, follicular EV-MMPs could be particularly relevant in the context of ART. Delving into this field could therefore open new perspectives both in understanding the physiological mechanisms underlying human reproduction and in identifying new prognostic biomarkers.

Several recent studies have focused on the miRNAome analysis of FF-derived EVs. Zhao et al. (2022) analysed the expression of miR-143-3p in FF-derived exosomes of patients with polycystic ovary syndrome (PCOS), showing that this miRNA promotes apoptosis in granulosa cells via inhibition of the BMPR1A gene and the Smad pathway, impairing follicular development. The expression of FF-derived exosomal miR-4449 is also significantly downregulated in patients with PCOS (M. Wang et al., 2023). Furthermore, FF-EVs from patients with PCOS or reduced ovarian reserve show morphological alterations and significant deregulation of miRNAs (e.g. miR-29a, miR-200c and miR-483) that are key for granulosa cell function (Ducarre et al., 2025). Finally, according to another study, the combination of miR-16-2-3p, miR-378a-3p and miR-483-5p has a high predictive ability for a positive pregnancy outcome (Muraoka et al., 2024). Indeed, considering the great potential of EVs in cell-cell interaction even within the ovarian follicle microenvironment, further study of them could be a stimulating source for new prognostic but especially therapeutic strategies applicable in ART.

2.2 MMPs in Follicle Development

Primordial follicles are located in the ovarian cortex. The cortical stroma of the ovary mainly consists of ECM components (types I, III, IV collagens, laminins, fibronectin and proteoglycans), which provide structural and mechanical support. Changes in ECM composition occur during all phases of folliculogenesis, indicating its important role in follicle development. Within the follicle, the basal lamina consists of extracellular matrix and separates the granulosa cells from theca cells, contributing to cell proliferation through intra- and extracellular signalling, as well as providing mechanical and structural support. The basal lamina acts as a selectively permeable barrier (blood follicle barrier, BFB), regulating the transit of molecules and contributing to oocyte development and maturation (Hennet & Combelles, 2012). Furthermore, the permeability of the basal lamina increases near the ovulatory phase, allowing the entry of serum proteins into the follicular fluid, which are necessary for the

expansion of the cumulus-oocyte complex. Indeed, during ovulation, the COC is also surrounded by extracellular matrix that is essential for ovulation and oocyte maturation. In addition to the serum component, the ECM in the COC is enriched by the secretions of cumulus and granulosa cells. Moreover, the COC matrix is crucial for fertilisation because it is the first point of contact between the sperm and oocyte gametes (Hennet & Combelles, 2012). Communication between the matrix component and the oocyte is of considerable importance in the follicular microenvironment.

ECM remodelling is involved in follicle growth, ovulation, angiogenesis, and corpus luteum formation, but also in embryo implantation: dysregulation of MMP levels could be a relevant predictor of oocyte fertilisation, and gelatinases play a critical role in this balance. In their bioinformatics analysis using DAVID and MetaCore tools on n=617 proteins identified in the FF, Bianchi et al. (2016) highlighted MMP-2 and MMP-9 as the main central nodes in the protein network, establishing 95 and 84 interactions with other molecules, respectively, accounting for more than 40% of the proteins in human follicular fluid. Their data suggested that gelatinases exert a regulatory role not only on ECM reorganisation but also on several FF factors. Over the years, several studies have confirmed MMPs as the most represented components, analysing FF from patients undergoing ART treatment. MMP-9 levels were found to be higher and positively correlated with ovarian quality and fertilisation rate in women undergoing ovarian stimulation, analysing the FF of a cohort of 60 women with idiopathic infertility (Bilen et al., 2014). MMP-2 was strongly correlated with the rate of oocyte maturation and fertilisation, analysing n=150 women undergoing ART treatment cycles (Yang et al., 2015). Other studies have focused on FF gelatinase activity in relation to embryo quality as well as fertilisation rate, confirming that MMP-9 may indicate a favourable follicular environment for oocyte maturation and fertilisation (Atabakhsh et al., 2018). In conclusion, therefore, the literature shows that gelatinases in follicular fluid could be considered as possible predictors of fertilisation success or oocyte quality. This is why further studies are needed to consider them as possible predictors of fertilisation success potential in ART.

Inflammation could be a concomitant cause of infertility (Weiss et al., 2009). An interesting aspect to explore concerns the possible correlation between inflammation and infertility. For this purpose, C-reactive protein (CRP) has been chosen as a biomarker of systemic inflammation during IVF treatment. CRP is an acute-phase opsonin and is produced by the liver; previous studies have shown increased CRP levels in follicular fluid in women after controlled ovarian hyperstimulation (COH) treatment and especially during the pick-up phase

(Gonzalez et al., 2018; Orvieto, 2004; Wunder et al., 2005).

2.3 FF and Endometrial Receptivity

During the menstrual cycle, the endometrial receptivity refers to the period in which the endometrium acquires the molecular and structural characteristics necessary to allow implantation of the embryo. This phase, known as the implantation window, typically occurs between days 19 and 21 of the menstrual cycle and is tightly regulated by steroid hormones, particularly oestrogen and progesterone (Figure 10).

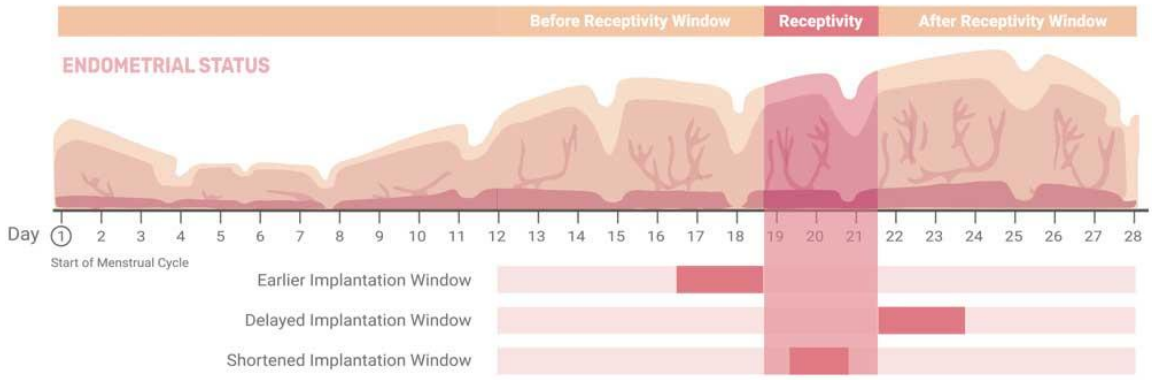

Figure 10 **Implantation window during the menstrual cycle.** The figure illustrates the endometrial status during the menstrual cycle and represents the implantation window, as the period when the endometrium is physiologically receptive to embryonic implantation (days 19-21).

During the receptivity period, the endometrial epithelium undergoes profound changes to support adhesion, invasion and interaction of the blastocyst with the stromal and vascular compartments (Lessey & Young, 2019). This receptive state is defined by coordinated gene expression, hormone receptor modulation, immune tolerance and ECM remodelling. Aberrations in this process, such as progesterone resistance or chronic inflammation, can lead to impaired implantation, infertility or pregnancy loss (Lessey & Young, 2019; Neykova et al., 2022). Ageing belongs to factors that may affect endometrial receptivity. Indeed, it has been shown that endometrial ageing, through molecular, cellular, inflammatory and epigenetic alterations, impairs endometrial receptivity and may contribute significantly to female infertility in later life (Pathare et al., 2023).

To allow embryo invasion, the endometrium transforms into a receptive tissue. This process requires the loss of cell polarity of endometrial epithelial cells, which normally present an

asymmetrical structure functional to maintain a repulsive apical surface. This leads to a reduction in the expression of adhesion junction proteins, such as E-cadherin, resulting in a weakening of lateral connections between cells of the basement membrane. These changes make the endometrium temporarily permissive to embryonic adhesion and invasion, creating a favourable microenvironment for implantation. Taken together, these processes contribute to plasma membrane remodelling (PMT), a phenomenon that shows similarities to the epithelial-mesenchymal transition (EMT) described in the invasive mechanisms of cancer cells during metastasis (Amack, 2021; Whitby et al., 2020) (Figure 11).

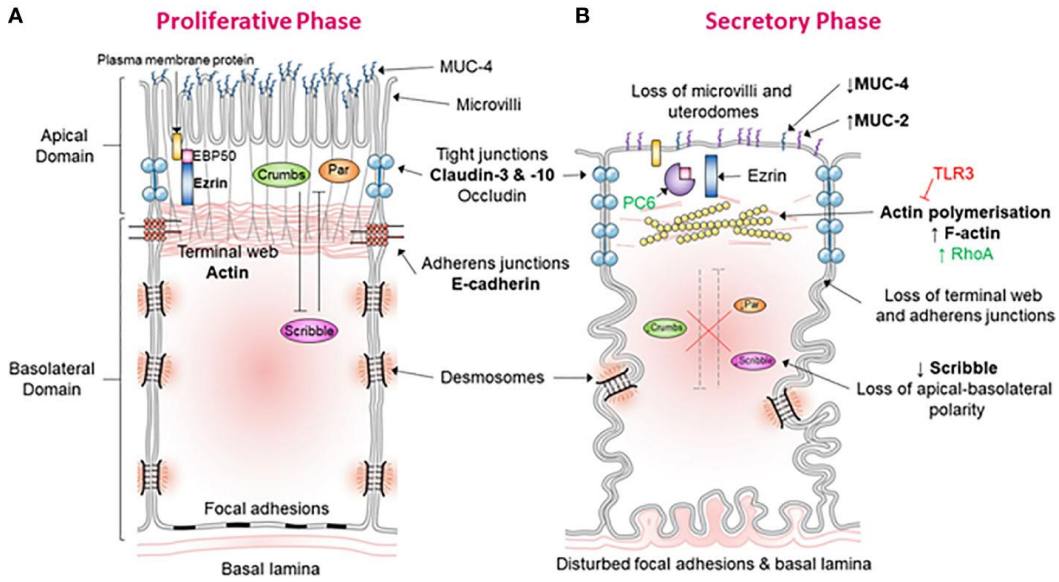

Figure 11 **Endometrial epithelium remodelling during PMT.** Schematic representation of the main morphological and molecular changes that characterise plasma membrane remodelling in the human endometrium. (a) Endometrial epithelium in the proliferative phase, with polar cells, adherent junctions and compact structure. (b) Endometrial epithelium in the secretory phase, in which loss of polarity, reduction of intercellular junctions and increased permissiveness to embryonic adhesion are observed (Whitby et al., 2020).

The importance of follicular fluid and its content not only affects the follicular environment, maturation and oocyte quality, but has also been shown to influence embryo implantation. Its components (hormones, growth factors, nucleic acids and EVs) can mediate changes in the endometrial microenvironment, enhancing endometrial receptivity and promoting embryo implantation. During ovulation, follicular fluid is released with the oocyte into the peritoneal cavity and reaches the fallopian tubes with it. A study shows that pre-ovulatory follicular fluid has an effect on CBF of Fallopian tube epithelial cells, increasing the frequency of ciliary beats, in n=13 women undergoing ART (Lyons et al., 2006). Although there is no evidence yet, some studies support the hypothesis that, after ovulation, part of the FF may reach the fallopian tube

and then the uterus and play a physiological role in the implantation of the embryo in the endometrium. Some pilot studies have evaluated the effect of autologous intrauterine injection of FF in female volunteers undergoing ART could have a beneficial effect on implantation rates and clinical pregnancy (Hamdi et al., 2018; Hashish et al., 2014).

3 Neurological Diseases: Focus on Multiple Sclerosis

MMPs are involved in several physiological processes in the central nervous system (CNS) such as neuronal development, cell migration, synaptic plasticity, and learning (Brzdak et al., 2017). However, their aberrant expression has been associated with several pathological conditions, such as multiple sclerosis (MS) (Behl et al., 2021; Lopez-Navarro & Gutierrez, 2022). In MS, MMPs, including gelatinases MMP-2 and MMP-9, are mainly implicated in blood-brain barrier (BBB) impairment, as they favour the infiltration of immunocompetent immune cells into the brain parenchyma (Rempe et al., 2016). Furthermore, MMPs contribute directly to demyelination and neuroinflammation. This session explores the involvement of MMPs in MS pathogenesis, analysing the potential of MMPs as a prognostic biomarker of the neurodegenerative progression.

3.1 Pathogenesis of Multiple Sclerosis

Multiple sclerosis is a chronic inflammatory disorder of the central nervous system. The disease is associated with blood-brain barrier injury, which allows leukocyte infiltration, demyelination, and neuronal damage. In MS, demyelination induces damage to both axonal and neuronal soma, resulting in characteristic lesions that can develop throughout the CNS, including white and grey matter, brainstem, spinal cord, and optic nerve. These lesions are typically disseminated in both space and time, reflecting the multifocal and progressive nature of the disease (Lassmann, 2018).

Although several hypotheses have been proposed, MS pathogenesis is still not fully understood. Nonetheless, the activation of the autoimmune response against myelin is currently recognised as the main pathogenetic mechanism of the disease (R. Liu et al., 2022). Tissue damage in MS results from a complex interaction between the immune system, glial cells, and neurons. Plaque formation is closely associated with a marked inflammatory response, involving immune system cells. In particular, T helper lymphocytes (CD4+) localise around the blood vessels in lesions, where they promote the extravasation of leukocytes and amplify the inflammatory process. In contrast, cytotoxic T lymphocytes (CD8+) are distributed in the brain parenchyma and can directly damage axons and oligodendrocytes. In addition to presenting antigens to T cells and producing pro-inflammatory cytokines, B lymphocytes also synthesize IgG immunoglobulins within ectopic germinal centres in the meninges (Lassmann, 2018). Since these antibodies are produced exclusively within the central nervous system, they are detectable in the cerebrospinal fluid (CSF) but not in the blood. For this reason, their detection is

considered a key diagnostic tool in multiple sclerosis (Link & Huang, 2006). During the acute phase of the disease, lesions are enhanced by the response of astrocytes and macrophages, which participate in the inflammatory process leading to the formation of dense glial plaques. Activated astrocytes produce pro-inflammatory mediators (e.g., IL-6, TNF-α, NO) that amplify the local inflammatory response, and also increase the synthesis of extracellular matrix proteins that contribute to the formation of glial plaques. Macrophages, together with activated microglia, phagocytose damaged myelin and release cytokines and proteases (including MMPs) that degrade the blood-brain barrier. Moreover, demyelination is associated with the loss and destruction of oligodendrocytes, contributing to the chronic progression of tissue damage and impairing the CNS capacity to restore myelin (Lassmann, 2018).

3.2 MS Diagnostic Criteria

Multiple sclerosis is a heterogeneous multifactorial disorder influenced by genetic and environmental factors. Among them, Epstein-Barr virus infection, gut microbiota, smoking, sex, and childhood obesity are particularly relevant (J. Howard et al., 2016; Zierfuss et al., 2024). Indeed, it is the most common neuronal disease in young adults (often between the ages of 20 and 40 years), with a higher incidence in the female population (Magyari & Sorensen, 2019). The clinical presentation of MS is highly variable, and its course can follow several patterns, which are significant for prognosis and therapeutic decisions. According to McDonald's criteria, four clinical courses of MS are distinguished: relapsing-remitting MS (RRMS), secondary progressive MS (SPMS), primary progressive MS (PPMS), and progressive relapsing MS (PRMS); instead, first clinical episodes are classified as clinically isolated syndrome (CIS). RRMS is the most common initial form of the disease, with 85% of MS cases at onset. It is assessed by episodes of acute neurological symptoms (relapses), followed by periods of recovery and neurological stability (remission). Most RRMS patients eventually transit to SPMS, which consists of a gradual and progressive worsening of the disease, with or without ongoing relapses. In 15% of cases, the disorder begins later in life (around the age of 40) with the PPMS condition, which is characterized by a steady decline in neurological function and motor disability, with no relapses. In contrast, progressive-relapsing MS is characterized by a steady neurological decline from the onset, accompanied by occasional relapses. However, PPMS is the rarest form of the disease. Finally, CIS term refers to an isolated episode of neurological symptoms lasting at least 24 hours, and is considered a possible early manifestation of multiple sclerosis (Yamout et al., 2024).

To date, diagnosis and monitoring of the disease are mainly performed by magnetic resonance imaging (MRI), which is still the gold standard in MS diagnosis (Hemond & Bakshi, 2018) and by detection of IgG oligoclonal bands (OCBs) in the CSF (Link & Huang, 2006). However, in the majority of cases of multiple sclerosis, there usually exists a mismatch between radiological and clinical findings, with patients sometimes having major symptoms based on very minimal MRI evidence, or presenting with widespread lesions in the absence of any overt clinical deficits (Gaetani et al., 2017). Therefore, new novel, reliable, and minimally invasive biomarkers still need to be found to allow better diagnosis timelines and monitor relapse and progression.

3.3 MMPs in Blood–Brain Barrier Dysfunction

The blood-brain barrier is a selectively permeable structure that regulates the exchange of molecules between blood and the central nervous system. Its main function is to protect the brain against pathogens and toxins, preventing them from crossing the barrier and reaching the CNS. The BBB consists primarily of brain capillary endothelial cells linked by tight junctions, which block the diffusion of potentially toxic molecules and a basal membrane, a specialized ECM that connects endothelial cells to pericytes and astrocytes (Zierfuss et al., 2024) (Figure 12a). Pericytes modulate microvasculature contractility, while astrocytes largely secrete basal membrane proteins and, together with their end-feet, almost completely line the cerebral vasculature. Moreover, astrocytes contribute to CNS homeostasis, also modulating BBB permeability. Indeed, the BBB is critical for maintaining brain homeostasis and an optimal environment for neuronal function. Endothelial cells regulate blood flow and ensure the supply of oxygen and nutrients to neurons, in part through their interaction with nervous system cells. In turn, metabolically active neurons stimulate astrocytes to release compounds (e.g., glutamate, ATP, prostaglandin, and nitric oxide) that induce vasodilation of the smooth muscle cells lining the vessels of the BBB. This combined process is known as neurovascular coupling (Zierfuss et al., 2024) (Figure 12b).

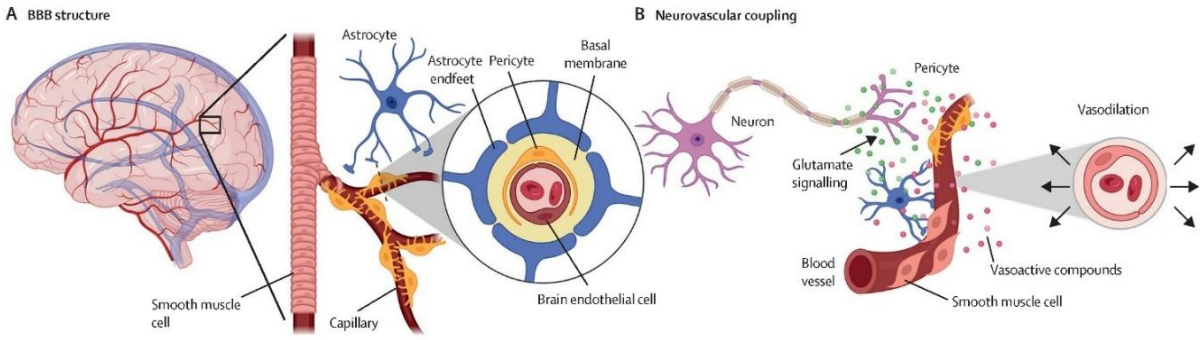

Figure 12 **Schematic representation of the blood-brain barrier and the neurovascular coupling process.** (a) Arteries penetrate the brain and branch into arterioles and capillaries. Smooth muscle cells surround arterioles, while pericytes surround capillaries. The BBB (cross-section) consists of endothelial cells of the brain capillaries connected by tight junctions, surrounded by a basal membrane and astrocytes. Astrocytes represent the cellular connection with neurons, while pericytes are embedded within the basal membrane. (b) The process of neurovascular coupling, in which the communication between neurons and BBB cells, ensures homeostasis in the brain.

In MS, neuroinflammation, demyelination, and neuroaxonal degeneration are mainly triggered by the infiltration of immunocompetent cells (CD4+ and CD8+ T cells, B cells, and myeloid cells) into the CNS parenchyma (Zierfuss et al., 2024). This process is called extravasation and consists of leucocyte migration from blood vessels into the tissue as an inflammatory response. It's tightly controlled by numerous molecules, including chemokines, cytokines, integrins, and cell adhesion molecules (CAM). Extravasation takes place mainly in post-capillary vessels, even under physiological conditions, to reach inflamed peripheral tissues. In CNS, leukocyte migration requires the opening of the blood-brain barrier due to the remodelling of the basal membrane ECM. Matrix metalloproteinases mediate the BBB breakdown and leukocyte infiltration (Gerwien et al., 2016) (Figure 13). In this context, MMPs are secreted by several immune and brain cells, such as macrophages and T cells, as well as BBB endothelial cells, astrocytes, and oligodendrocytes, respectively.

3.3.1 MMP-2 and MMP-9 as potential biomarkers in MS

MMP-2 and MMP-9 play a central role in the immunopathogenesis of MS through the disruption of the BBB and the recruitment of inflammatory cells into the CNS (Gerwien et al., 2016; Hannocks et al., 2019; Rempe et al., 2016). Indeed, MMP-2 and MMP-9 have been previously proposed as candidate biomarkers for MS progression, and higher levels of MMP-2

and MMP-9 in CSF, serum, and plasma of MS patients have also been reported.

Fainardi et al. (2009) analysed gelatinase activity in the CSF and serum of 74 MS patients by zymography and observed a significant increase in active MMP-2 in the CSF of RRMS patients in the remitting phase, indicating a potential role of MMP-2 in the reparative phase of the disease. The same group had found that levels of active MMP-9 in CSF and serum were significantly higher in MS patients than in noninflammatory controls (Fainardi et al., 2006). In another study, plasma levels of MMP-9 were measured in n=35 patients with RRMS, n=24 healthy controls, and n=26 high-risk first-degree relatives using an ELISA test. The results showed that RRMS patients had significantly higher levels of MMP-9 than healthy controls (p= 0.0203), while in high-risk family members the increase was not statistically significant (p = 0.208). Furthermore, a significant correlation between MMP-9 and cytokine levels (IL-17 and IL-23) in the serum of MS patients compared with healthy subjects was demonstrated, suggesting an interaction between MMPs and specific pro-inflammatory cytokines in disease progression (Trentini et al., 2016).

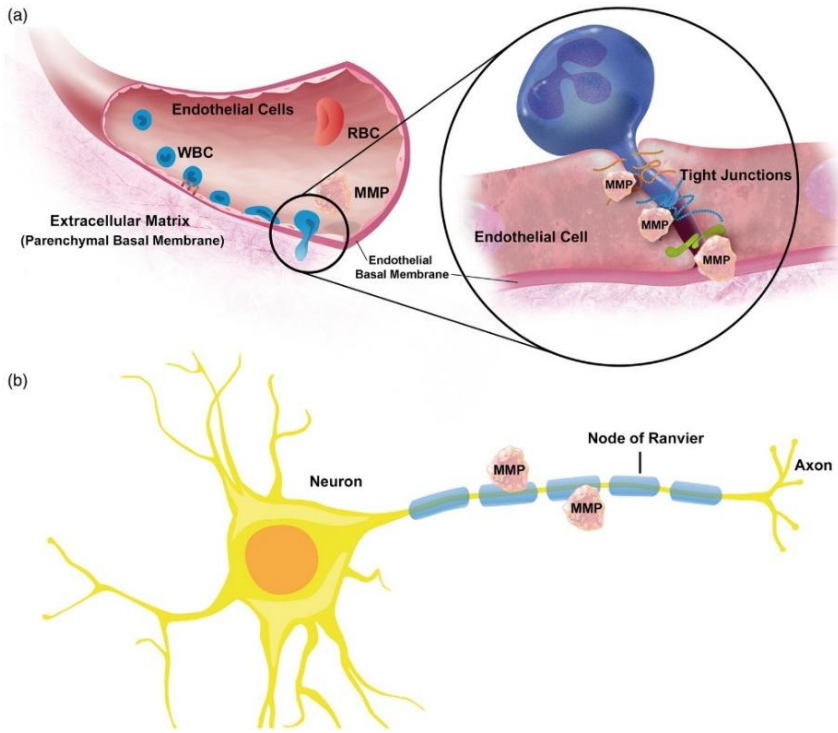

Figure 13 **MMPs in multiple sclerosis.** (a) Endothelial cells and leukocytes release MMPs, which are believed to contribute to the degradation of tight junctions and ECM basal membrane proteins of the BBB, thereby promoting extravasation of immune cells. Under pathological conditions, the activity of MMPs contributes to the impairment of BBB integrity. (b) MMPs also directly participate in the process of demyelination of neuronal axons (Rempe et al., 2016).

The production of extracellular vesicles by nervous system cells is widely recognised. Indeed, EVs may have essential roles in both physiological and pathophysiological processes. Their function has also been investigated for multiple sclerosis; it is possible to isolate EVs from CSF and MS patients' serum (Barreca et al., 2017; Geraci et al., 2018). In addition, some studies have detected MMP-2 and MMP-9 and their inhibitors as cargo in EVs produced by CNS cells (Sbai et al., 2009). EVs appear to participate in destroying the BBB during the infiltration of immune system cells (Sáenz-Cuesta et al., 2014). Accordingly, it would be interesting to investigate any possible matrix metalloproteinase enrichment within EVs in multiple sclerosis patients' CSF and plasma samples.

4 Fibrosis: Focus on Dupuytren's Disease

Dupuytren's contracture is a fibroproliferative disorder affecting the connective tissue of the hand. It consists of collagen deposition in the palmar fascia, primarily triggered by the formation and proliferation, and differentiation of myofibroblasts, forming fibrotic nodules and cords (Figure 14). This condition contributes to generating contractile forces and abundant extracellular matrix proteins such as type I and III collagen and fibronectin, causing tissue stiffening and loss of hand function. The disease can involve one or both hands and leads to progressive finger flexion contractures that significantly impair daily activities in patients with advanced disease (Layton et al., 2023; Verjee et al., 2009).

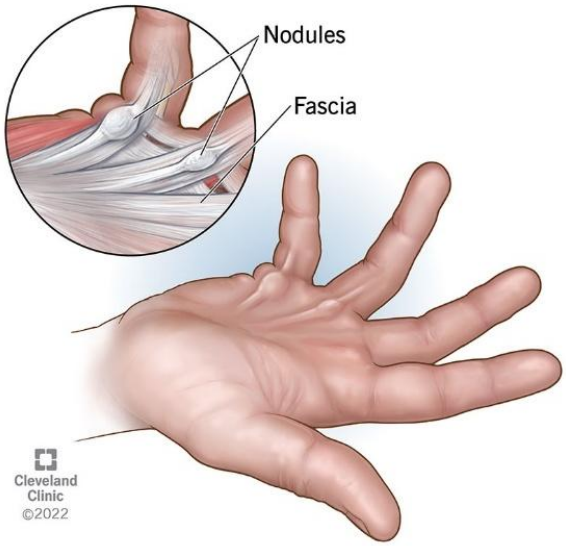

Figure 14 **Dupuytren contracture.** Dupuytren contracture is the most common localized fibrotic disease of the hand, causing fixed flexion deformity of the digits and finger contracture. It is a progressive fibroproliferative disorder that involves collagen deposition, mediated by the formation and proliferation of myofibroblasts, resulting in fibrotic nodules and cords on the palmar fascia.

4.1 Epidemiology, Aetiology and Histopathogenesis

The prevalence of Dupuytren's disease varies widely globally, ranging from 3% to 42%, depending on the population studied. It is significantly more common in northern European countries, from which it derives the historical association with the Northern European population (hence the nickname "Viking disease"). However, more recently, numerous cases have also been reported in Africa, Asia, and South America, often without a family history, suggesting an important role for environmental factors as well (Hindocha, 2018; Riesmeijer et

al., 2019a).

Aetiologic risk factors that have been postulated so far include genetic and environmental conditions. The disease shows a marked familial predisposition, based on multifactorial and polygenic mechanisms. It predominantly affects males over the age of 50, although in older women the prevalence tends to increase (Hindocha, 2018; Pineda et al., 2024). Statistical analyses have shown that men are more likely to develop the disease in both hands and with involvement of several fingers (Alencar et al., 2021). The main associated environmental factors include manual labour, chronic alcohol abuse, liver disease, smoking, and diabetes mellitus (Riesmeijer et al., 2019b). However, the exact mechanisms of the disease pathogenesis remain unclear to date.

Histologically, Dupuytren's tissue consists of nodules with high cellularity, α-SMA-positive myofibroblasts and hypocellular collagen-based cords. These nodules, visible in the early stages of the disease, result from active cell proliferation and intense production of extracellular matrix, mainly composed of collagen. The disease progresses in three distinct phases: a proliferative phase, dominated by highly cellular nodules with a predominance of α-SMA-positive myofibroblasts; an involutional phase in which nodules begin to mature into chordae; and a residual phase, with predominantly hypocellular fibrous chordae composed of densely organised collagen (Verjee et al., 2009). The study by Layton et al. (2020) revealed, through single-cell analysis, a complex heterogeneity of fibroblasts in Dupuytren's disease, identifying functionally distinct subpopulations, including highly proliferative myofibroblasts, implicated in the processes of contraction, inflammation, and matrix remodelling. Indeed, nodules are also sites of intense remodelling activity, as evidenced by the dynamic expression of metalloproteinases (Johnston et al., 2007).

Currently, the main intervention to release contracted palmar fascia and restore mobility of the hand is surgery (Karbowiak et al., 2021). The rate of recurrence, however, is high, and no pharmacological treatment has yet demonstrated efficacy in preventing disease progression or recurrence following surgery (Ruettermann et al., 2021).

4.2 *MMP14* Gene Expression and rs1042704 Variant in DD

Gene expression analysis has revealed upregulation of several genes involved in cell contraction, matrix production, and extracellular matrix remodelling in DD’s patients. Among

these genes, *MMP14* has emerged as a leading mediator in the disease process (Johnston et al., 2007). In Dupuytren's contracture, MMP-14 is mainly expressed in response to increased tissue stiffness due to collagen deposition in fibrotic lesions. This increase in stiffness results in a mechanical stimulus that triggers intracellular signalling pathways (mediated by cytokines such as TGF-β1) in fibroblasts that lead to transcriptional induction of *MMP14* (Ratajczak-Wielgomas et al., 2012; Verjee et al., 2009). Recent research in single-cell transcriptomics has confirmed that the *MMP14* gene is selectively upregulated in a subpopulation of α-SMA-positive myofibroblasts within Dupuytren's nodules (Layton et al., 2020). At a protein level, immunohistochemical studies show that MMP-14 localises predominantly on the leading edge of invading myofibroblasts, allowing focused matrix degradation and promoting contracture formation (Johnston et al., 2007).

A genome-wide association study (GWAS) identified 17 *loci* significantly associated with Dupuytren's disease risk. Among them, the rs1042704 SNP was localised in the *MMP14* gene (Ng et al., 2017). This SNP results in a non-synonymous substitution (Asp273Asn or D273N) within the catalytic domain, away from the enzymatic cleft but in a structurally critical region that influences the orientation of the domain on the plasma membrane. Functional studies have shown that this D273N variant shows markedly reduced collagenolytic activity and a negative effect when co-expressed with the wild-type enzyme (Itoh et al., 2021). Itoh et al. (2021) also show that the D273N variant of MMP-14 does not interfere with the production or localisation of the enzyme, which is nevertheless present on the cell membrane. Indeed, *in silico* studies suggest that the presence of the polymorphism leads to a conformational change in the protein such that it no longer binds properly to collagen. This hinders the normal degradation of the extracellular matrix and promotes the abnormal accumulation of collagen in tissues.

4.3 MMP-14/MMP-2 Axis and Therapeutic Implications

The functional interplay between MMP-14 and MMP-2 constitutes a central proteolytic axis in the pathogenesis of Dupuytren's disease and in the expansion of fibrotic lesions. MMP-14 activates the latent form of MMP-2 (proMMP-2) at the cell surface through a TIMP-2-dependent mechanism, thereby amplifying pericellular matrix degradation. Beyond its direct collagenolytic capacity, MMP-14 facilitates extracellular matrix remodelling and promotes fibrotic cell invasion by triggering proMMP-2 activation, reinforcing its dual role in both matrix breakdown and lesion progression (Ng et al., 2017; Suojanen et al., 2009). This dual role is crucial in shaping the pericellular matrix architecture and enabling fibrotic invasion in

Dupuytren’s lesions.

In Dupuytren's nodules, MMP-2 is upregulated at both transcriptional and protein levels, and its activation ratio is significantly higher than in the normal range, as demonstrated by zymography analysis (Ratajczak-Wielgomas et al., 2012). However, this activation is not homogeneous throughout all stages of the disease: while early lesions show high enzyme activity, advanced stages show persistence of MMP-2 accompanied by reduced collagen degradation capacity, probably due to increased TIMP-2 and altered MMP-2/TIMP-2 ratio (Ratajczak-Wielgomas et al., 2012).

From a therapeutic point of view, this axis represents both a challenge and an opportunity. The high expression of MMP-2 and its co-localisation with α-SMA-positive cells suggest that intervening upstream, at the level of MMP-14 regulation, could change the course of the disease. Preclinical studies have shown promising results in this regard. In particular, a humanised, high-affinity inhibitory antibody developed against an MMP-14 exosite able to selectively block the collagenolytic activity of the enzyme, while sparing the ability to activate proMMP-2, and thus preserving essential physiological remodelling functions (Botkjaer et al., 2016). This selective inhibition strategy is particularly relevant in the context of the rs1042704 polymorphism, which already impairs the collagenolytic activity of MMP-14 without altering its MMP-2 activation function (Itoh et al., 2021).

AIMS

The main objective of this PhD project was to systematically investigate the activity, regulation, and application potential of matrix metalloproteinases, with a focus on gelatinases (MMP-2 and MMP-9), in different physiological and pathological contexts: human reproduction, neuroinflammation, and fibrosis. For this purpose, three models were included, respectively: follicular fluids and FF-derived extracellular vesicles from women undergoing assisted reproductive techniques, biological fluids from patients diagnosed with multiple sclerosis, and primary myofibroblasts derived from Dupuytren's disease patients undergoing surgery.

Going into detail, the work trailed the following objectives for each specific context analysed.

1 In Reproduction Physiology: Follicle Microenvironment

- To analyse the gelatinolytic activity of MMP-2 and MMP-9 in follicular fluid samples and FF-EVs from women undergoing ART treatment.
- To characterise FF-derived EVs in size and content, especially to evaluate the possible mechanism of functional compartmentalisation in the targeted regulation of gelatinases.
- Assessing correlations between MMP activity, considering their compartmentalisation, with maternal age, to understand the role of gelatinases in follicular ageing.
- To assess the correlations between gelatinase activity and the inflammatory status of patients, by assaying CRP protein expression as a marker of systemic inflammation to understand the role of gelatinases in oocyte quality, again considering possible age-related factors.
- To functionally characterise FF by assessing its effect on endometrial receptivity, using *in vitro* models of receptive and non-receptive endometrium that mimicked tissue receptivity through cell migration. To test whether the effect on endometrial receptivity mirrored changes in follicular fluid as a function of ageing.
- To analyse whether and to what extent follicular fluid treatment affected the expression of the adhesion protein E-cadherin, reflecting the loss of cell polarity typical of the endometrium in the receptive phase, and to highlight any age-dependent changes in the fluid's ability to modulate endometrial plasticity.

2 In Neurological Disease: Multiple Sclerosis

- Apply high-resolution proteomic analysis (LC-MS/MS) on CSF to identify differential profiles between MS and controls and, using bioinformatics and machine learning tools, identify potential diagnostic biomarkers.
- To analyse the activity of MMP-2 and MMP-9 in CSF, serum, and plasma of MS patients and subjects with other neurological diseases, by zymography, to assess their differential expression in central nervous system and peripheral nervous system compartments.
- To correlate the expression of MMPs with the inflammatory status of patients by assessing CRP protein as a reference of inflammation, both localised in the CNS and peripheral in circulating blood.
- To explore associations between MMP activity and disease status, stratifying patients according to clinical stages of disease (relapse vs remission) and inflammatory parameters, in order to assess the validity of gelatinases as dynamic biomarkers of disease.
- Assessing the presence of MMP isoforms as a possible perspective in investigating their fine-tuning for matrix degradation.
- To investigate the presence of MMPs within EVs isolated from CSF, hypothesising their targeted role in blood-brain barrier damage and neuroinflammatory communication.

3 In Fibrosis: Dupuytren's Disease

- To evaluate MMP-2 activation in primary myofibroblasts derived from DD patients stratified into GG homozygotes, GA heterozygotes, and AA homozygotes according to the allelic presence of the rs1042704 polymorphism of the MMP14 gene.
- To study the effect of anti-MMP-14 inhibitory antibody on the genotype-dependent regulation of MMP-2 activity to explore a possible personalised therapeutic approach.
- Apply proteomic analyses on conditioned media from antibody-treated myofibroblasts, in order to identify treatment-specific responses and novel proteins involved in extracellular matrix remodelling.

The multidisciplinary approach adopted allowed for an integrated exploration of the functional role of MMPs in three distinct biological systems, to evaluate their use as diagnostic biomarkers and therapeutic targets in reproductive medicine, neurodegenerative, and fibrotic diseases.

MATERIALS AND METHODS

1 Follicular Fluid Collection from Women Undergoing ART

Follicular fluid (FF) samples were obtained from a cohort of n=126 female patients undergoing assisted reproductive technology (ART) cycles at the Centre of Reproductive Medicine of the Papardo Hospital (Messina, Italy). FF was collected during transvaginal oocyte retrieval, following standard clinical protocols. After oocyte retrieval, FF samples were centrifuged at 1,500×g for 15 minutes to remove cellular debris, aliquoted, and stored at –20°C until further processing. Complete clinical data were available for n=114 patients, including age, infertility diagnosis, hormonal profiles, and ART outcomes, including β-human chorionic gonadotropin (β-hCG) positivity and live birth rates.

4 CSF, Serum, and Plasma Collection from Multiple Sclerosis Patients

Biological samples were collected from a cohort of n=62 patients diagnosed with multiple sclerosis (MS) and n=31 subjects with other neurological diseases (OND), as part of their diagnostic work-up at the Department of Biomedicine, Neurosciences and Advanced Diagnostics (BiND, University of Palermo). Samples of 15–20 mL of CSF were collected through lumbar puncture in polypropylene tubes. Each sample was centrifuged at 432×g for 15 min at room temperature. The supernatant was centrifuged at 1,730×g for 15 minutes at room temperature, aliquoted, and then frozen at -80°C until use. Blood samples were collected by venipuncture procedures. Blood was immediately processed to separate serum and plasma by centrifugation at 1,500×g for 10 minutes at room temperature. Serum was obtained after coagulation and centrifugation; plasma was prepared from EDTA-treated blood to preserve coagulation factors. All samples were aliquoted and stored at –80°C. Clinical features were recorded to enable correlation analyses. Among them, age, sex, disease status (relapse vs remission), disease onset, and expanded disability status scale (EDSS) scores were available.

5 Tissue Collection from Patients with Dupuytren's Disease

The collection of surgical tissue specimens, isolation of primary myofibroblasts, and subsequent cryopreservation were performed by Miss Mira Pecheva, PhD student at the University of East Anglia (UEA) in Norwich, as part of her doctoral research project conducted

under the supervision of Dr. Rosemary Davidson and Prof. Ian Clark. The collection of Dupuytren's tissue samples from patients undergoing surgical intervention at the Norfolk and Norwich University Hospital commenced in November 2021 as part of a dedicated research project. Patient participation has been consistently high, with all individuals approached since the initiation of the study consenting to tissue donation. A subset of n=12 patients was selected to represent the three genotypic groups associated with the MMP14 rs1042704 polymorphism (GG, GA, and AA), with n=4 individuals per genotype. Each patient was assigned a unique anonymised identifier to ensure compliance with ethical and confidentiality standards. The identifiers corresponding to the selected cohort were: 24BR027N, 24BR028N, 24BR030N, 24BR092N, 24BR094N, 24BR095N, 22BR100N, 24BR172N, 21BR475N, 23BR748N, 22BR872N, and 22BR873N. Excised palmar fascia tissues were processed under sterile conditions for the isolation and expansion of primary myofibroblasts. Cells were subsequently cryopreserved to establish a biobank suitable for functional and molecular analyses.

6 Extracellular Vesicles Isolation by Ultracentrifugation

Before ultracentrifugation, FF samples (n=117) were centrifuged at 1500×g for 15 minutes to remove cellular debris. The recovered volume varied between 2 and 9 mL and was adjusted to a final volume of 12 mL with desalted phosphate-buffered saline (PBS) in 13.2 mL Beckman Coulter® tubes. Plasma samples from MS patients (n=90), collected in volumes ranging from 2 to 8 mL, were processed using the same protocol as for FF. The CSF was diluted in PBS to a final volume of 40 ml, and CSF-derived EVs (CSF-EVs) were purified by ultracentrifugation. EVs were isolated from all sample types by ultracentrifugation using a Beckman Optima™ L-90K ultracentrifuge at 105,000×g for 90 minutes at 4 °C. EV pellets from FF and plasma samples were resuspended in 20 µL of desalted PBS, while CSF-EV pellets were resuspended in 10 µL of PBS. All the obtained EV samples were quantified using the Bradford assay and stored at –80 °C until use.

7 Characterization of FF-EVs by Dynamic Light Scattering

Aliquots of FF-derived extracellular vesicles (FF-EVs) were diluted 1:1000 in Milli-Q water and transferred into disposable cuvettes for measurement. The hydrodynamic diameter (Z-Average), polydispersity index (PdI), and zeta potential were assessed using dynamic light scattering (DLS). Measurements were performed at the ATeN Center (Advanced Technologies

Network) using a Malvern Zetasizer Nano ZS instrument, equipped with a 632.8 nm laser operating at a fixed backscatter angle of 173°.

8 Gelatin Zymography

Aliquots of 10 µL FF previously diluted (1:20) or 3 µL FF-derived EVs were mixed with Bolt™ LDS Sample Buffer (4X; Thermo Fisher Scientific) and Milli-Q water, under non-reducing conditions. Aliquots of 30 µL of CSF samples were evaporated to dryness using a vacuum concentrator (SpeedVac). CSF dried pellets were then rehydrated in 20 µL of Bolt™ LDS Sample Buffer (1X), under non-reducing conditions. CSF-derived EV aliquots of 3 µL were mixed with Bolt™ LDS Sample Buffer (4X) under non-reducing conditions. Aliquots of 10 µL of serum and plasma previously diluted (1:25) were mixed with Bolt™ LDS Sample Buffer (4X) and Milli-Q water, under non-reducing conditions. Aliquots of 10 µL of Foetal bovine serum (FBS) previously diluted (1:25) were mixed with Bolt™ LDS Sample Buffer (4X). Aliquots of 7.5 µL of conditioned medium from DD primary fibroblasts were mixed with loading buffer. Recombinant protein rProMMP-2 (9 ng) and rProMMP-9 (0.03675 ng) were diluted in 0.05% Brij [prepared from a 30% w/v stock solution of Brij (Acros Organics) in PBS].

Sample aliquots, prepared as described above, were loaded onto a 7.5% polyacrylamide SDS-PAGE gel copolymerized with 0.1% gelatin. The run was performed at 150 V in Tris-glycine running buffer (25 mM Tris-HCl, 200 mM glycine, and 0.1% w/v SDS). After electrophoresis, gels were incubated at room temperature for 1 h in a wash buffer (50 mM Tris-HCl, pH 7.5 and 2.5% Triton X-100) to allow partial renaturation of proteins by removing the SDS and then incubated for 18 h at 37°C with an activation buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; and 5 mM $CaCl_2$), to allow the activation of the gelatinases. Gels were stained with Coomassie Blue G-250 (0.2% Coomassie Blue, 40% methanol, 10% acetic acid) for 50 minutes at room temperature. After gel destaining in distilled water, gelatinolytic activity was visualized as clear bands against a dark background. Gel acquisition was performed using the BioRad Molecular Imager VersaDoc™ MP or Odyssey CLx Imager (Li-Cor). Densitometric analysis was performed using ImageJ software (NIH). Band intensities were normalized to FBS positive controls. All experiments were performed in triplicate.

9 Two-Dimensional Gelatin Zymography

To characterize isoforms of gelatinases, a subset of cerebrospinal fluid (CSF) samples (n=12) was analysed by two-dimensional gelatin zymography (2D-IPG Zymography), combining isoelectric focusing (IEF) with SDS-PAGE separation under non-reducing conditions. After brief centrifugation to remove cellular debris, 30 µL of each CSF sample was dried using a SpeedVac concentrator and rehydrated in ISOT buffer (4% CHAPS, 40 mM Trizma base, 65 mM DTE, and a trace of bromophenol blue in 8 M urea). Rehydrated proteins were loaded onto 7 cm IPG strips (pH 4–7, linear) and separated by isoelectric focusing using a Protean IEF system. For the second dimension, strips, after incubation for 15 min in equilibration buffer, were placed on 7.5% SDS-PAGE gels copolymerized with 0.1% gelatin. After electrophoresis gels were processed as previously described.

10 Cell Cultures and Treatments

Two human endometrial epithelial cell lines were employed as in vitro models to investigate endometrial receptivity. The RL95-2 cell line, derived from human adenosquamous endometrial carcinoma, was used to represent a receptive endometrial phenotype, while the HEC-1-A cell line, derived from human endometrial adenocarcinoma, was used as a model of non-receptive endometrium. Both cell lines were cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated FBS and 1% penicillin–streptomycin. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. RL95-2 and HEC-1-A cells were employed in the wound healing assay, as detailed below.

Primary myofibroblasts from Dupuytren's disease patients were cultured under the same conditions, except that phenol red-free DMEM and Foetal Calf Serum (FCS) were used. For treatment experiments, cells were seeded at a density of 83,333 cells/mL in 6-well plates and cultured in serum-free medium (0% FCS) for 24 or 48 hours. Cells were treated with either an anti-MMP14 inhibitory antibody (E_2C_6, 3.113 mg/mL) or an isotype control antibody (DI_3, 2.625 mg/mL). Untreated cells served as controls. Conditioned media were collected after treatment and stored at –80 °C for downstream analysis.

11 Wound Healing Assay

RL95-2 and HEC-1-A cells were seeded in 48-well plates at a density of 200,000 and 80,000 cells per well, respectively, and incubated at 37 °C until reaching approximately 80% confluence. To synchronize the cell population, the culture medium containing serum was removed, and cells were gently washed with 300 µL of sterile PBS. The medium was then replaced with serum-free DMEM, and cells were maintained under starvation conditions for approximately 15 hours at 37 °C in a humidified incubator with 5% CO_2. After starvation, the wound healing assay was performed by creating a linear scratch across the cell monolayer using a sterile 200 µL pipette tip. Detached cells were removed by PBS washing: three washes with 300 µL for RL95-2, and one wash with 200 µL for HEC-1-A cells. Each well was then supplemented with serum-free DMEM containing 1% (v/v) follicular fluid. For both cell lines, every well was treated with a distinct FF sample derived from one of n=42 female patients undergoing ART procedures, aged between 26 and 38 years. Control wells received serum-free DMEM alone. Cell migration was monitored at 0h, 6h, and 24h using a phase-contrast optical microscopy at 4x magnification. The scratch area at each time point was quantified using ImageJ software. To quantify migration, the wounded area of each well was divided into two separate fields, which were measured individually, and the mean area was calculated. The percentage of wound closure was calculated by subtracting the open area remaining at 6h and 24h from the initial wound area measured at time 0h. Data were then normalized to the untreated controls (serum-free DMEM only) to account for baseline motility. All wound healing assays were performed in triplicate for each cell line.

12 Cell lysis and protein extraction

Protein lysate was obtained from HEC-1-A cells and DD primary myofibroblasts. Cells were washed with PBS to remove residual culture medium. Cell lysis was then performed by adding 100 µL of RIPA buffer (50 mM Tris-HCl, pH 8.0; 150 mM NaCl; 1% NP-40; 0.5% sodium deoxycholate; 0.1% SDS; and 1% protease inhibitor cocktail) directly to each well. Cells were incubated on ice for 30 minutes under gentle agitation to ensure efficient extraction of total proteins. Cell lysates were then centrifuged at 15,000×g for 20 minutes at 4 °C to remove cellular debris and genomic DNA. The resulting supernatant, containing soluble total proteins, was collected and used for subsequent analysis. Protein concentration was determined by Bradford assay or BCA Assay (Abcam) according to the manufacturer's protocol.

13 Western Blotting

Follicular fluid, cerebrospinal fluid, serum, plasma, and DD fibroblast-derived medium samples were processed as previously described for zymography in terms of dilution protocols. For Western blot analysis, all samples were further prepared under reducing conditions by adding Bolt™ Sample Reducing Agent (10X) and Bolt™ LDS Sample Buffer (4X), followed by denaturation at 95 °C for 5 minutes.

Samples were loaded in 10% polyacrylamide gel and separated at 150 V in Tris-glycine running buffer. After electrophoresis, proteins were electrotransferred for 1h at 100V into a nitrocellulose membrane (Amersham^{TM}) using wet conditions in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). To verify efficient transfer, membranes were briefly stained with Ponceau S solution (0.2% Ponceau S in 3% trichloroacetic acid), followed by destaining in distilled water. Non-specific binding sites were blocked by incubating membranes for 1 hour at room temperature in TBS-T buffer (Tris-buffered saline containing 0.05% Tween-20) supplemented with 5% non-fat dry milk (ChemCruz). Membranes were then incubated overnight at 4 °C with the appropriate primary antibodies. Primary antibodies used were as follows: rabbit polyclonal anti-E-cadherin antibody (1:3000; sc-7870, Santa Cruz Biotechnology); mouse monoclonal anti-β-actin antibody (1:5000; A5441, Sigma-Aldrich); and mouse monoclonal anti-C-reactive protein (CRP) antibody (1:1000; sc-69770, Santa Cruz Biotechnology); rabbit monoclonal anti-MMP2 antibody (1:2500; ab92536, Abcam); rabbit monoclonal anti-MMP9 antibody (1:10,000; ab76003, Abcam); rabbit monoclonal anti-MMP14 antibody (1:5000; ab51074, Abcam), rabbit monoclonal anti-β-actin antibody (1 µg/mL; ab213262, Abcam). After six washes in TBS-T, membranes were incubated for 1 hour at room temperature with the corresponding HRP-conjugated secondary antibodies. Secondary antibodies used were as follows: goat anti-rabbit IgG (1:5000; W4018, Promega); goat anti-mouse IgG (1:5000; A17352, Antibodies); goat anti-mouse IgG (1:5000; #31430, Invitrogen); and polyclonal goat anti-rabbit IgG (1:2000 to 1:10,000; P0448, Dako Agilent). Membranes were then washed for additional six times in TBS-T before signal detection. Antigen–antibody complexes were detected using the SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific). Chemiluminescent signals were acquired with the ChemiDoc XSR imaging system (Bio-Rad) or GeneSys image acquisition software (Syngene) and analysed using Quantity One software (Bio-Rad Laboratories). Band intensity was quantified using ImageJ software.

14 Proteomic Analysis of CSF

14.1 Sample Preparation and LC-MS/MS Analysis

Cerebrospinal fluid samples collected from patients diagnosed with multiple sclerosis (n = 14) and from a control group with other neurological diseases (n = 13) were subjected to proteomic analysis. Protein concentration was determined by Bradford assay, and 30 µg per sample. CSF sample preparation was performed using the iST kit (PreOmics, Germany), according to the manufacturer's protocol at the laboratory of Dr. Simone D. Scilabra (Ri.MED Foundation).

Conditioned media from primary fibroblast cultures were subjected to proteomic analysis using a standard protocol. The media were derived from cells obtained from n=4 patients for each genotype of the *MMP14* SNP (GG, GA, AA), with each cell line exposed to three experimental conditions (NoTx, DI_3, and E_2C_6). A total of n=36 samples were analysed and subjected to filter-aided sample preparation (FASP). Each sample was prepared in technical triplicate. Samples were loaded onto Vivacon spin columns (Sartorius) and concentrated by centrifugation at 14,000×g for 10 minutes. Subsequently, proteins were reduced with dithiothreitol (DTT; Sigma-Aldrich) in 200 µL of UA buffer (8 M urea in 0.1 M Tris-HCl, pH 8.5) and then alkylated with 50 mM iodoacetamide (IAA; Sigma-Aldrich) in the dark. After reduction and alkylation, samples were washed three times with UB buffer (8 M urea in 0.1 M Tris/HCl, pH 8.0). Sequentially, samples were digested with Lys-C protease (1:50 enzyme to protein ratio; Promega) in UC buffer (2 M urea in 25 mM Tris/HCl, pH 8.0) and incubated overnight at 37°C in a wet chamber. Second digestion was performed by trypsin (1:100 enzyme to protein ratio; Promega) in ammonium bicarbonate (ABC; 3.95 mg/mL) and incubated for 4h at 37°C in a wet chamber. Peptides were subsequently eluted from the filter columns by centrifugation at 14,000×g for 1h and immediately acidified with 20 µL of 8% formic acid. The resulting peptides were sent to Ri.MED Foundation for the next steps. Peptides were desalted using stop-and-go extraction (STAGE) tips, prepared in-house with Empore C18 disks (Sigma-Aldrich). Before use, the C18 resin was activated with 100 µL of methanol, then washed four times with 0.1% formic acid (FA). Peptide samples were loaded onto the C18-packed STAGE-tips, washed again with 0.1% FA to remove contaminants, and finally eluted with 40 µL of 60% acetonitrile (ACN) and 0.1% FA in MS-grade water. The eluted peptides were subsequently dried using speed vacuum centrifugation until all ACN was evaporated. Finally, peptides were resuspended in 20 µL of 0.1% FA and incubated in a sonication bath to dissolve peptides. Samples were stored at -20°C until MS analysis. Peptide concentrations were determined using a NanoDrop 2000

spectrophotometer (Thermo Scientific), and 1 µg of peptides was injected for separation by nano-liquid chromatography (nanoLC) on a Vanquish Neo UHPLC system (Thermo Scientific) equipped with an Acclaim PepMap C18 analytical column (25 cm×75 µm ID, Thermo Scientific). Peptides were separated over a 130-minute binary gradient consisting of water and acetonitrile, both containing 0.1% formic acid. The eluted peptides were ionized via nano-electrospray ionization (nano-ESI) and analysed on an Exploris 480 Orbitrap mass spectrometer (Thermo Fisher Scientific) for tandem mass spectrometry (MS/MS) analysis.

The acquired mass spectra were processed for protein identification using MaxQuant software, with database searching performed against the *Homo sapiens* (Human) reference proteome from UniProt. Trypsin was specified as the digestion enzyme. The "first search" function was enabled to recalibrate precursor ion masses within a 20-ppm window. For the main search, the precursor and fragment mass tolerances were set to 4.5 ppm and 20 ppm, respectively. Carbamidomethylating of cysteine residues was set as a fixed modification, while oxidation of methionine and N-terminal protein acetylation were considered variable modifications. A protein was considered confidently identified only if it was detected in all three technical replicates analysed.

14.2 Proteomic Analysis and Machine Learning Classification

Following LC-MS/MS analysis was performed at the laboratory of Dr. Simone D. Scilabra. The total proteins were identified across the CSF samples from the MS and OND groups. Label-free quantification (LFQ) protein intensities were Z-score normalized within each group (MS and OND) using Perseus software (v1.6.1.3). Differential expression analysis was performed using a two-tailed Student's t-test, and results were corrected for multiple testing using the false discovery rate (FDR) method. FDR significance threshold was >1.3, equivalent to $p < 0.05$. Statistical results were reported as –log(*p*-value) and $\log_2$ fold change (OND/RRMS).

The supervised machine learning approach was implemented using the Random Forest classification algorithm (Svetnik et al., 2003). The analysis was performed on the set of proteins found to be differentially expressed between the MS and OND groups following LFQ. After model training, its predictive performance was assessed via confusion matrix analysis. A feature importance analysis was conducted within the Random Forest framework to rank the contribution of each protein to the model's prediction process. All machine learning analyses were carried out using RStudio.

14.3 Bioinformatic Functional Enrichment Analysis

Functional enrichment analysis was performed using the database and integrated tools of the STRING platform (stringdb.org) on proteins identified in the proteomic analysis. Tissue-specific expression analysis (TISSUE) was conducted to assess the anatomical origin of the identified proteins. Gene Ontology analysis was performed, and particularly focused on the Biological Process category (GO: BP). KEGG and Reactome pathway annotations were used to investigate metabolic and signalling pathway enrichment. Statistical significance was determined based on false discovery rate correction (FDR ≤ 0.05).

15 Enzyme-Linked Immunosorbent Assay (ELISA) for IGFBP-2

Quantification of IGFBP-2 levels in cerebrospinal fluid (CSF) samples from MS patients (n = 15) and OND controls (n = 9) was performed using a commercial Human IGFBP-2 ELISA kit (Thermo Fisher Scientific), according to the manufacturer's instructions. Absorbance was measured at 450 nm using a microplate reader (Tecan), and IGFBP-2 concentrations were calculated based on the corresponding standard curve and normalized for protein content calculated by Bradford assay.

16 Statistical Analysis

Statistical analyses were conducted using GraphPad Prism. Depending on data distribution and sample size, comparisons were performed using the Mann–Whitney *U* test, one-way ANOVA, or simple linear regression analysis. Pearson's correlation coefficients were used for association studies. Coefficients of variation (CV) were calculated to assess interindividual variability.

RESULTS AND DISCUSSION

1 Characterization of Follicular Fluid in Women Undergoing ART

The characterisation of follicular fluid (FF) is a fundamental way to understand processes that govern oocyte maturation, ovarian quality, and fertilization. Indeed, FF reflects the ovarian microenvironment in which the oocyte develops and matures and is mainly composed of granulosa cells and theca cell secretions and exudates of blood serum (Pan et al., 2024). Its composition, therefore, mirrors not just the functional status of the follicle but also the endocrine, paracrine, and autocrine signalling within the follicle. Thus, FF can yield useful information regarding the health of the oocyte and the prospects of success of ART techniques. Indeed, investigation of FF composition may reveal valuable biomarkers in the prediction of ovarian responsiveness, oocyte competence, or embryo quality.

This study aimed to characterise the follicular fluid of women undergoing ART treatment to identify potential biomarkers associated with oocyte quality and endometrial receptivity. In particular, the gelatinolytic activity of MMPs and their relationship with maternal age, inflammatory status, and the ability of FF to modulate endometrial cell migration and receptivity were investigated.

1.1 Demographic and Clinical Characteristics of Patient Cohort

The study involved a total of n=126 patients undergoing assisted reproductive technology (ART) treatment at the Papardo Hospital of Messina (Italy). Among patients with available clinical data (n=114), the mean age was 37 years, ranging from 25 to 44 years (Table 2). The cohort had a heterogeneous age distribution and was divided into four age groups, distinguishing patients aged 25-30, 31-35, 36-40, and 41-44 years. The 25-30 years old group accounted n=11 patients (9.6% of the total), n=31 (27.2%) patients belonged to the 31-35 age group, n=46 (40.4%) made up the largest group of 36-40 years old, and women in the older age group of 41-44 years accounted n=26 (22.8%) individuals. The percentages varied significantly between the groups, analysing the response to treatment in terms of β-hCG test positivity, which indicates the establishment of pregnancy, and cases with live birth (successful ART). Of the n=4 patients in the 25-30 years group who tested positive for β-hCG, n=3 carried the pregnancy to term with delivery. In the 31-35 year old group, there were n=9/16 β-hCG positive births;

n=18/29 in the 36-40 year old group; while in the 41-44 year old group, only n=3/8 had a positive outcome with live birth (Table 2). These findings are consistent with existing literature, which reports a general decline in fertility with advancing maternal age, often attributed to impaired endometrial receptivity and reduced oocyte competence (J. Zhao et al., 2023).

Table 2 **Demographic and Clinical ART Outcomes**

	age ranking				
	total	**25–30**	**31–35**	**36–40**	**41–44**
No. of patients (n, %)	114	11 (9,6%)	31 (27.2%)	46 (40,4%)	26 (22,8%)
β-hCG positivity (n)	57	4	16	29	8
Cases with birth (n)	33	3	9	18	3
Female age °	38; 25÷44	—	—	—	—
Male partner age °	40; 29÷58	—	—	—	—

° Values are expressed as medians with minimum and maximum ranges indicated.

Particular attention was paid to the three age subgroups (25-30, 31-35, 36-40) because, despite being in an age generally associated with higher reproductive potential, the success rate in terms of live births was unexpectedly modest (Figure 15).

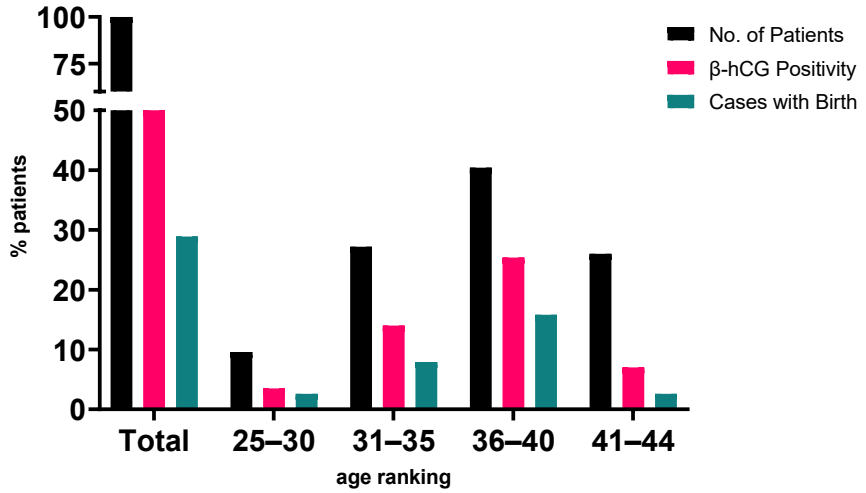

Figure 15 **Number of patients with positive β-hCG test results and number of births, divided by age group**. The graph illustrates reproductive outcomes according to maternal age, comparing pregnancy test positivity with final live birth.

This has stimulated interest in exploring which factors may induce infertility. Infertility can be caused by a variety of factors, which may affect one or both partners. The main female causes include tubal disease, reduced ovarian reserve, ovulatory or endocrine disorders, endometriosis,

and idiopathic forms of infertility. The male factor, on the other hand, is mainly represented by abnormalities in the seminal fluid, which may relate to sperm quantity or motility. In the cohort under analysis, a unique cause of infertility was identified in most cases (n=73). Among these, n=54 cases of infertility were attributable to a female factor, while only n=19 cases were exclusively related to a male factor. In cases of multifactorial infertility (n=19), the trend is reversed: only n=3 cases involved exclusively female factors, while n=16 cases had at least one male component (Figure 16). Thus, single causes were more frequently of female origin, whereas in the case of multifactorial causes, a male component was more frequent. These results emphasise the complexity of infertility, a condition often characterised by overlapping independent causes, which may hinder successful ART treatment. The presence of multiple factors highlights the need for an integrated and personalised clinical approach in this field. Furthermore, in the cohort under analysis, 20.18% experienced ART failures. This additional finding underlines the importance of finding biomarkers that predict the outcome of ART practices.

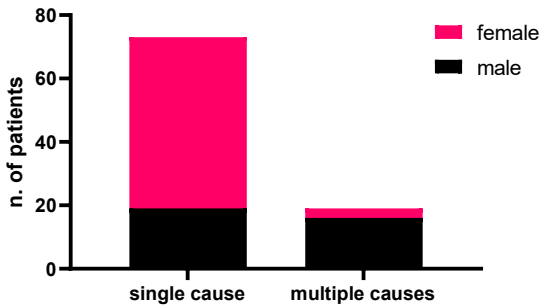

Figure 16 **Distribution of patients by sex-dependent infertility factors.** In the analysed cohort, most infertility cases (n=73) were associated with a single identifiable cause, predominantly of female origin (n=54), while only a minority were due exclusively to male factors (n=19). In contrast, among multifactorial cases (n=19), the presence of a male component was prevalent (n=16), with exclusively female-related multifactorial infertility being rare (n=3).

1.2 Gelatinolytic Activity in Follicular Fluid

The ECM remodelling is significantly implicated in follicle growth, ovulation, corpus luteum formation, angiogenesis, and embryo implantation. Dysregulation of MMPs, such as gelatinases, may be a significant factor in oocyte fertilization and may also serve as a possible predictive marker of oocyte quality or fertilization success. To assess gelatinase activity in the

ovarian microenvironment, zymographic analysis was conducted on follicular fluids (n=126) from women undergoing ART (Figure 17). From the zymograms, it was possible to visualize the activity of the two inactive forms of MMP-9 (92 kDa) and MMP-2 (72 kDa). ProMMP-2 is more highly expressed than proMMP-9. No active forms were detected through zymography, probably due to low concentrations in FF. There is no presence of complexed forms such as dimers of proMMP-9 (200 kDa) and proMMP-9/TIMP-1 (130 kDa), despite being commonly found in serum, from which follicular fluid is partly derived (Figure 17).

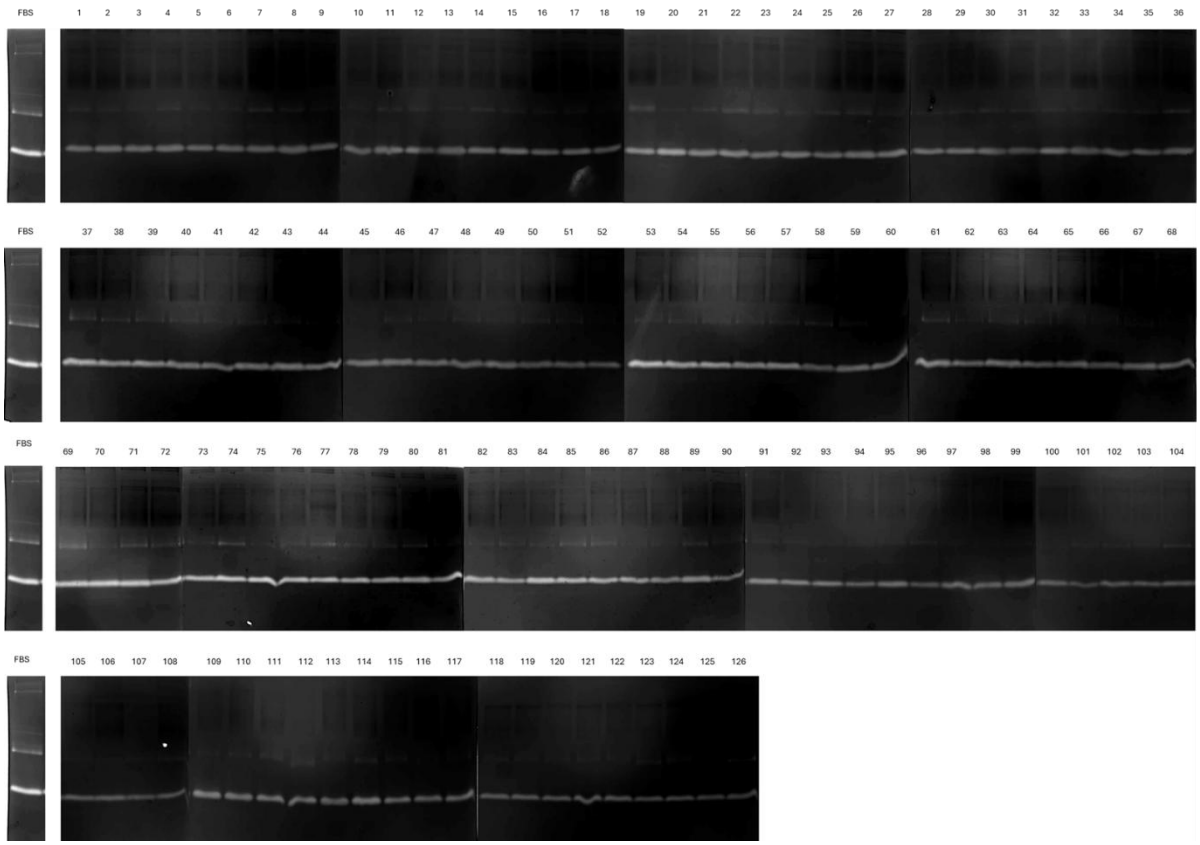

Figure 17 **Gelatin zymography of follicular fluids from women undergoing ART.** Zymograms show proteolytic activity corresponding to the latent forms of MMP-9 (92 kDa) and MMP-2 (72 kDa). Among these, proMMP-2 is more abundantly expressed than proMMP-9. Any active form was detected. No complexed forms of MMP-9, such as proMMP-9 homodimers (200 kDa) or proMMP-9/TIMP-1 complexes (130 kDa), are detectable in FF samples. *ImageJ* software was used to quantify the densitometric bands corresponding to the MMP gelatinolytic activity. FBS, foetal bovine serum (as positive control).

The lytic bands were quantified and normalized to FBS used as a positive control. The measurements were used for subsequent analyses of gelatinolytic activity. The expression of proenzyme forms is consistent with the high level of regulation in matrix degradation,

especially in the ovarian microenvironment. Moreover, the activity of MMP-2 is rather consistent and homogenous between the samples compared to MMP-9. Indeed, the variability of MMP-2 is lower compared to that of MMP-9, reflecting significant inter-individual differences. For instance, the gelatinolytic activity of MMP-2 is consistently present in FF with 2-fold variability among patients and a coefficient of variation (CV) of 20,16% (Figure 18b). On the contrary, MMP-9 shows greater variability (16-fold variability, 68,95% CV), suggesting a more regulated and transient role (Figure 18a).

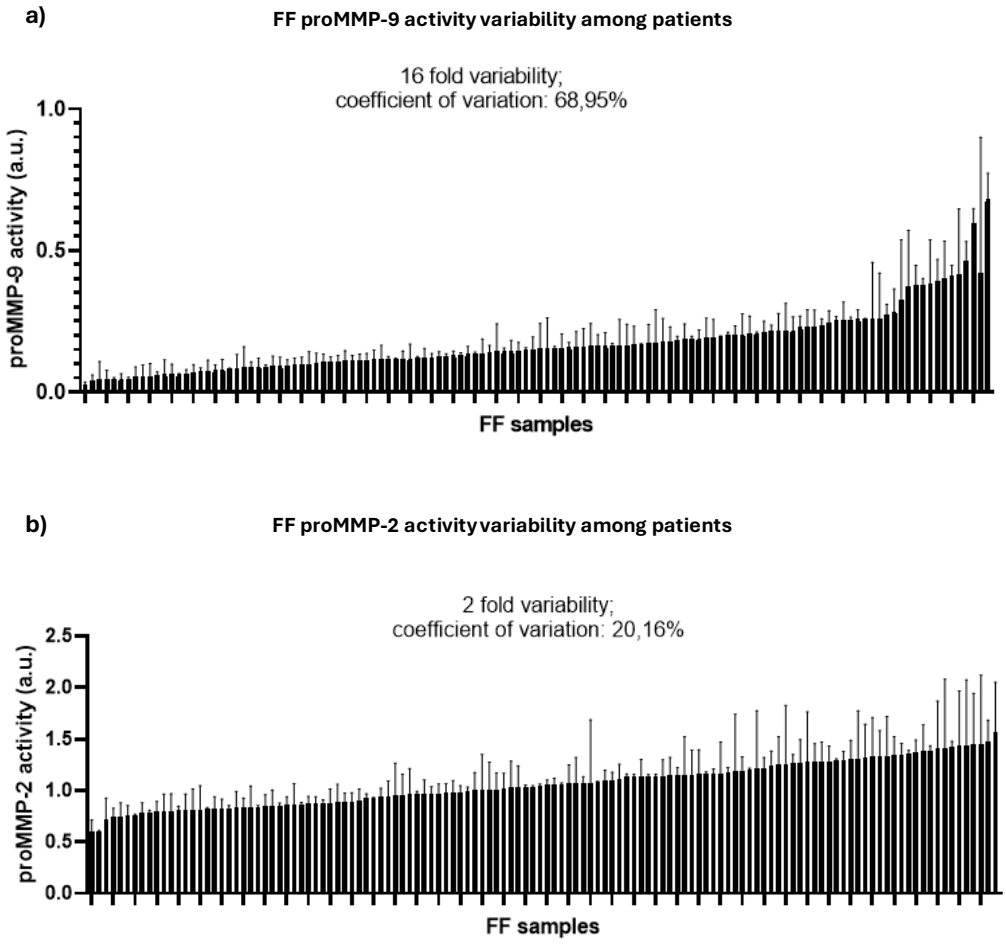

Figure 18 **Variability in MMP gelatinolytic activity among FF samples.** The graphs show the distribution of gelatinolytic activity of proMMP-9 (a) and proMMP-2 (b) in individual FF samples. MMP-2 activity is consistently detected across all samples, exhibiting a 2-fold variability and a coefficient of variation (CV) of 20.16%. In contrast, MMP-9 activity is more heterogeneous, showing a 16-fold variability and a CV of 68.95%, suggesting that MMP-9 expression should be more dynamic and finely regulated.

This finding probably results from the different origin of the two gelatinases. ProMMP-2 is constitutively expressed in follicular fluid at higher and constant levels, involved in the

degradation of the follicle basement membrane during ovulation. MMP-2 is also involved in remodelling the ECM surrounding cumulus oophorus and theca cells, which together with granulosa cells, constitutively produce MMP-2. In contrast, MMP-9 tends to be induced only during ovulation or in response to local inflammatory stimuli, such as LH or cytokine spikes. Thus, in pre-ovulatory follicular fluid or nondominant follicles, MMP-9 is low or absent (Curry & Osteen, 2003).

1.2.1 Correlation between FF MMP activity and pregnancy

The activity of the gelatinases MMP-2 and MMP-9 in the follicular fluid was assessed for a possible correlation with the outcome of fertility treatment. From the results obtained, no significant differences were observed in the activity of these metalloproteinases, neither in relation to β-hCG test positivity (Figure 19a), nor considering the outcome of the pregnancy (abortion vs live births, Figure 19b).

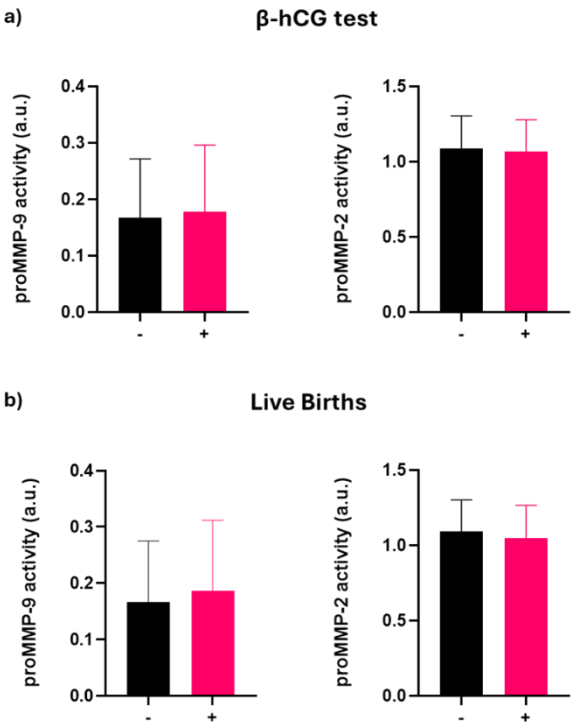

Figure 19 **Gelatinolytic activity of MMP-2 and MMP-9 in FF in relation to ART treatment outcome.** (a) Comparison between patients with a negative (-) and positive (+) β-hCG test. (b) Comparison between patients with a positive β-hCG test who did not carry a pregnancy to term (-) and those who had a birth. No significant differences were observed between the groups.

These data indicate that, in the cohort analysed, MMP-2 and MMP-9 activity detected by zymography is associated neither with successful embryo implantation nor with continuation

of pregnancy to term. These results contrast with those reported by Lee et al. (2005), who, analysing a smaller group of patients (n = 54), had observed greater MMP-9 activity in patients who had achieved pregnancy. However, in the present analysis, conducted on a larger cohort of n=114 patients, this association was not confirmed, suggesting further analysis. In this regard, to more accurately correlate MMP levels with pregnancy rates, it would be appropriate to collect FFs from all follicles in each patient and assess whether MMP levels are consistent across all follicles or vary according to oocyte maturity or competence.

1.2.2 Age-dependent MMP activity in follicular fluid

It is well known that in serum samples, MMP expression correlates with age (Cancemi et al., 2020). Dysregulation of MMPs in the follicle microenvironment can affect ovarian quality, oocyte maturation, fertilisation, and embryo development (Atabakhsh et al., 2018; Bilen et al., 2014; Yang et al., 2015). Ovarian ageing could be associated with a dysregulation of MMP activity, including gelatinases MMP-2 and MMP-9, in the follicular fluid. This imbalance may impair the quality of the follicular microenvironment and lead to reduced oocyte competence, negatively affecting outcomes in ART treatment. To investigate the relationship between ovarian ageing and MMP expression, it was assessed whether advancing age in the n=114 patients was associated with significant changes in MMP-9 and MMP-2 levels in follicular fluid. Simple linear regression analyses revealed a significant inverse association between MMP-9 and MMP-2 expression and age advancement. In both cases, a statistically significant negative correlation emerged, confirming the hypothesis that advancing age is associated with a reduction in gelatinolytic activity in the follicular microenvironment. Specifically, for proMMP-9, the slope of the regression line (p = 0.0318) suggests a gradual but constant decrease with age (Figure 20a). Similarly, the expression of proMMP-2 shows a decreasing trend (p = 0.0409) (Figure 20b). However, the observed variability in proMMP-2 and proMMP-9 levels can only partly be explained by age, considering that 2.5% of the variation in proMMP-2 (r^2 = 0.025) and 2.8% (r^2 = 0.028) of proMMP-9 can be directly associated with age (Figure 20). These results indicate that although age is a relevant factor, it is not the only determinant of MMP regulation, and other hormonal, metabolic, or environmental factors may have a significant impact on their activity in the follicular microenvironment.

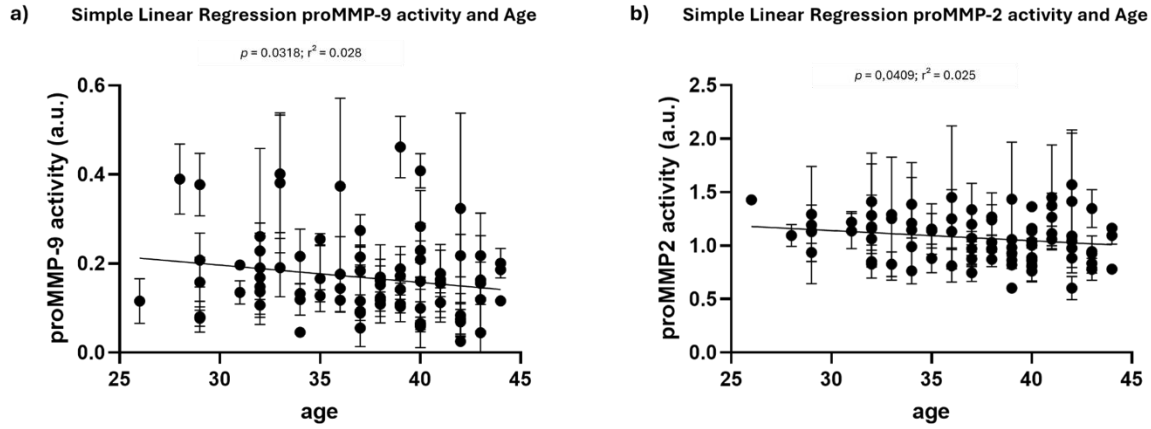

Figure 20 **Simple linear regression analysis between age and MMP-2 and MMP-9 expression in FF.** Scatter plots with simple linear regression show a significant negative correlation between age and the expression of both gelatinases. A statistically significant negative correlation was observed between patient age and the expression of (a) proMMP-9 (p = 0.0318) and (b) proMMP-2 (p = 0.0409). R^2 values (0.028 and 0.025, respectively) suggest that age accounts for only part of the variability, indicating the influence of additional regulatory factors.

However, stratifying the patients by age groups (25-30, 31-35, 36-40, 41-44) showed no difference or trend in the expression of gelatinases MMP-2 and MMP-9 when comparing the groups (Figure 21). This finding confirms that age is not the only determining variable in the variation of gelatinase activity, which can be influenced by multiple factors. Furthermore, the high variability between individuals could mask the differences between the separated groups, which are more visible when considering age as a continuous variable in linear regression.

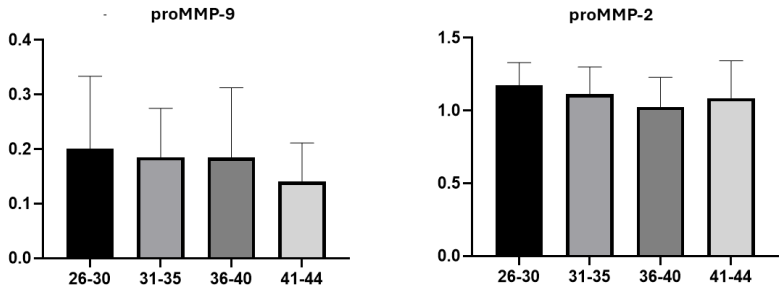

Figure 21 **Expression of gelatinases in FF- across age groups.** Graphs show levels of (a) proMMP-9, (b) proMMP-2 in FF- from women undergoing ART stratified by age. No difference or trend in the expression of gelatinases MMP-2 and MMP-9 was found among the groups

1.2.3 Expression of MMPs in Extracellular Vesicles from Follicular Fluid

Extracellular vesicles are key components of intercellular communication and represent a fundamental mechanism by which follicular cells modulate the ovarian microenvironment. Within the follicular fluid, EVs transport a variety of bioactive molecules, including proteins, lipids, and RNA, which profoundly influence oocyte maturation and the quality of the cumulus-oocyte complex (Machtinger et al., 2015; Neyroud et al., 2022; Shomali et al., 2020; Soares et al., 2023). Although the presence of MMPs in follicular fluid is widely studied, there is still little evidence on their presence and function within EVs. Therefore, n=117 EVs were isolated from FFs of women undergoing ART by ultracentrifugation. The EVs derived from the FF (FF-EVs) were analysed through zymography to assess whether MMP-2 and MMP-9 could be targeted within vesicles (Figure 22). The aim was to explore the possible role of these proteases as markers of follicular and oocyte quality and to assess their potential implication in reproductive mechanisms.

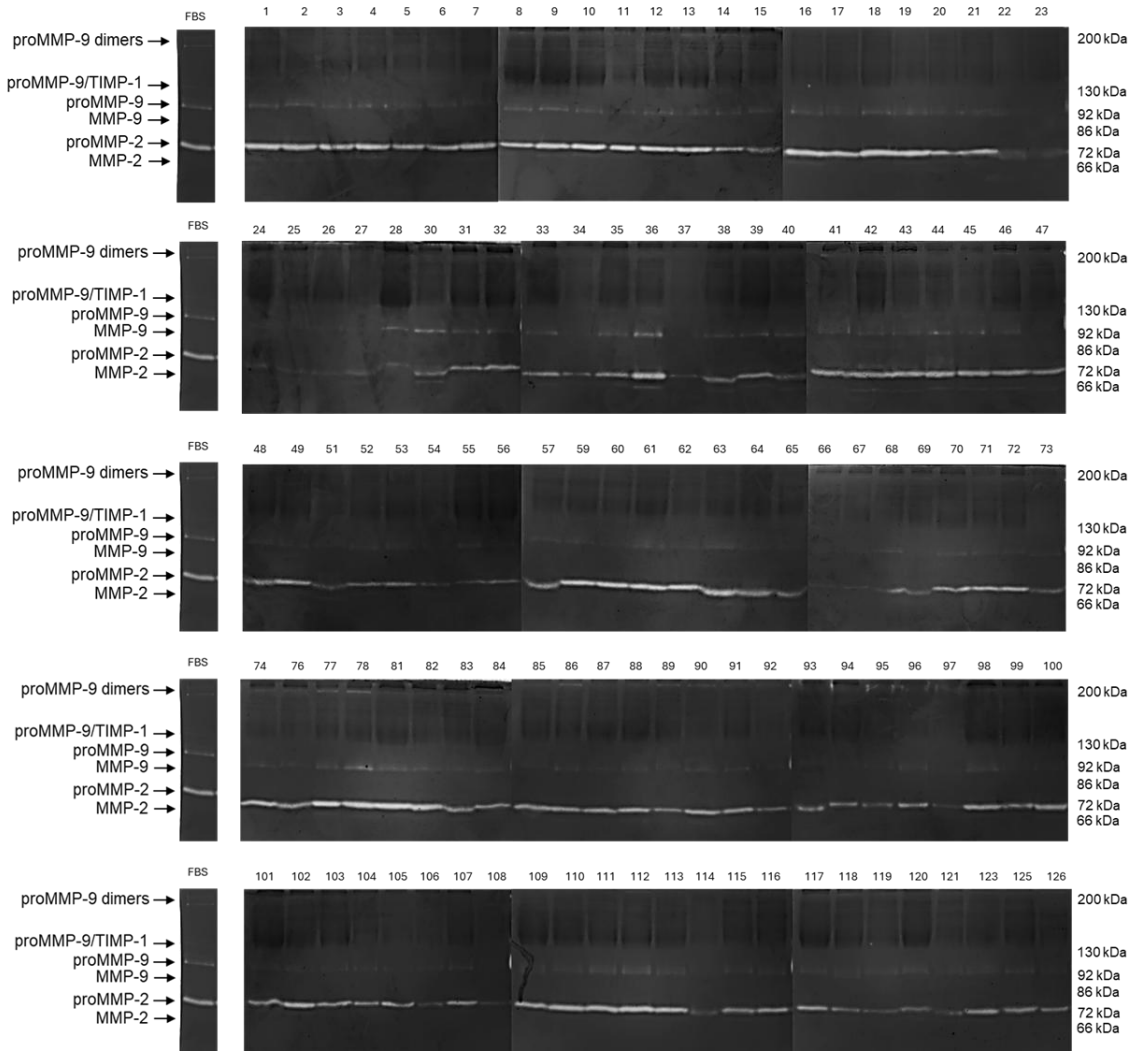

Figure 22 **Gelatin zymography of gelatinase activity in extracellular vesicles isolated from follicular fluid.** Gelatin zymograms show the presence and enzymatic activity of MMP-2 and MMP-9 in EVs isolated from n=117 FF samples of women undergoing ART. Bands corresponding to the molecular weights of proMMP-9 (92 kDa) and proMMP-2 (72 kDa) are visible. Interestingly, MMP-2 (66 kDa) active form is detected, indicating functional activation and compartmentalisation within the EVs. No high-molecular-weight complexes such as proMMP-9 dimers (200 kDa) and proMMP-9/TIMP-1 (130 kDa) are observed. Numbers assigned to each lane correspond to FF samples, according to the internal numbering of the n=126 total patients included in the study. *ImageJ* software was used to quantify the densitometric bands corresponding to the MMP gelatinolytic activity. FBS, foetal bovine serum (as positive control).

The results showed the presence of both proMMP-2 (72 kDa) and proMMP-9 (92 kDa) in the FF-EVs. As in the whole FF, qualitatively also in the vesicles, the expression of proMMP-2 is higher and more constant than proMMP-9. Interestingly, in the majority of the FF-EVs analysed, activity attributable to the active form of MMP-2 (66 kDa) was observed. This finding indicates a possible further degree of regulation of the enzyme, which, compartmentalised in the active form, can act in a more targeted and controlled manner. The absence of high-molecular-weight complexes such as proMMP-9 dimers (200 kDa) and proMMP-9/TIMP-1 (130 kDa) was also observed (Figure 22).

Quantitatively, the intensity of the lytic bands was measured and normalised against the positive control to reduce the technical variability of zymography. In addition, the enzyme activity was also normalised to the starting volumes of each FF sample to reduce the impact of variability related to the different initial quantities and mitigate the differences related to the heterogeneity of the source material among patients. However, overall, the variability of MMPs is greater in FF-EVs than in FFs. Specifically, the gelatinolytic activity of proMMP-2 is present in FF-EVs with 18-fold variability among patients and a coefficient of variation of 48,24% (Figure 23b). The active form of MMP-2, although not expressed in all EVs analysed, is compartmentalised in 70% of the cases. Interestingly, the fold variability of active MMP-2 is identical to that of proMMP-2 (18-fold variability), although with a slightly higher coefficient of variability (58.15%) (Figure 23c). ProMMP-9 shows greater variability (31-fold variability), but a CV of 70.73% (Figure 23a) comparable to that of proMMP-9 in FF (Figure 18a).

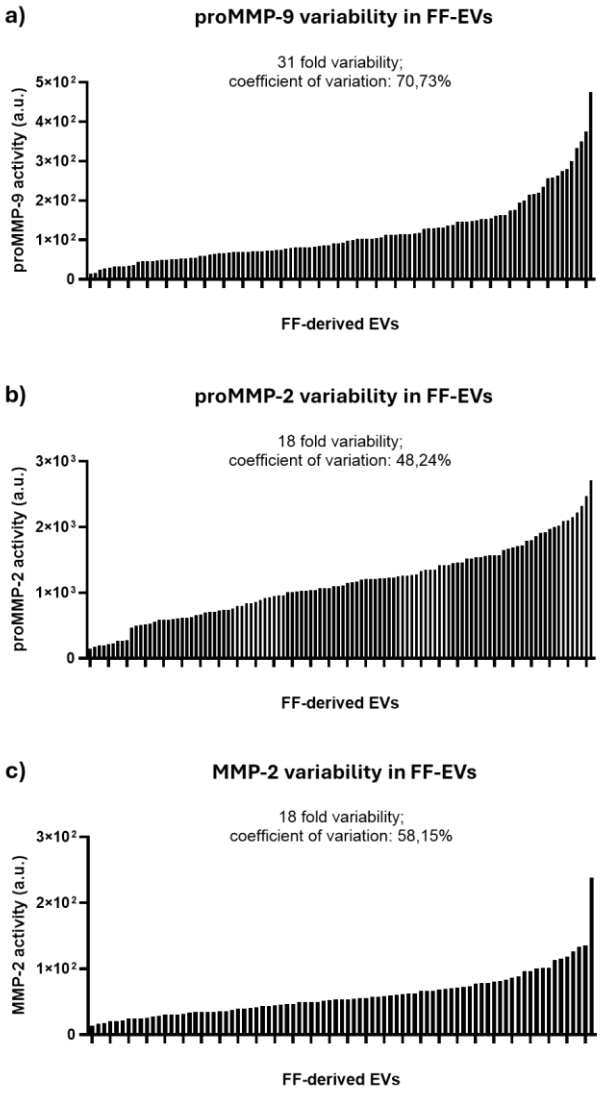

Figure 23 **Variability in MMP gelatinolytic activity among FF-derived EVs.** The graphs show the distribution of gelatinolytic activity of proMMP-9 (a) and proMMP-2 (b), and MMP-2 (c) in FF-EVs. MMP-2 activity is consistently detected across all samples, exhibiting 18-fold variability in both (b) pro- and (c) active forms. However, the two forms present different CV: (b) 48, 24% for proMMP-2 and (c) 58,15% for MMP-2. MMP-9 activity is more heterogeneous, showing a 31-fold variability and a CV of 70.73%, suggesting higher control.

The greater variability observed in MMP expression could be partially related to the complex regulation of their compartmentalisation into vesicles. Indeed, the selection of cargo to be incorporated into EVs is a highly regulated process, and EVs' release mechanisms are governed by multiple signalling pathways. In the ovarian follicle, the content and quantity of EVs can be influenced by numerous factors, such as oocyte competence, oocyte quality, but also by other factors related to the individual patient (e.g., age, inflammatory status, or oxidative stress). Finally, methodological limitations, such as the ultracentrifugation procedure to isolate EVs, may introduce additional variability due to sample handling.

Analysing the expression of gelatinases as cargo of EVs, it was interesting to note that the proMMP-9 content was positively and significantly associated with that of the active form MMP-2. The correlation analysis indeed highlighted a Pearson coefficient r = 0.677 and $p < 0.0001$ (Figure 24). This finding suggests that the inclusion and transport of these two gelatinases in EVs could be co-regulated and released in a coordinated manner to act synergistically in the follicular microenvironment.

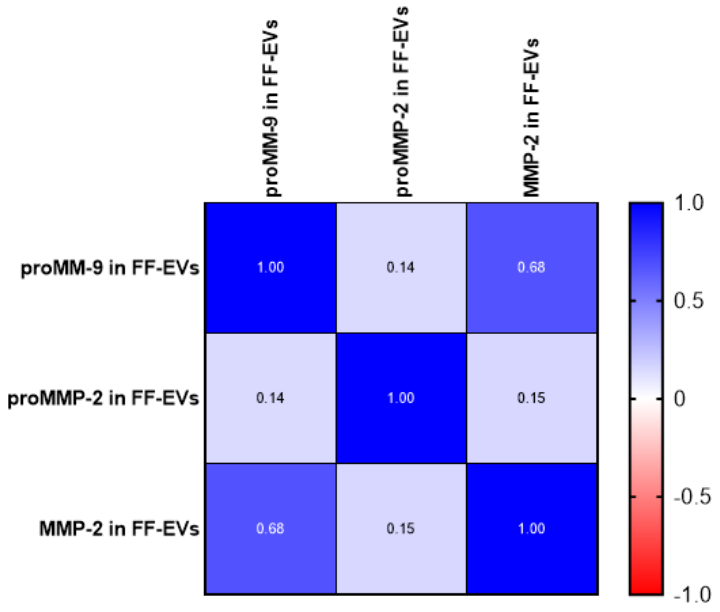

Figure 24 **Correlation matrix between gelatinase levels in FF-EVs.** The graph shows the Pearson correlation coefficients between proMMP-2, the active form of MMP-2, and proMMP-9. A positive and significant correlation between the active form of MMP-2 and proMMP-9 ($r = 0.68$, $p < 0.0001$) was observed, supporting the hypothesis of co-regulation of their loading in EVs. The other correlations are weak and not significant. The colouring of the map reflects the intensity and direction of the correlations, with more intense shades indicating higher values of r.

1.2.3.1 FF-EVs' physico-chemical characterisation

Physico-chemical characterisation by dynamic light scattering (DLS) was performed on FF-derived EVs to obtain information on the hydrodynamic radius (Z-Average), polydispersity index (PdI), and zeta potential of the suspended particles (Khan et al., 2022). The EVs characterised derived from FFs of n=12 patients, divided into four age groups described above (three patients per group). For each sample, a triple reading was taken, and the hydrodynamic radius values were then averaged.

Figure 25 shows an example graph of size distribution by intensity of a FF-EV sample (each peak refers to a single recording of the same sample).

Figure 25 **Size distribution of FF-EVs by DLS.** Graph representing the size distribution by intensity of EVs derived from three replicates of the same sample (Records 49, 50, 51). All samples show a main peak centred around 200-300 nm. The similar trend of the curves between the replicas suggests a good reproducibility of the measurement.

Although the values obtained per individual sample were heterogeneous, the mean hydrodynamic radius of the EVs was approximately 100 nm, a value that places the vesicles in the category of small EVs (d < 200 nm) according to the MISEV 2023 guidelines (Welsh et al., 2024). Moreover, such a size distribution is consistent with the application of the ultracentrifugation technique, which allows the isolation of a heterogeneous population of EVs (Khan et al., 2022). In general, the PdI per reading ranged from 0.2 to 0.7 (mean PdI = 0.5). This factor can be attributed to the variability of biological samples and the presence of different subpopulations of vesicles in each sample. However, the values remain compatible with DLS analyses of complex biological samples, where a PdI below 0.7 is still considered interpretable in the literature (Khan et al., 2022). The detected zeta potential was negative in all samples, confirming the electrostatic repulsion between the particles and the consequent absence of spontaneous aggregation tendency.

The analysis conducted in relation to the age of the patients did not reveal any significant variations in the size of the FF-EVs. However, the negative Pearson correlation (r = -0.41) between the hydrodynamic radius of the vesicles and age suggests the possible presence of an inverse association, although not statistically significant (Figure 26). This preliminary finding requires evaluation on a larger number of samples in order to draw more robust conclusions.

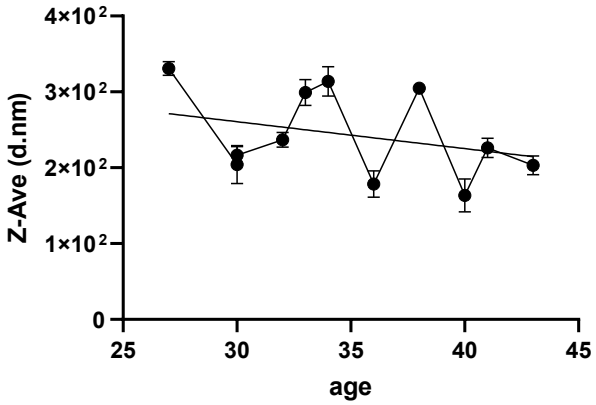

Figure 26 **Correlation between patient age and FF-EVs.** A scatter plot represents the hydrodynamic radius (Z-ave) of FF-EVs related to the age of patients. The linear regression line shows a negative trend, although the observed variability and non-significant statistics.

1.2.3.2 Comparative analysis of gelatinase expression between FF and FF-EVs

A comparative analysis was performed to compare gelatinase activity in FF-EVs vs. follicular fluid. A modest positive but significant correlation was found in the expression of proMMP-9 in the two compartments, with a Pearson coefficient r = 0.35 and $p < 0.0001$. Linear regression analysis between proMMP-9 levels in follicular fluid and those in FF-EVs confirmed the statistically significant positive correlation ($p < 0.0001$) (Figure 27a). This finding suggests that the inclusion of proMMP-9 in EVs reflects the total levels present in the FF.

No significant correlation was found for proMMP-2. The content of proMMP-2 in FF-EVs does not reflect the concentration of the proenzyme in the FF (Figure 27b). This finding reinforces the hypothesis that the regulation and activation of MMP-2 is highly regulated and selective, considering the detection of the active form in the FF-EVs but not in the fluid. This could indicate a mechanism of selective and localised enzymatic activation through EVs secreted by follicle cells. Since granulosa and theca cells constitutively produce MMP-2 (Curry & Osteen,

2003), the inclusion of the protein in EVs could represent an additional level of regulation for matrix remodelling during processes such as ovulation and oocyte maturation.

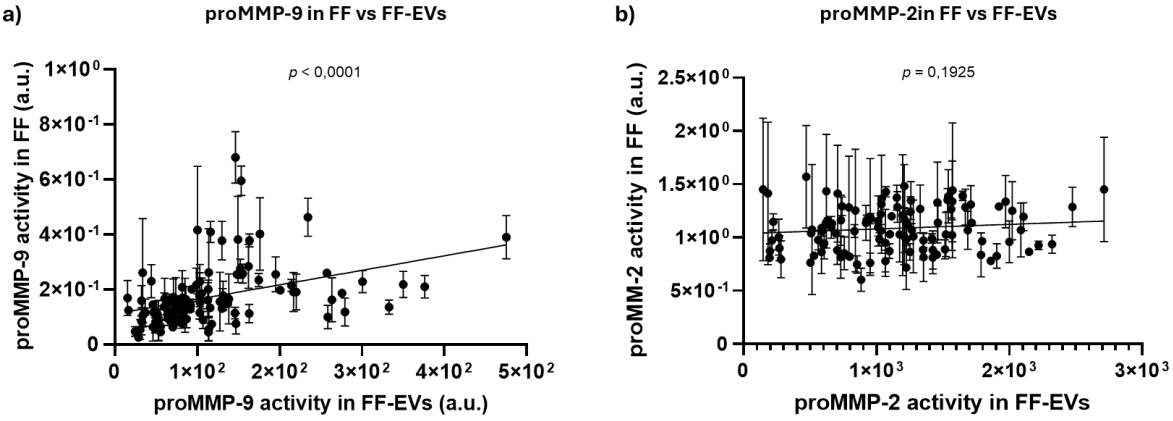

Figure 27 **Simple linear regression of MMP-2 and MMP-9 expression in FF vs FF-EVs.** (a) Scatter plots with simple linear regression show a significant positive correlation between proMMP-9 activity in FF and FF-EVs ($p < 0.0001$). (b) No statistically significant correlation in proMMP-2 expression was found between FF and FF-EVs ($p = 0.1925$).

1.2.3.3 Age-dependent MMP activity in FF-EVs

In order to investigate the influence of maternal age on MMP compartmentalisation, a comparative analysis of MMP-2 and MMP-9 levels within the FF-EVs was conducted according to age ranking. Women undergoing ART were stratified and divided into four age groups (25-30, 31-35, 36-40, 41-44). Although there were no significant differences in expression for any of the gelatinases between the groups, a tendency for the mean content of proMMP-9 and proMMP-2 to decrease in the three youngest age groups was observed. The correlation is reversed for the oldest age group (41-44), where the average level of the proenzymes increases. No trend is observed for MMP-2 expression, confirming the high variability between samples (Figure 28). Although these data are only insights, more research is warranted to evaluate the age-dependent modulation of MMPs content in EVs on communication within the ovarian microenvironment.

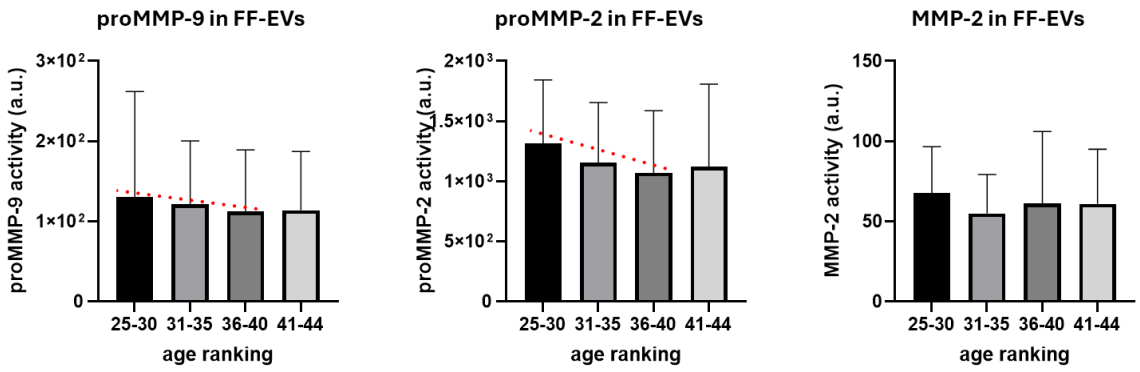

Figure 28 **Expression of gelatinases in FF-EVs across age groups.** Graphs show levels of (a) proMMP-9, (b) proMMP-2, and (c) active MMP-2 in FF-EVs from women undergoing ART stratified by age. A decreasing trend (dashed red line) is observed for both proMMP-9 and proMMP-2 with increasing age in the three youngest groups, while active MMP-2 levels remain unchanged.

1.2.4 Correlations with the Inflammatory Status of Women Undergoing ART

Chronic, subclinical and systemic inflammation can negatively affect fertility by impairing reproductive function. Moreover, inflammation can alter the follicular microenvironment, interfering with oocyte maturation and reducing oocyte quality, with a direct impact on the possibility of successful ART outcomes. C-reactive protein (CRP) is a known marker of systemic inflammation. It is produced in the liver in response to stimulation by inflammatory mediators, such as pro-inflammatory cytokines. Recent studies have shown that elevated levels of CRP in serum are associated with an increased risk of pregnancy failure, because of an altered maternal immune tolerance towards the embryo (Weghofer et al., 2020), also in women with unexplained infertility undergoing ovarian stimulation (Zhang et al., 2023). A study on n=875 women undergoing ART also showed that higher serum CRP levels (>3 mg/L) were associated with a significantly lower pregnancy rate than in women with lower CRP values (<1 mg/L) (Gavrizi et al., 2022).

Since FF is largely derived from serum exudate (Pan et al., 2024), the systemic inflammatory state may also be reflected locally in the follicular microenvironment. Consequently, CRP has been proposed as a potential indicator of inflammation also at the level of the FF. To assess the protein expression of CRP, n=126 FF samples were analysed by western blotting (Figure 29). The immunoreactive bands were quantified to assess the relative signal intensity corresponding to CRP protein levels and compare the expression between different FF samples. The marked

variability in band intensity may be attributable to a combination of individual biological factors that influenced CRP expression. Moreover, the high heterogeneity in CRP levels in patients' FFs reflects the complexity of the follicular environment, influenced by numerous patient-specific variables.

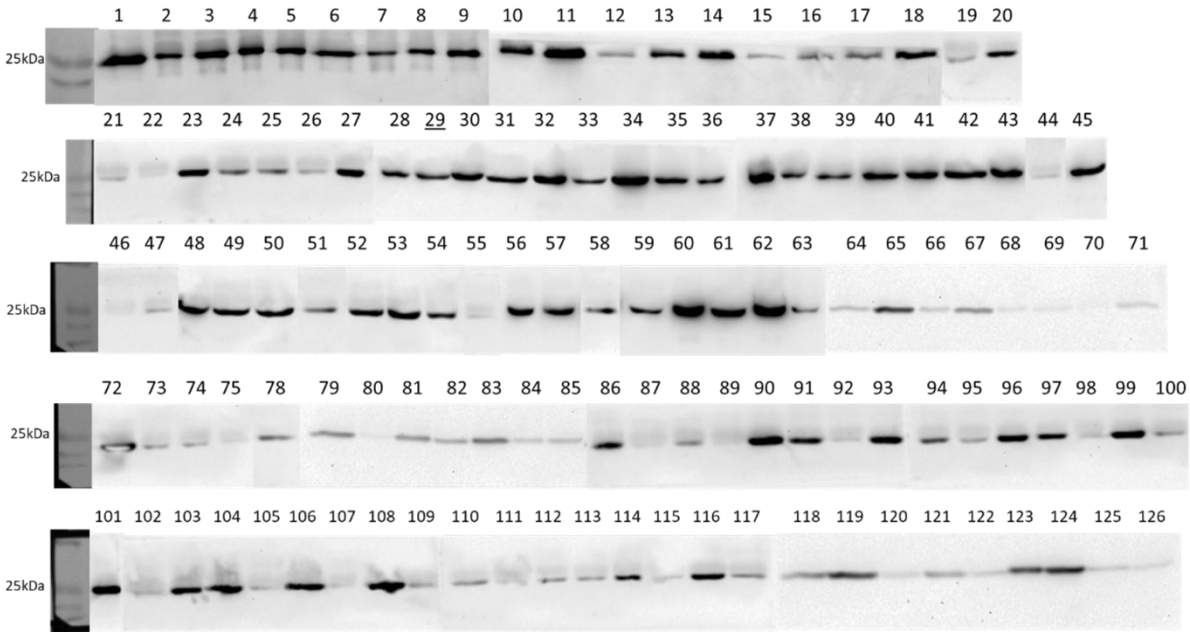

Figure 29 **CRP protein expression levels in FF of women undergoing ART.** Western blottings were performed to detect C-reactive protein expression in n=126 follicular fluid samples from women undergoing ART. The immunoreactive bands correspond to CRP (25 kDa). *ImageJ* software was used to quantify the densitometric immunoreactive bands.

1.2.4.1 Correlation between CRP expression levels in FF and pregnancy rate

CRP expression levels in FF were analysed to investigate a possible association with pregnancy rate. As shown in Figure 30a, CRP levels were not significantly different between patients with a negative β-hCG test and those with a positive test, suggesting no correlation between the levels of the protein and the outcome of embryo implantation. This result may be consistent with the study by Gavrizi et al. (2022), which showed that high levels of CRP in the blood did not influence the clinical pregnancy rate. However, when stratifying the patient cohort with a β-hCG positivity, no differences in CRP levels were observed between those who carried a pregnancy to term and those who had an abortion (Figure 30b). This result is in contrast with the same work mentioned above, which showed that high serum CRP levels were associated with an increased risk of pregnancy loss in women with unexplained infertility. Thus, CRP levels in follicular fluid may not reflect systemic levels, and FF-CRP fluid CRP may not directly

influence pregnancy outcomes. Further research is needed to clarify the significance of CRP presence in the follicular environment.

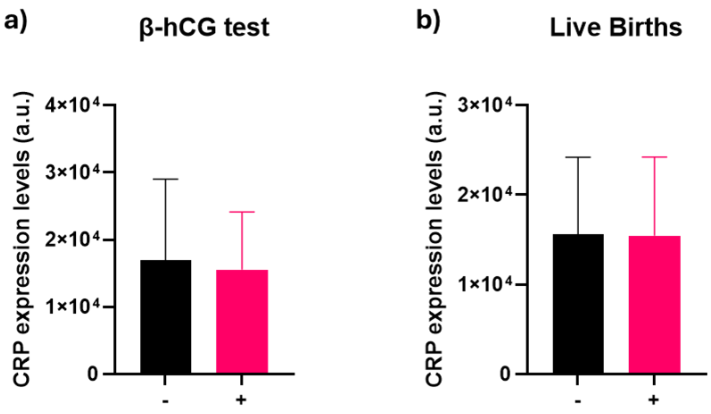

Figure 30 **CRP levels in the FF in relation to fertility treatment outcome.** (a) Comparison between patients with a negative (-) and positive (+) β-hCG test. (b) Comparison between patients with positive β-hCG who did not carry a pregnancy to term (-) and those who had a birth (+). No significant differences were observed between the groups.

1.2.4.2 Age-dependent C-reactive protein expression levels in follicular fluids

Increased CRP correlates with ageing (Tang et al., 2017). In women undergoing ART, an increase in serum CRP is related to age (Khancha & Nosenko, 2024). To investigate whether the inflammatory status is correlated with age, n=126 patients were stratified into four age groups (25-30, 31-35, 36-40 and 41-44 years). A positive trend between increasing CRP levels and increasing age was observed (Figure 31a). Unfortunately, statistical significance did not emerge (Figure 31b), probably due to the high individual variability in expression levels and the sensitivity of the analytical method. These insights suggest a possible link between reproductive ageing and high inflammation. Further studies are needed to determine the association between CRP expression within the follicular microenvironment and its impact on age-dependent reproduction.

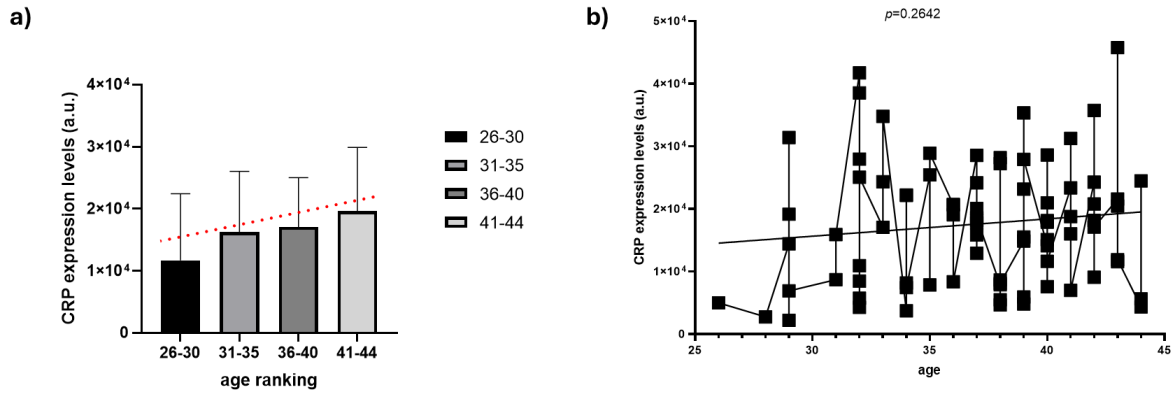

Figure 31 **Age-dependent CRP expression levels in FF samples.** (a) To assess whether inflammatory status varied with age, patients (n=126) were stratified into four groups (25-30, 31-35, 36-40, 41-44 years). A trend (red dashed line) was observed for CRP levels to increase with age. (b) Despite the positive trend, simple linear regression analysis between CRP expression and advancing age did not reveal statistical significance (p=0.2642).

1.2.4.3 Correlation between gelatinase activity and inflammatory status

Gelatinase activity generally correlates with an increased inflammatory state, especially with advancing age (Cancemi et al., 2020). A correlation analysis was conducted to test whether the activity levels of MMP-2 and MMP-9 assessed by zymography correlated with the expression of CRP in follicular fluid. Linear regression analysis showed a positive and statistically significant correlation (p=0.0149) between the expression of MMP-9 in its zymogenic form and CRP levels in the FF (Figure 32). However, no co-dependency correlation was observed between proMMP-9 and CRP (Pearson coefficient r = 0.1510, p = 0.0942). This indicates that the expression of the two proteins is positively correlated but may depend on different and independent mechanisms. From a biological point of view, the significance of this result could be associated with the function of MMP-9, which is closely associated with the immune response. MMP-9 is, in fact, secreted and regulated predominantly by immune cells (Al-Roub et al., 2023), thus, its expression could be a marker for oocyte quality related to the inflammatory state in the follicular microenvironment. In contrast, no significant correlation was observed between the expression of proMMP-2 and CRP, suggesting a lack of dependency between the two proteins. This finding is consistent with the fact that proMMP-2 is constitutively produced by follicular cells and therefore not closely related to inflammation levels. Finally, no significant correlation emerged between CRP levels and gelatinolytic activity detected in EVs isolated from follicular fluids (data not shown), suggesting that the expression and compartmentalisation of MMPs within EVs are not dependent on the inflammatory state.

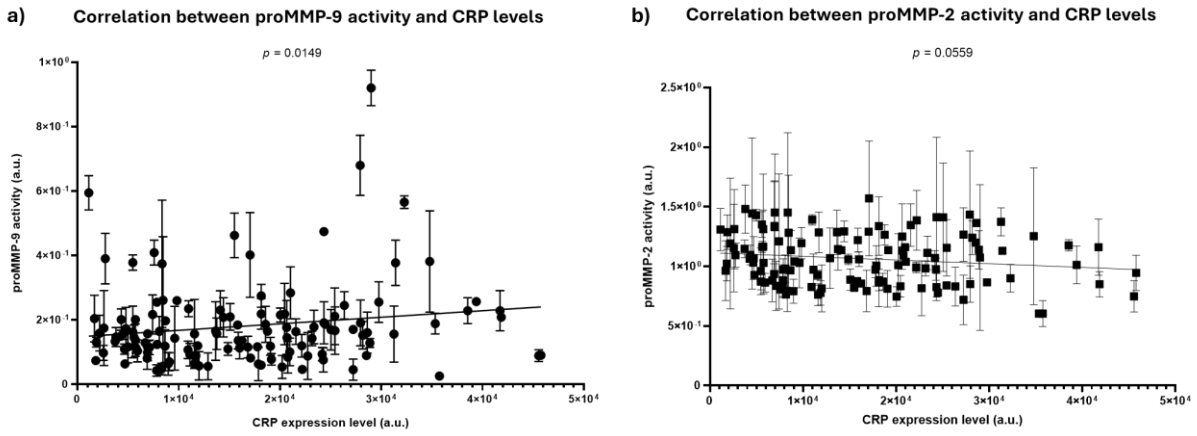

Figure 32 **Correlation between gelatinase activity and CRP expression in FF.** (a) Scatter plots with simple linear regression show a significant positive correlation between proMMP-9 activity and CRP levels in FF (p=0.0149). (b) No statistically significant correlation between proMMP-2 expression and CRP expression was found (p = 0.0559).

1.3 Influence of Follicular Fluid in Endometrial Receptivity

Follicular fluid may be involved not only in oocyte maturation but also in the post-ovulatory phases. Some studies support the hypothesis that FF may have positive influences on embryo implantation by modulating endometrial receptivity. Intrauterine injection of autologous FF in subfertile women undergoing ART, although showing no adverse effects in clinical pregnancy, no significantly higher implantation rate was found (Hamdi et al., 2018; Hashish et al., 2014). It would be particularly relevant to functionally investigate, under controlled conditions, whether FF directly contributes to enhancing embryo implantation by modulating endometrial receptivity. *In vitro* studies on endometrial cells could provide a controlled and effective experimental model for analysing the functional effects of follicular fluid on endometrial receptivity. Moharrami et al. (2020) showed that treating primary cultures of endometrial stromal cells with FF resulted in increased expression levels of genes (e.g. *HOXA10, HOXA11, LIF, ITGB3,* and *ITGAV)* involved in the successful implantation of blastocysts. These genes are implicated in stromal adhesion and remodelling, regulated by a complex network of endocrine signals (e.g. progesterone and oestrogen) and paracrine/autocrine mediators, including cytokines (Du & Taylor, 2015; Kimber, 2005). Considering the complex composition of FF, which includes growth factors (IGF-1, IGF-2 and VEGF), hormones (FSH, LH, oestrogen and progesterone) and cytokines (Pan et al., 2024), it is plausible that FF may

significantly influence cell proliferation and cell migration at the uterine level, hereby contributing to endometrial remodelling.

To assess whether FF can influence endometrial receptivity by modulating cell migration, two cell lines were used as a model of receptive (RL95-2) and non-receptive (HEC-1-A) human endometrial epithelium (Figure 33). The two models have been employed in studies on cell adhesion, as they are particularly well suited to simulate embryonic rooting using trophoblastic cell spheroids (Harduf et al., 2007). The major morphological difference between the two lines lies in epithelial polarity, which is absent in RL-95 and marked in HEC-1-A. RL-95-2 are therefore considered a receptive model as the receptive endometrium transiently loses cell polarity to favour interaction and trophoblastic adhesion. In contrast, the polarised epithelial phenotype of HEC-1-A is typical of a proliferating endometrium that is repulsive to embryo implantation (Harduf et al., 2007).

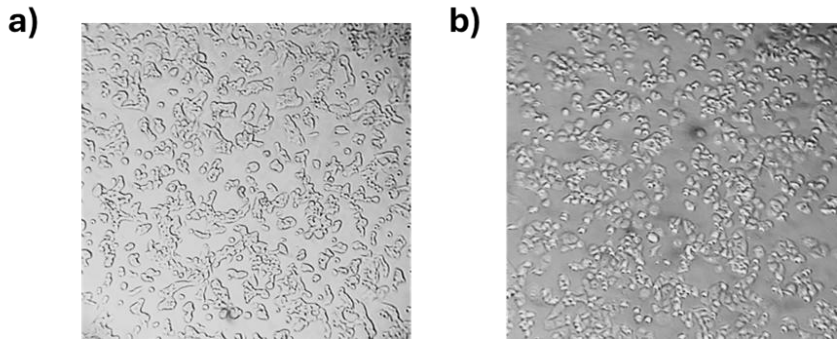

Figure 33 **Morphology of human endometrial epithelial RL95-2 and HEC-1A cell lines**. a) RL92-5 and b) HEC-1-A cell lines observed under optical microscopy. RL95-2 cells exhibit a non-polarised phenotype as a receptive endometrium model. HEC-1A cells display a polarised epithelial morphology as a non-receptive endometrium model.

The results obtained from the analysis of gelatinolytic activity in follicular fluid suggested a negative correlation between MMP-2 and MMP-9 and advancing age. Interestingly, in FF-EVs, this trend was only maintained in the younger groups of women (28-39 years) and lost in older women. The higher MMP activity in younger women may positively affect a possible FF-effect on endometrial receptivity. In contrast, the inflammatory status assessed by CRP expression followed a positive trend line with increasing age of women undergoing ART. Thus, a lower level of inflammation in younger women could favour endometrial receptivity. These data led to the consideration of the effects of FF on endometrial cell receptivity in younger women.

To evaluate the effect of young women FFs on endometrial receptivity, the two cell lines RL95-2 and HEC-1-A were treated with follicular fluids derived from a cohort of n=42 ART patients aged between 25 and 38 years. In this age range, ovarian function is generally considered optimal and associated with sufficient ovarian reserve. Fluid composition is also less affected by age-related factors such as oxidative stress and altered hormone and cytokine production, making interpretation of the functional effects of FF treatment more reliable. To assess the effects on cell migration following FF treatment, cell lines were subjected to wound healing assay (or scratch assay) and treated for 24h with follicular fluid (n=42). To assess the migration rate independent of cell-specific traits, wound closure rates, monitored at 6h and 24h, were normalised to untreated controls, thereby reducing technical variability.

Comparing qualitatively the effects on the two cell lines, FF treatment appears to promote gap closure over time in HEC-1-A but not in RL95-2, suggesting a promotion of cell migration in the epithelial non-receptive endometrium model (Figure 34).

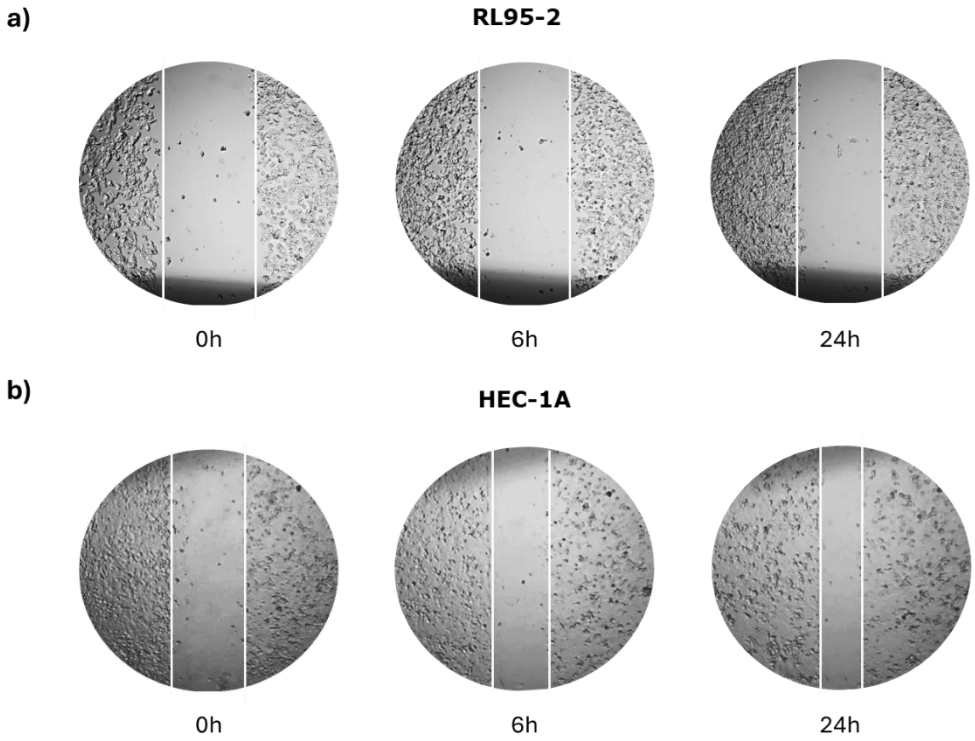

Figure 34 **Analysis of cell migration using the wound healing assay.** Representative images of the cell-free area (outlined by parallel black lines) in endometrial a) RL95-2 and b) HEC-1-A cell lines, treated with follicular fluid at 0h, 6h, and 24h. Each of n=42 FFs was employed as treatment on both cell lines. The experiment was performed in triplicate for each FF and both cell lines. FF treatment appears to promote gap closure over time in the non-receptive endometrium model HEC-1-A but not in the receptive RL95-2 cell line. Images were acquired

under identical field and magnification conditions at each time point. The wound surface was measured using ImageJ software.

This finding is confirmed by the quantification of wound closure: the graphs in Figure 35 show the pattern of cell migration (represented as % wound healing normalised to untreated controls) in HEC-1-A and RL95-2 after 6h and at 24h. After 6h of treatment, no significant differences were observed between the two cell lines, suggesting that in the initial phase, FF exerts no significant differential effects on migration. On the contrary, at 24h, HEC-1-A cells show significantly greater migration than RL95-2, indicating a more pronounced and prolonged response to FF treatment.

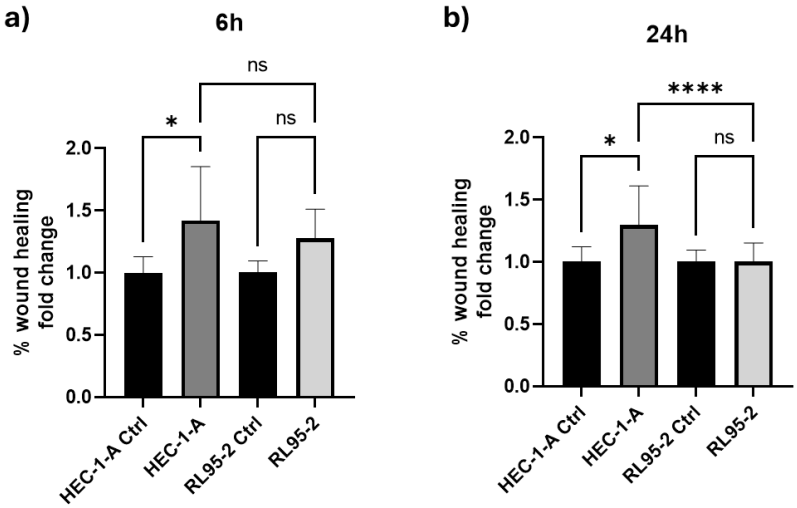

Figure 35 **Quantification of cell migration after FF treatment in HEC-1-A and RL95-2 using the wound healing assay.** The bar graphs show the percentage of wound closure at 6h (a) and 24h (b) after FF treatment, normalised to control (fold change). In HEC-1-A cell line, migration increased significantly compared to the control at both 6h and 24h. RL95-2 cell migration showed no variation relative to controls at either time point. No significant difference is observed between the two cell lines at 6h. At 24h, HEC-1-A cells exhibit a significantly greater wound closure compared to RL95-2 cells, indicating an enhanced migratory response. * $p < 0.05$ **** $p < 0.0001$

1.3.1 Correlation between stimulation of cell migration and patient age

To explore a possible correlation between the ability of follicular fluid to stimulate cell migration and maternal age, the cohort of n=42 patients was divided into three age groups: 26-30 years (n=7), 31-35 years (n=21) and 36-38 years (n=14).

Analysis of the data shows a general trend of decreasing follicular fluid-induced migratory capacity (expressed as a % of wound closure) with increasing maternal age. This negative trend can be observed in both the RL95-2 and HEC-1A cell lines after 6 hours of treatment and is also evident in HEC-1A at 24 hours. In particular, the trend line is more pronounced in HEC-1A, being consistent across the two observation times. In contrast, in RL95-2, the negative trend is present at 6 hours but tends to disappear at 24 hours (Figure 36).

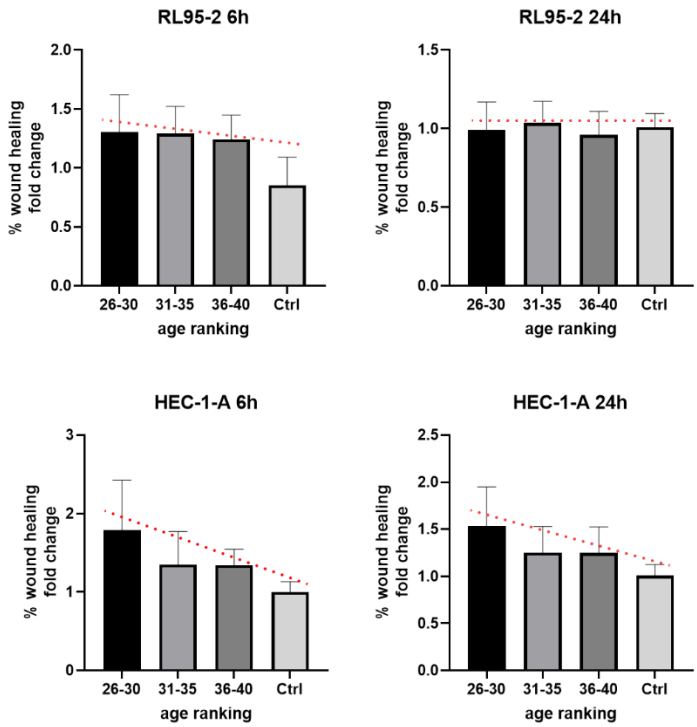

Figure 36 **Cell migration in RL95-2 and HEC-1A endometrial cells treated with FF according to age ranking.** The graphs show the percentage of wound closure (% wound healing) at 6h and 24h after FF treatment from patients clustered in three age groups: 26-30, 31-35 and 36-40. The control group (Ctrl) consists of untreated cell lines. A general trend of reduced cell migration (red dashed lines indicate the negative trend) with increasing maternal age is observed, lost in RL95-2 and maintained in HEC-1-A at 24h after treatment. Data are expressed as mean ± standard error.

These results indicate that FF from younger women exerts a greater pro-migratory effect on endometrial cells, with a more consistent and prolonged effect observed in the HEC-1A cell line. In contrast, FF from older women gradually loses its ability to promote cell migration. Overall, the data show a negative association between increasing maternal age and the ability

of follicular fluid to promote endometrial cell migration, potentially reflecting age-related differences in its molecular composition or signalling capacity.

1.3.2 Follicular fluid-induced modulation of E-cadherin in endometrial cells

It is well known how endometrial remodelling involves the progressive loss of cell polarity of endometrial epithelial cells. Endometrial remodelling and plasma membrane transformation (PMT) involve processes closely related to those of the well-known epithelial-to-mesenchymal transition (Whitby et al., 2020). During this process, the degradation of the extracellular matrix increases, enhancing the migratory and invasive capacity of the cells. Moreover, EMT is a biological process in which epithelial cells lose their intercellular junctions and cell polarity. This event leads to a downregulation of epithelial markers and an upregulation of mesenchymal markers. E-cadherin (E-CAD) is an adhesion molecule involved in the assembly and stabilisation of adherens junctions, and its expression decreases upon induction of EMT (Fang & Kang, 2021).

Having a markedly polarised phenotype, HEC-1-A endometrial cells are characterised by high basal E-CAD expression (Harduf et al., 2007). As shown by wound healing assays, FF stimulated cell migration in HEC-1-A cells, maintaining the effect for up to 24h. To test whether this increase in cell migration also corresponds to the induction of EMT processes, E-CAD (135 kDa) expression was assessed by immunoblotting in the cell lysate of HEC-1-A cells subjected to scratch assay, after 24h of FF treatment. Each cell lysate was obtained from cells treated with one of the n=42 FF from women previously selected based on age (Figure 37).

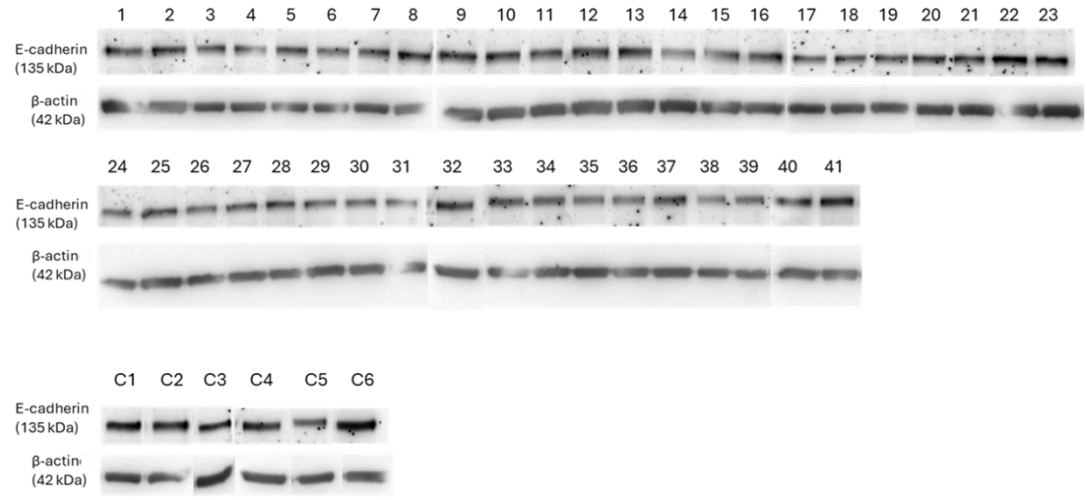

Figure 37 **Western blot analysis of E-cadherin expression in HEC-1A cell lysates after FF treatment.** Each lane represents an HEC-1-A lysate from cells exposed to a distinct FF sample

(n=41), numbered accordingly and arranged in order of increasing maternal age. The blot was probed with an anti-E-cadherin (E-CAD) antibody to assess protein expression levels, and β-actin was used as a housekeeping control. Band intensities were quantified using ImageJ software, and E-CAD expression values were normalised to the corresponding β-actin levels and the untreated controls.

Treatment with the different FFs led to a reduction in E-cadherin expression in HEC-1A cells (Figure 38). This result supports the hypothesis that the increase in migratory capacity observed in HEC-1A cells after 24h of exposure to FFs is supplemented by a downregulation of E-cadherin (Figure 38a, b), suggesting a possible transition to a mesenchymal phenotype. Interestingly, a positive correlation was observed between E-CAD expression and the age of women undergoing ART (Pearson r = 0.3103, p=0.0455). Moreover, E-CAD expression was significantly higher in cells treated with FF from the older age group (36–40 years) compared to those treated with FF from younger women (25–30 years) (p=0.0114) (Figure 38).

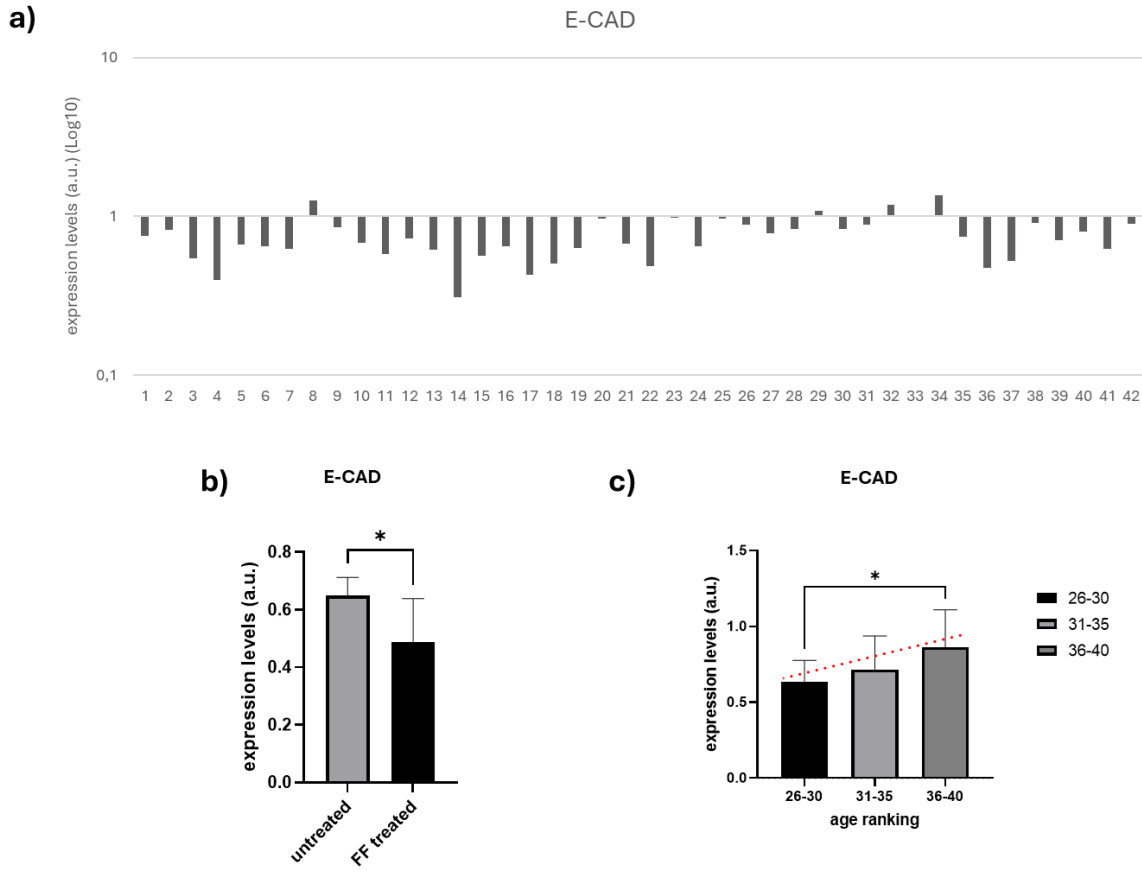

Figure 38 **Expression levels of E-CAD in HEC-1A cell lysates after 24h FF treatment for wound healing assay**. E-CAD expression levels in HEC-1A cells treated with FF from n=42 women undergoing ART. a) Each bar represents E-CAD level normalised to β-actin and normalised to untreated control (dashed line at value = 1). Data are displayed on a logarithmic scale (log_{10}), and FF treatment is ordered by increasing maternal age. b) The general

comparison between FF treated and untreated HEC-1-A cell line shows a reduction in E-CAD expression following treatment, suggesting a downregulation of epithelial markers. c) Bar graph of the average E-CAD expression levels in HEC-1-A cells treated with FF from n=42 women divided into three age groups (26-30; 31-35; 36-40): a significant difference in protein expression is observed between samples with FF from the youngest group (26-30) and the oldest group (36-40). The red dashed trend line indicates a positive correlation between E-CAD expression and the age of FF from donors. * $p < 0.05$

In summary, the collected results support the hypothesis that Follicular fluid affects cell migration and E-cadherin expression in endometrial cells. After 24 hours of treatment, HEC-1-A cells, a model of nonreceptive endometrium, show greater migratory capacity than RL95-2 cells, considered a receptive model of endometrium. HEC-1-A are less differentiated cells, and FF could stimulate their plasticity and predisposition for structural reorganisation of the epithelium, compared with an already differentiated epithelium. This suggests the potential of FF to stimulate favourable functional mechanisms for fertilisation and implantation, especially in less differentiated tissues. Furthermore, analysing the results on cell migration according to women's age, it appears that FF from younger women stimulates more intense and consistent migration in the same cell type. In contrast, follicular fluid from older women induces a weaker effect. These data indicate an association between maternal age and the ability of follicular fluid to promote endometrial remodelling. Finally, to explore the effect of FF from a molecular perspective, it was found that it induces a significant reduction in E-cadherin expression, suggesting the activation of processes similar to the epithelial-mesenchymal transition. This finding strengthens the hypothesis that FF can induce greater cell differentiation. However, the most interesting finding is that residual E-CAD expression correlates positively with maternal age: FFs from older women result in higher levels of E-CAD than those from younger women, indicating a greater ability to induce loss of epithelial phenotype in younger samples, consistent with the increased migration observed.

2 Analysis of Biological Fluids from Multiple Sclerosis Patients

The analysis of multiple biological matrices from patients with multiple sclerosis offers the opportunity to explore several aspects of the immunopathology of the disease and its progression. Indeed, a multifactorial approach was applied to analyse biological fluids, such as cerebrospinal fluid (CSF), serum, and plasma, considering their different biochemical composition as well as different physiological and functional roles in the central nervous system. The combined evaluation of the three fluids allowed us to assess how the different tissues reflected the heterogeneity of MS related to central and peripheral inflammatory processes.

The CSF is in direct contact with the brain parenchyma and the spinal cord, then it is the most representative of the processes involved in the breakdown of the blood-brain barrier and the infiltration of leukocytes. Therefore, CSF analysis can provide information closely related to disease activity within the CNS, including MMP-mediated tissue remodelling and inflammation, and thus can enable a more accurate prognosis of the disease. However, lumbar puncture for CSF sampling remains an invasive practice, so the common goal is to search for new biomarkers from more accessible sources and ensure reproducibility. Serum represents the most comprehensive sample type from a clinical point of view, but also in translational research. Although not strictly related to the CNS, serum can provide information on the systemic immune response that characterises the development and progression of multiple sclerosis. In addition, the activity of circulating MMPs, regulated by peripheral mediators potentially involved in the disease, may shed light on the bidirectional communication between the peripheral and central immune system, including the recruitment and activity of immunocompetent leukocytes at the level of the BBB. Plasma, while similar to serum, preserves coagulation factors and is optimally suited for extracellular vesicles isolation and the analysis of soluble proteins. Since plasma is an active pool of circulating biomarkers, it can report both systemic immune activation and, through vesicular cargo, signals from the CNS, thus being a useful candidate for liquid biopsy techniques in MS.

Through the comparative analysis of these three biological compartments, collected from the same subjects where suitable, the present study aims to identify both common and distinct molecular signatures, enhancing the search for reliable biomarkers of disease activity, clinical status, and pathophysiological processes in MS.

2.1 Demographic and Clinical Characteristics of Patient Cohort

The study included subjects with a planned lumbar puncture for their diagnostic work-up at the Department of Biomedicine, Neurosciences and Advanced Diagnostics (BiND) of the University of Palermo. It was conducted on a cohort of patients diagnosed with multiple sclerosis (MS, n=62) who were systematically compared to subjects with other neurological diseases (OND, n=31), collectively considered as controls. The cohort was well-characterised according to clinical and demographic features (Table 3). Clinical diagnosis, based on the 2017 McDonald criteria (Thompson et al., 2018), was applied to classify patients in RRMS (n=59), PPMS (n=3), and CIS (n=2). Further clinical stratification was applied according to disease phase where feasible, distinguishing patients in relapse (n=36) and in remission (n=23). Moreover, NC subset was divided in two subgroups: subjects with other inflammatory neurological disorders (OIND, n=4) and non-inflammatory neurological disorders (ONIND, n=25). The analysis involved biological fluids collected from the patients, including cerebrospinal fluid (CSF), serum, and plasma. Sample sizes were as follows: n=49 MS vs. n=27 OND CSF samples, n=25 MS vs. n=12 OND serum samples, and n=30 plasma samples in total.

Table 3 **Clinical and demographic features of the studied cohort**

	Patients (MS)		**Controls (OND)**		
	n=62		n=31		
	RRMS	**PPMS**	**CIS**	**OIND°°**	**ONIND°°°**
No. of patients	59	3	2	4	25
Sex (female)	42	0	1	3	13
Age°	34; 17-54	36; 27-44	35; 29-41	55	54; 24-84
EDSS°	2; 0.0-5,5	3; 1,5-6,0	NR	NA	NA
Disease duration (months)°	30; 1-300	16; 12-23	NR	6; 2-9	15; 1-96
No. of subjects in relapse	36	0	1	NA	NA

RRMS, relapsing-remitting multiple sclerosis; PPMS, primary progressive multiple sclerosis; CIS, clinically isolated syndrome; OIND, other inflammatory neurological disorders; ONIND, other non-inflammatory neurological disorders; EDSS, Expanded Disability Status Scale; NR, not reported; NA, not applicable. ° Values are expressed as medians with minimum and maximum ranges indicated. °° The OIND cases correspond to patients diagnosed with myelitis. °°° ONIND subjects presented with a heterogeneous group of non-inflammatory neurological conditions, including chronic cerebrovascular diseases, ischaemic stroke, hydrocephalus, parkinsonism, diabetic polyneuropathy, degenerative ataxia, dementia, conversion disorder, brain tumour, and autoimmune vasculitis.

2.2 CNS Compartment: Cerebrospinal Fluid Analysis

Cerebrospinal fluid is one of the main diagnostic matrices for inflammatory diseases of the central nervous system, such as multiple sclerosis. CSF analysis allows the detection of oligoclonal bands, which indicate immune system activation in the central nervous system, and often complements clinical and radiological analyses for MS diagnosis (Link & Huang, 2006). To date, there is no single pathognomonic test that alone confirms the diagnosis of MS, and this often complicates the correct interpretation of results, contributing to frequent diagnostic errors (Kennedy et al., 2024). A retrospective study conducted by Kaisey et al. (2019) reported that around 20 per cent of patients referred to specialist centres with a previous diagnosis of MS did not have the disease, but other pathological conditions. Misdiagnosis can mean unnecessary treatments, avoidable side effects and a delay in treating the real disease. For this reason, a multisystem approach to CSF analysis could open new directions for the discovery of biomarkers, contributing to greater accuracy in the diagnosis and prognosis of the disease.

Cerebrospinal fluid is a biological compartment that directly reflects pathological processes occurring within the CNS. In this study, multiple complementary techniques were applied to explore the molecular composition of CSF and identify potential biomarkers of disease. Proteomic profiling by LC-MS/MS revealed MS-specific expression patterns, with IGFBP-2 emerging as a promising biomarker signature for MS diagnosis. Gelatin zymography was used to assess the activity of matrix metalloproteinases (MMP-2 and MMP-9), uncovering expression differences associated with inflammation and disease subtype. Further analyses investigated gelatinase isoforms and MMP compartmentalisation in extracellular vesicles, offering new insights into their role in neuroinflammation. Altogether, these approaches highlight the relevance of CSF analysis in understanding MS pathogenesis and improving diagnostic accuracy.

2.2.1 CSF Proteomic Profiling

Omics analyses are effective tools for identifying novel molecular pathways and quantifying differentially expressed molecules, thus enabling the identification of multiple markers potentially predictive of disease course. Mass spectrometry is an ultimate analytical technique for characterising biological samples. Proteomic analysis of CSF offers a more specific representation of alterations related to CNS damage than serum, and it allows the exploration of molecular alterations relevant to disease immunopathogenesis, to identify new diagnostic or prognostic biomarkers of disease progression.

LC-MS/MS mass spectrometry was applied to analyse CSFs from n=14 MS patients and n=13 ONDs. Proteomic analysis identified a total of n=892 proteins across all analysed CSF samples. Comparison between MS subjects and neurological controls revealed n=118 proteins differentially expressed (fold change as $\log_2$(OND/MS)) in a statistically significant manner, based on false discovery rate (FDR) threshold and significance cutoff of $-\log_{10}(p\text{-value}) > 1.3$ (equivalent to $p < 0.05$) (Figure 39).

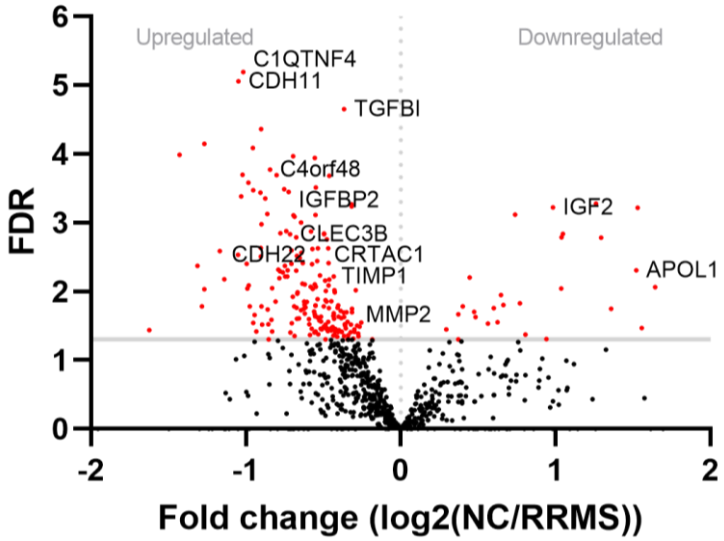

Figure 39 **Volcano plot of proteomic analysis in CSFs from MS and OND patients.** Volcano plot representing the differential expression of proteins identified in a proteomic analysis. The X-axis represents the fold change ($\log_2$(OND/MS)) between the MS and OND groups. The Y-axis shows the false discovery rate (FDR), where the horizontal line corresponds to a significance threshold of $-\log_{10}(p\text{-value}) > 1.3$ (equivalent to $p < 0.05$). Red dots represent proteins with significantly different expression.

Of the 892 proteins identified, 820 were found to be commonly expressed in both the MS and OND groups. Among them, n=34 proteins were significantly upregulated and n=12 downregulated in MS patients, listed in Fig. 21, b. Moreover, n=72 proteins were uniquely detected in MS samples and absent in controls. Interestingly, no proteins were uniquely expressed in the controls (Figure 40).

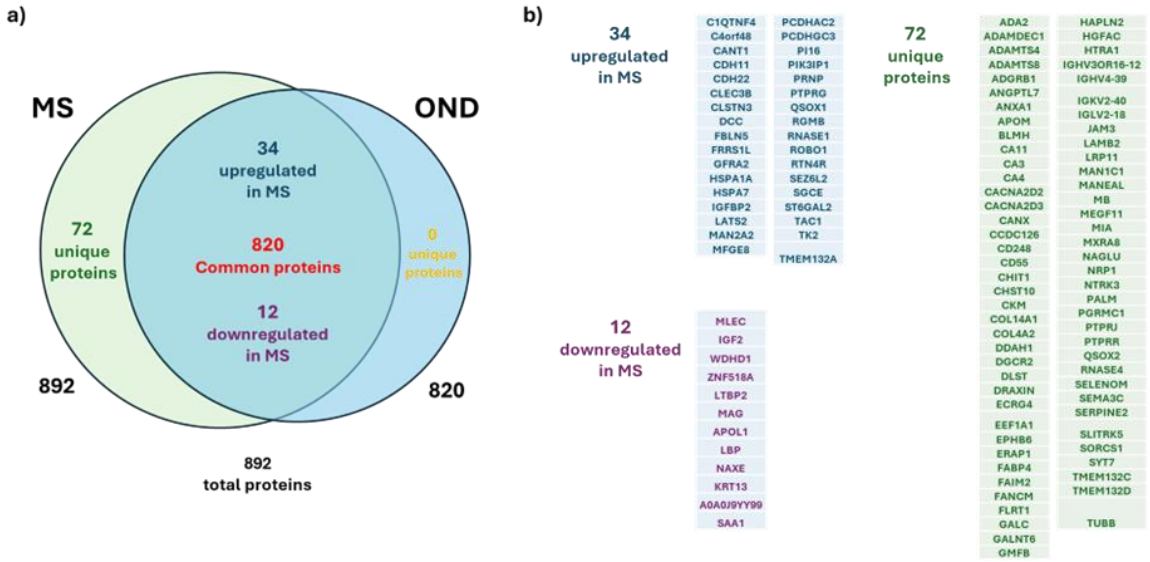

Figure 40 **Venn diagram of differentially expressed proteins between MS and OND groups.** (a) The Venn diagram summarises the proteomic comparison between CSFs from MS and OND patients. Out of n=892 total proteins identified, n=820 were commonly expressed in both groups. Among these, n=34 proteins were significantly upregulated and n=12 were downregulated in the MS group; n=72 proteins were uniquely detected in the MS group, while no unique proteins were found in OND. (b) The corresponding lists of upregulated, downregulated, and MS-unique proteins is reported.

2.2.1.1 Protein Enrichment Analysis

To further investigate the biological functions of the proteins identified in the CSF samples in the proteomic analysis, a Gene Ontology (GO) enrichment analysis was performed, with emphasis on the ontology on biological processes (BP), KEGG and Reactome pathways enrichment using the database and integrated tools of the STRING platform (stringdb.org). This approach allowed us to assess which functional categories were overrepresented relative to all identified proteins (n=892) in the CSFs of MS patients and ONDs.

An initial enrichment analysis based on tissue-specific expression profiles was performed to derive information on the potential tissue origin of the proteins (Figure 41). The outcome of the analysis was n=182 tissues significantly enriched. As expected, the analysis revealed a significant association (FDR≤0.05) between the proteome and CNS-specific compartments, including subarachnoid space, cerebrospinal fluid, and spinal column. Interestingly, proteins typically associated with plasma cells and generally originating in the bone marrow were detected. It is well known that B cells are involved in the destruction of the BBB during neuroinflammatory processes, facilitating the increase in its permeability (G. Kumar & Axtell,

2023). Overall, the enrichment of bone marrow-related proteins, including B lymphocytes, in CSF proteomics supports the hypothesis of a strong immunological component in the MS disease context.

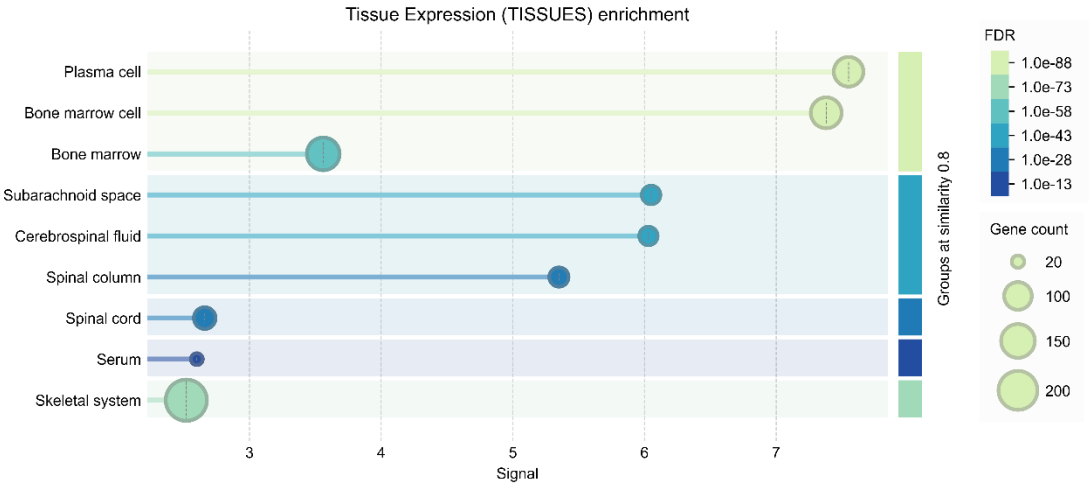

Figure 41 **Bubble plot of Tissue Expression (TISSUE) enrichment.** Functional enrichment analysis was performed on n=892 total proteins from proteomic profiling on CSFs from MS and OND patients. Bubble size corresponds to the number of proteins associated with each tissue (gene count), and bubble colour indicates statistical significance by False Discovery Rate (FDR≤0.05) where lighter colours are associated with higher enrichment. Most notably, many highly significant enrichments are found in immune tissues such as bone marrow and plasma cells, suggesting that there is a significant immunological signature in the proteomic profile.

The Gene Ontology (GO) analysis indicated that there was an extremely high overrepresentation of n=821 GO terms for biological processes (BP) (Figure 42). Among them, the most prominent representation was observed for proteins involved in complement activation and cell adhesion. The same biological themes were observed in the KEGG pathway enrichment analysis, where there were 26 significantly overrepresented pathways, further supporting the involvement of structural and immune processes (Figure 43). The complement system, as a constituent of innate immunity, is involved in numerous neuroinflammatory and neurodegenerative disorders. There have been recent studies describing the bidirectional function of the complement system, wherein on one side it facilitates the removal of myelin debris through phagocytosis by myeloid cells, but conversely, there is abnormal activation of complement with demyelination and neuronal damage (Saez-Calveras & Stuve, 2022). Histological analysis of plaque sites (n=35) in tissue samples obtained from progressive MS patients (n=17) and control donors (n=16) was positive for proteins (regulatory and effector)

engaged in the classical complement pathway (e.g. C3, factor B and C1q) (Ingram et al., 2014). Levels of complement component and activation product in CSF and serum correlate with the severity and progression of MS, as shown in a study that related them to clinical ratings of disease severity, such as the EDSS scale (Oechtering et al., 2024).

The increased expression of adhesion proteins in the CSF can be related to immune activation, especially with the migration processes of immunocompetent lymphocytes and their infiltration into the CNS. Adhesion proteins allow immune cells to bind to the endothelial lining of blood vessels and penetrate the BBB. Elevated numbers of lymphocytes that carry adhesion molecules such as VLA-4 and LFA-1 (three to four times normal) have been reported in a CSF study in patients with multiple sclerosis (Elovaara et al., 2000). This supports the view that adhesion molecules play a part in neuroinflammation by facilitating immune cell migration into the CNS.

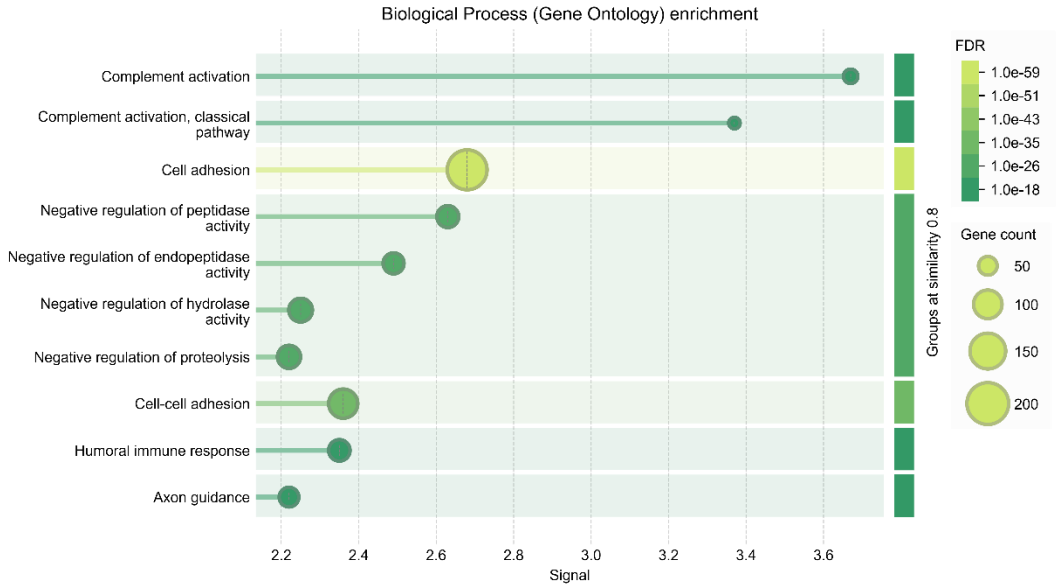

Figure 42 **Bubble plot of Gene Ontology Biological Process enrichment.** Functional enrichment analysis was performed on n=892 total proteins from proteomic profiling on CSFs from MS and OND patients. Bubble size corresponds to the number of proteins associated with each Biological Process (BP), and bubble colour indicates statistical significance by False Discovery Rate (FDR≤0.05), where lighter colours are associated with higher enrichment. Most notably, many highly significant enrichments are found in complement activation and cell adhesion.

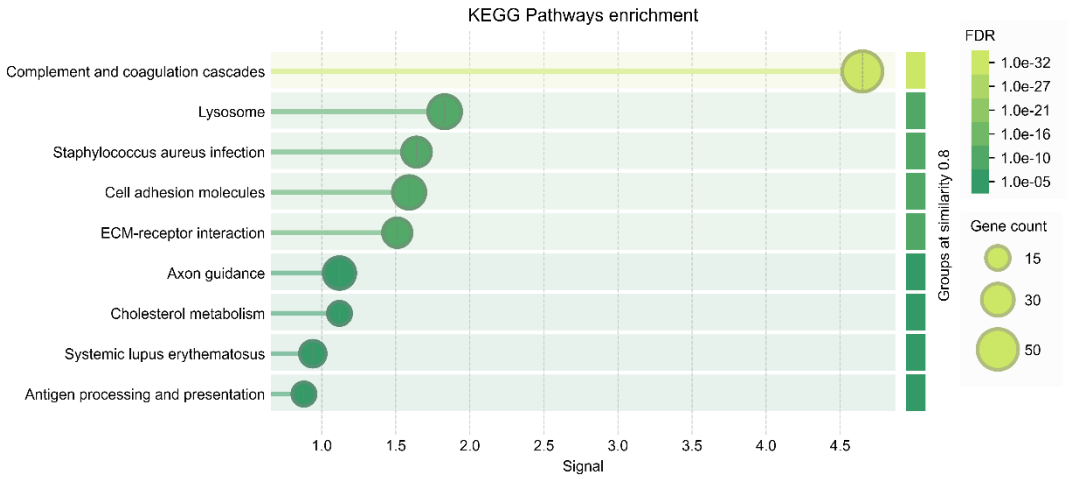

Figure 43 **Bubble plot of KEGG Pathways enrichment.** Pathway enrichment analysis was performed on n=892 total proteins from proteomic profiling on CSFs from MS and OND patients. Bubble size corresponds to the number of proteins associated with each pathway on the KEGG database, and bubble colour indicates statistical significance by False Discovery Rate (FDR≤0.05) where lighter colours are associated with higher enrichment. Most notably, many highly significant enrichments are found in complement and coagulation cascades, encouraging GO:BP analysis.

Finally, the Reactome pathway enrichment analysis revealed n=153 significantly enriched pathways. Among these, particularly interesting is the one related to the regulation of insulin-like growth factor (IGF) transport and uptake by insulin-like growth factor binding protein 2 (IGFBP-2) (Figure 44).

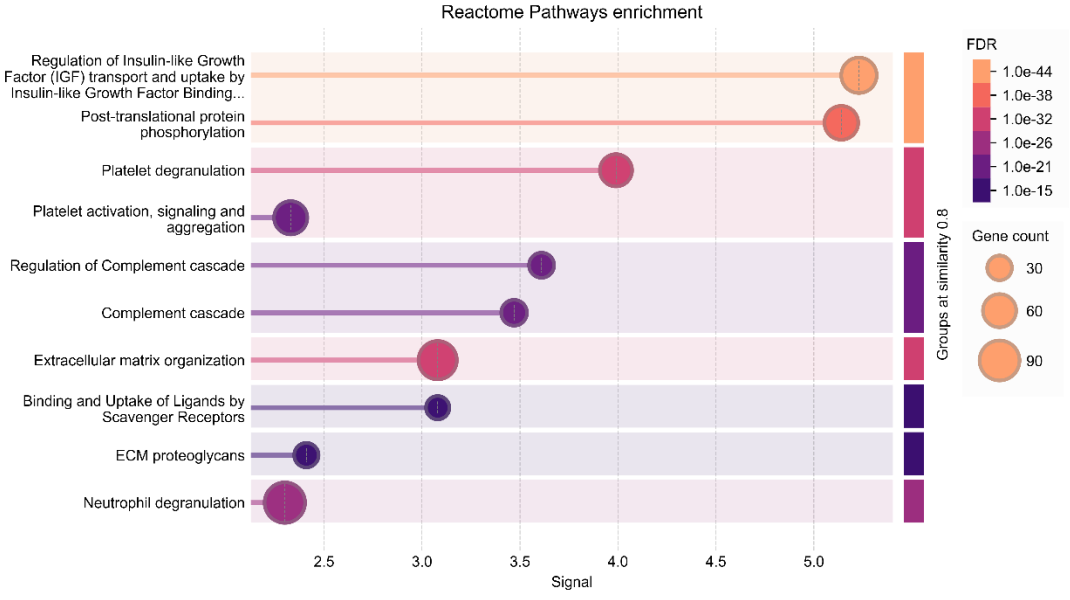

Figure 44 **Bubble plot of Reactome Pathways enrichment.** Functional enrichment analysis was performed on n=892 total proteins from proteomic profiling on CSFs from MS and OND

patients. Bubble size corresponds to the number of proteins associated with each reactome pathway, and bubble colour indicates statistical significance by False Discovery Rate (FDR≤0.05), where lighter colours are associated with higher enrichment. Most notably, many highly significant enrichments are found in the regulation of insulin-like growth factor (IGF) transport and uptake by insulin-like growth factor binding protein 2 (IGFBP-2).

2.2.1.2 Classification of MS by machine learning

Machine learning was applied to evaluate the diagnostic potential of the CSF proteomic profile in distinguishing patients with multiple sclerosis from those with other neurological diseases using a classification model based on the Random Forest algorithm (Svetnik et al., 2003). The algorithm was employed to identify which proteins within the identified CSF proteome could most effectively predict disease status, distinguishing MS from ONDs. Feature importance analysis identified the proteins that contributed most to the classification of the patients (Figure 45). The first most significantly relevant protein that showed high discriminating ability was IGFBP-2, suggesting a role as a diagnostic biomarker and confirming previous Reactome pathway enrichment analyses. IGFBP-2 is involved in cell growth regulation and immune responses; it was previously reported to be implicated in multiple sclerosis (Chesik et al., 2007). Given its considerable significance, the differential expression of this protein deserves further investigation, as reported below.

Other relevant proteins include CRTAC1, CLEC3B, C4orf48 and TGFBI, which are involved in key biological processes highly related to multiple sclerosis, such as immune regulation, cell adhesion and inflammatory response. Cartilage acidic protein 1 (CRTAC1) is an extracellular matrix protein of chondrogenic tissue. Although its precise function in the CNS is still unclear, increased concentration of CRTAC1 in the CSF of patients with multiple sclerosis has been previously documented in proteomic studies (Hammack et al., 2004; Veenstra et al., 2005), suggesting its possible involvement in ECM remodelling and chronic inflammation. Similarly, CLEC3B, a protein involved in fibrinolysis modulation, is potentially involved in tissue remodelling processes during neuroinflammation. Despite the lack of functional studies linking CLEC3B to MS specifically, its consistent detection across proteomic datasets (Hammack et al., 2004; Veenstra et al., 2005) highlights a potential role in the neuroinflammatory microenvironment. C4orf48 is a poorly characterised secreted neuropeptide (Endele et al., 2011), and recent findings suggest its involvement in extracellular matrix deposition and fibrotic signalling (J. Yang et al., 2024). Its connection to multiple sclerosis has not been documented yet, but its relevance in the model could uncover a possible novel role in MS pathophysiology. Lastly, transforming growth factor beta induced (TGFBI) is a target of TGF-

β and is involved in cell adhesion and inflammatory responses. While TGF-β signalling is well known to be involved in MS immunopathogenesis (Esmaeilzadeh et al., 2023), more research is needed to fully understand a possible involvement of TGFBI in MS.

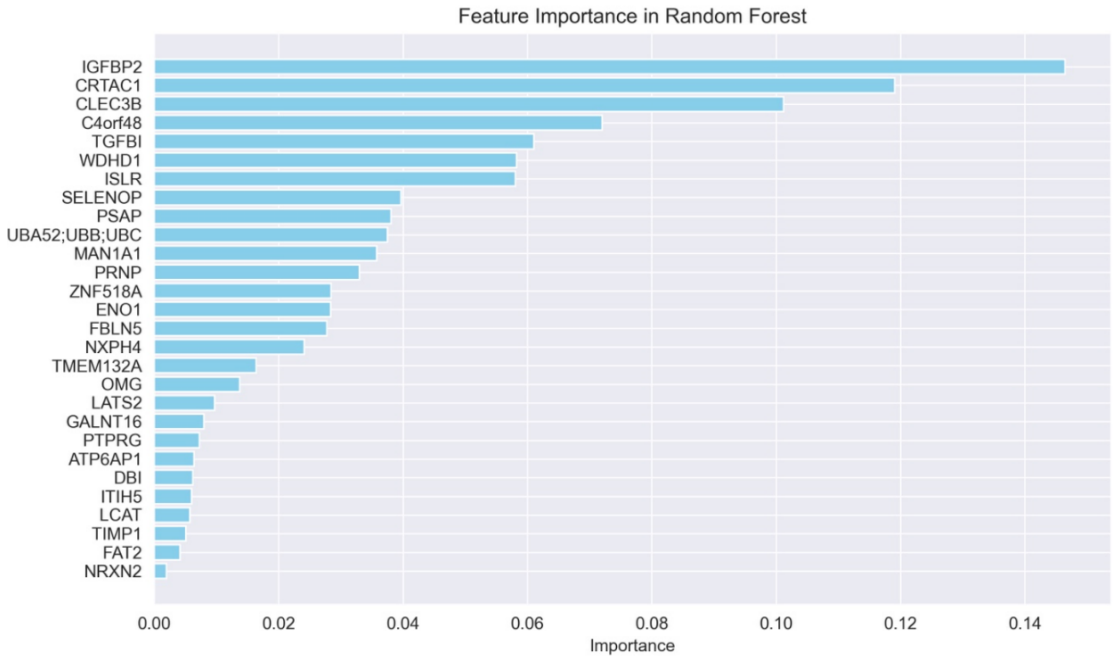

Figure 45 **Feature importance analysis from the Random Forest classification model.** The bar plot shows the relative importance of proteins in distinguishing patients with MS from OND controls, as determined by the Random Forest model. IGFBP2, CRTAC1, CLEC3B, C4orf48 and TGFBI emerged as the most informative proteins, suggesting their potential relevance as candidate biomarkers for MS.

Thus, the model was trained on differentially expressed proteins and evaluated by a confusion matrix, from which a high classification ability is evident. The model correctly classified n=6 MS patients (true positives), with no false negatives; n=2 patients were correctly classified as controls (true negatives), with only one false positive misclassified as MS (Figure 46). Thus, the results indicate an excellent sensitivity of the model in the specific classification of patients.

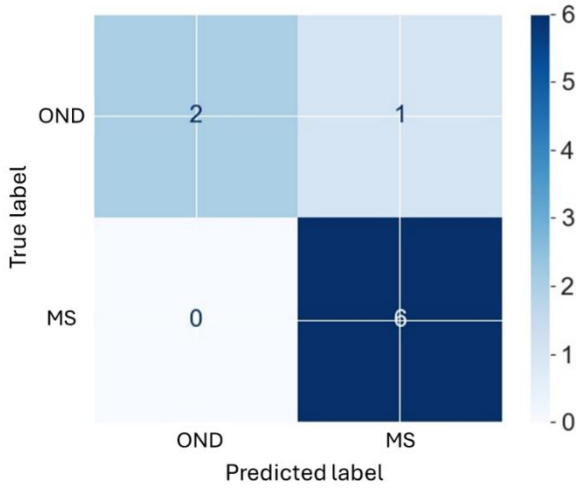

Figure 46 **Confusion matrix of the Random Forest classification model distinguishing MS from ONDs.** The matrix represents the reliability of the classifier algorithm in predicting MS from ONDs based on CSF proteomic data. The model demonstrated good specificity, identifying n=6 true positive MS, n=2 true negative ONDs, n=1 false positive, and no false negatives.

Altogether, these results demonstrate how high-throughput proteomic analysis, combined with the integrated support of artificial intelligence, can be a powerful tool for the development of novel diagnostic strategies for multiple sclerosis. Moreover, the outcome of a multisystem approach enables the discovery and study of greater depth new putative biomarkers needed to optimise a more accurate and earlier diagnosis. This improvement is essential in reducing the incidence of misdiagnosis, which still represents a major challenge in MS treatment.

2.2.2 Differential expression of IGFBP2

According to the proteomic profile, IGFBP-2 is counted among the upregulated proteins and IGF is downregulated in MS (Figure 39 and 40); moreover, the IGF pathways is among the highest relevance according to the Reactome enrichment analysis (Figure 44)and was the first most important feature used by the classifier algorithm to identify patients with MS (Figure 45). These results, therefore, led to the designation of IGFBP-2 as a putative diagnostic biomarker of the disease. To validate these findings, an ELISA test was performed to quantify IGFBP-2 levels in CSF samples, comparing MS patients to other neurological disease controls. Remarkably, IGFBP-2 levels were significantly higher in the MS group, confirming the upregulation observed in proteomic analysis (Figure 47).

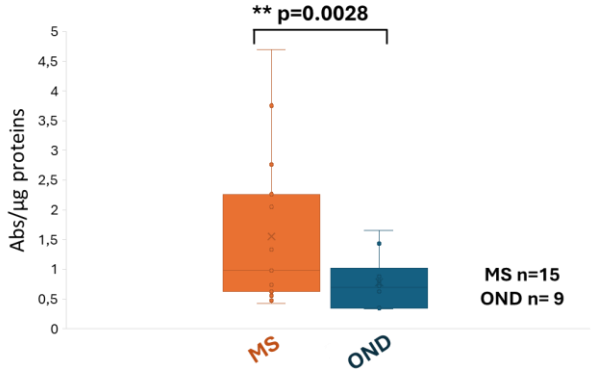

Figure 47 **Quantification of IGFBP-2 in CSF samples by ELISA.** Boxplot showing IGFBP-2 concentration levels in CSFs from patients with multiple sclerosis (MS, n=15) and other neurological diseases (ONDs, n=9), normalised for µg of proteins. Statistical analysis was performed using the Mann–Whitney *U* test; ** $p = 0.0028$.

In MS, IGFs and their binding molecules (IGFBPs) can control neurodegenerative and immunosensitive processes. Specifically, IGFBP-2, the richest IGF-binding protein in cerebrospinal fluid, is highly expressed by reactive astrocytes in the active lesion of MS and can modify cellular responses to IGFs through both independent and IGF-1 receptor-dependent mechanisms (Chesik et al., 2007; S. Khan, 2019). *In vitro* studies show that IGFBP-2 can activate astrocyte proliferation in the presence of IGF-1, but simultaneously prevent the survival of oligodendroglial progenitor cells (Chesik et al., 2007). IGF-2 is not overexpressed in MS compared with controls, it is known to play a role during brain development and in the regulation of neuronal metabolism, as well as having a moderate affinity for IGFBP-2, which regulates its bioavailability and interaction with cellular receptors (Russo et al., 2005). In summary, dysregulation of the IGF-IGFBP system, the overexpression of IGFBP-2, may impair the potential neuroprotective effects of IGFs.

Overall, the consistent upregulation of IGFBP-2 observed throughout the proteomic profiling, its central role in the enriched inflammatory and regulatory pathways, its importance at the top of the classification model and the confirmatory ELISA data support its potential utility as a reliable diagnostic biomarker for multiple sclerosis, reflecting both its mechanistic involvement in disease pathogenesis and its discriminatory power in distinguishing MS from other neurological conditions.

2.2.3 Gelatinolytic Activity in CSF of MS Patients

The enzymatic activity of gelatinases was quantified in CSFs of MS patients and neurological controls (ONDs). Gelatin zymography was employed to detect pro- and active forms of MMP-2 and MMP-9, allowing the characterisation of their expression patterns in MS clinical subgroups. A total of n=47 CSFs from MS patients and n=29 ONDs were analysed. From the zymograms, it is possible to observe the presence of gelatinolytic bands corresponding to the activity of MMP-2 and MMP-9 in the CSF samples analysed. Considering the molecular weight, pro- (72 kDa) and active (66 kDa) forms of MMP-2 can be distinguished, alongside proMMP-9 (92 kDa) and active MMP-9 (86 kDa). In addition to the monomeric forms of MMP-9, a higher molecular weight band is observed (around 130 kDa), consistent with the presence of the proMMP-9/TIMP-1 complex. Dimers of proMMP-9 are absent in CSF (Figure 48).

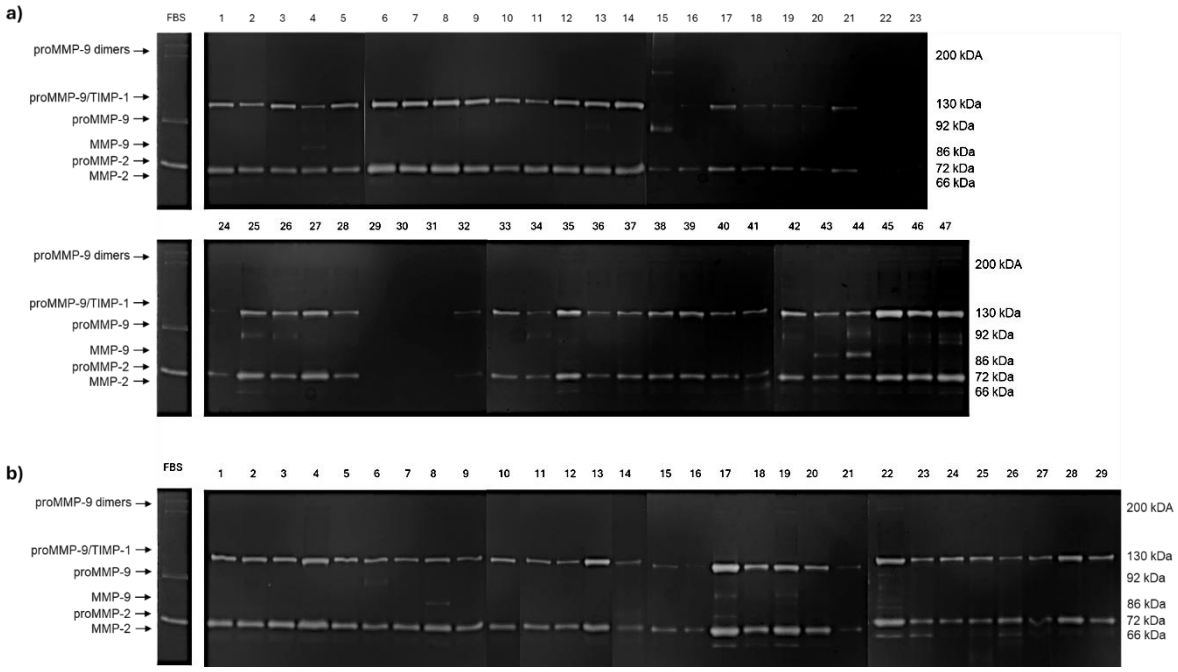

Figure 48 **Zymographic analysis of CSFs in MS and neurological controls**. Gelatin zymography of CSF samples from (a) 49 MS patients and (b) 27 ONDs showing gelatinolytic activity of MMP-2 and MMP-9. Pro (72 kDa) and active (66 kDa) forms of MMP-2, and pro- (92 kDa) and active (86 kDa) forms of MMP-9 were detected. A band around 130 kDa, corresponding to the proMMP-9/TIMP-1 complex, was also observed. No proMMP-9 dimers were detected. Experiments were performed in triplicate. *ImageJ* software was used to quantify the densitometric bands corresponding to the MMP gelatinolytic activity.

Analysis of zymograms showed distinct patterns in the expression of the two gelatinases, including between inactive and active forms. A predominant expression of the latent forms of

both MMP-2 and MMP-9 was observed in all samples. This finding may be motivated by the fact that in extracellular fluids, under both physiological and chronic conditions, MMPs are predominantly synthesised and secreted in their inactive form, since their activation is highly controlled and is a spatially and temporally limited event.

The expression patterns of MMP-2 and MMP-9 turn out to be very different, with a general higher expression of MMP-2 than MMP-9 in CSF. Qualitatively, proMMP-2 was detectable in all samples and exhibited greater expression than its active form, which, however, is observed to be more stable and with less pronounced differences among MS patients compared to controls, in whom MMP-2 expression is higher overall. In contrast, the activity of the proenzyme form of MMP-9 was generally low, as was the presence of its active form. This finding suggests a limited gelatinolytic activity of MMP-9 in the central compartment, consistent with further regulatory mechanisms by which the proteolytic potential of MMP-9 is limited, represented by increased intensity of bands corresponding to the proMMP-9/TIMP-1 complex.

In summary, MMP-2 expression indicates a ubiquitarian gelatinolytic activity among the clinical groups. In contrast, lower expression and a more pronounced interindividual variability are shown for MMP-9. This difference can be explained from a functional point of view, as MMP-2 is an enzyme constitutively expressed in several CNS cytotypes, including astrocytes (Ogier et al., 2006). Its presence in CSF is generally stable and reflects its constitutive role in matrix remodelling even under physiological conditions. In contrast, MMP-9 is an inducible enzyme, and its production is associated with the activation of leukocytes, which release MMP-9 during inflammatory responses.

To investigate the significance of the observed differences in gelatinolytic activity between patients with multiple sclerosis and other neurological diseases, a densitometric analysis of the lytic bands detected by zymography was performed by using Image J in triplicate experiments (Figure 49). Surprisingly, the activity levels of gelatinases are overall lower in MS patients than in ONDs. In particular, both pro-MMP-2 and MMP-2 showed lower levels in MS patients compared with ONDs, although the statistical significance was reached only for the active form. Similarly, the proMMP-9/TIMP-1 complex appears to be present in lower levels in CSFs from MS patients, although not significantly so (Figure 49).

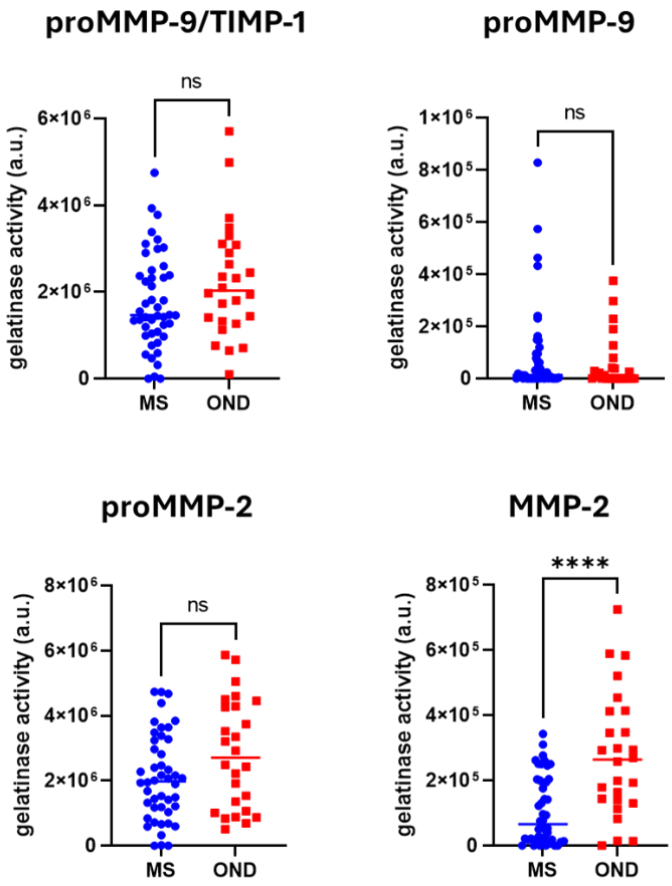

Figure 49 **Gelatinase activity in CSFs of MS patients compared to controls.** Scatter plots showing the expression levels of MMPs evaluated by gelatin zymography in n=76 CSF samples analysed from n=47 patients with multiple sclerosis (MS) and n=29 with other neurological disorders (OND). Each dot corresponds to a mean value of three replicates. The lines indicate the median. The densitometric analysis, performed by measuring the optical intensity of each band and the corresponding area, is referred to as gelatinase activity in arbitrary units (a.u.). Statistical analysis was performed using the Mann–Whitney U- nonparametric test. Graphics were obtained by GraphPad software. ns, not significant; **** p <0.0001.

2.2.3.1 Associations with the Inflammatory Status

C-reactive protein (CRP) is a biomarker of systemic inflammation. CRP is commonly assessed to monitor the therapeutic response and to predict long-term outcomes in patients with inflammatory diseases. During the acute phase of inflammation, it is synthesised in the liver upon stimulation by inflammatory mediators such as pro-inflammatory cytokines. In neurological disorders, measuring CRP levels in the CSF may give valid information on local inflammatory processes directly related to the CNS.

The inflammatory status was evaluated by analysing the expression levels of CRP through

immunoblotting in a subgroup of CSF samples from n=10 MS patients and n=12 ONDs (Figure 50b). Interestingly, a more variable pattern of CRP expression was detected for OND subjects, compared to MS patients. The quantitative comparison between the two groups showed a lower expression of CRP in MS patients compared to OND subjects (Figure 50c), suggesting the presence of a lower index of acute inflammation in MS patients compared to patients with other neurological diseases, including non-inflammatory ones. A possible explanation might lie in the fact that multiple sclerosis, unlike many neurological diseases, is characterized by chronic, localized, and less acute neuroinflammation, particularly in the early stages of the disease, generally associated with the RRMS stage.

In order to assess the relationship between gelatinolytic activity and inflammatory status in the CSF, a correlation analysis was performed between the expression of MMPs, assessed by zymography (Figure 50a), and CRP levels (Figure 50b) from the same patients. Interestingly, although the low number of analysed samples, there was a significantly positive correlation (p=0.0251) between the presence of proMMP-2 and CRP in patients with multiple sclerosis (Table 4). To support this finding, a simple linear regression analysis was conducted between CRP levels and proMMP-2 expression in MS patients, which confirmed a significant positive correlation. No correlation between MMP and CRP was found in control patients (Figure 50d).

Table 4 **Pearson Correlation between MMPs and CRP in CSFs of MS and controls**

	MS			**OND**		
CRP vs	**proMMP-9/ TIMP-1**	**proMMP-2**	**MMP-2**	**proMMP-9 /TIMP-1**	**proMMP-2**	**MMP-2**
Pearson r	0.2501	0.6971	0.2045	0.04925	0.1014	-0.07816
P (two-tailed)	0.4859	0.0251	0.5709	0.8792	0.7538	0.8092
P value summary	n.s.	*	n.s.	n.s.	n.s.	n.s.

n.s., not significant; * p=0.0251.

Notably, within the MS group, patients with higher expression of proMMP-2 still tend to have relatively higher CSF inflammation. These results indicate that, even in the context of relatively low local inflammation, proMMP-2-mediated gelatinolytic activity may play a relevant role in the chronic inflammatory processes of MS. Considering the role of MMPs in impairing the BBB and facilitating immune cell infiltration, the observed association suggests that proMMP-2 may be a key mediator of neuroinflammation in MS and not in other neurological diseases.

Moreover, the positive correlation between the MMPs and CRP could indicate an association between the lower inflammatory state of multiple sclerosis and the lower expression of MMPs. So, low levels of MMP in multiple sclerosis could be associated with the levels of inflammation that is chronic inflammation in the disease. To support this hypothesis, how MMP levels varied according to the clinical stages of MS associated with disease progression was evaluated.

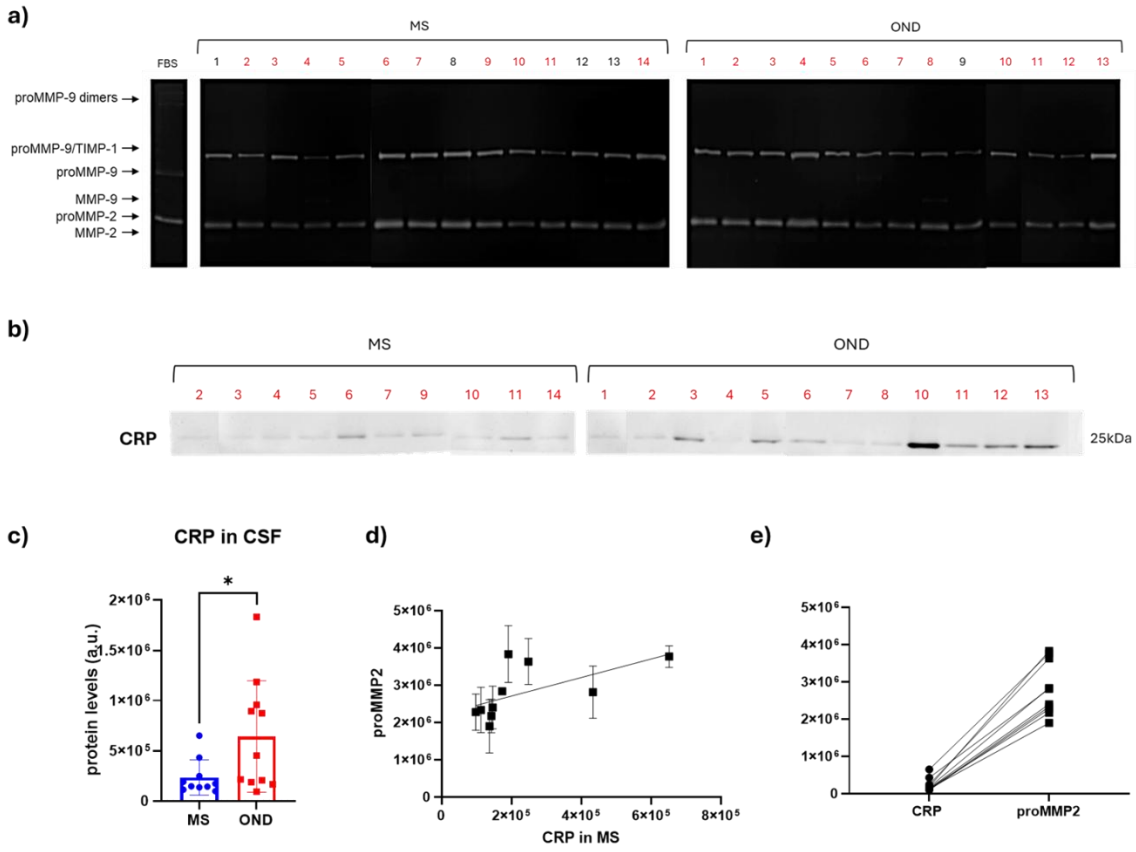

Figure 50 **Correlation between MMPs and inflammatory status of MS patients.** Gelatin zymography (a) and CRP immunoblotting (b) were performed on CSF samples from n=10 MS and n=12 OND patients. (c) Quantification of CRP expression shows lower levels in MS compared to OND. Statistical analysis was performed using the Mann–Whitney *U*-nonparametric test. * p=0.0430. (d, e) Significant positive correlation between CRP levels and proMMP-2 expression in MS patients was identified by linear regression analysis (p=0.0090). Graphics were obtained by GraphPad software.

2.2.3.2 Clinical correlations

To explore the clinical impact of different gelatinolytic activity, patients were further stratified according to clinical phases. Multiple sclerosis patients were divided between those with relapsing-remitting type (RRMS), primary progressive type (PPMS), and clinically isolated syndrome (CIS), while those within the OND category were divided between inflammatory

(OIND) and non-inflammatory neurological disorders (ONIND). This stratification was performed to assess whether the variability in MMP expression among MS patients was related to the clinical phase of the disease and the inflammatory status.

Statistical analysis confirmed that MMPs were overall less expressed in patients with multiple sclerosis than in controls. Specifically, in the RRMS patient group, proMMP-9/TIMP-1, proMMP-2, and MMP-2 were significantly less expressed than in patients with other noninflammatory neurological diseases (ONIND) ($p < 0.0001$) (Figure 51).

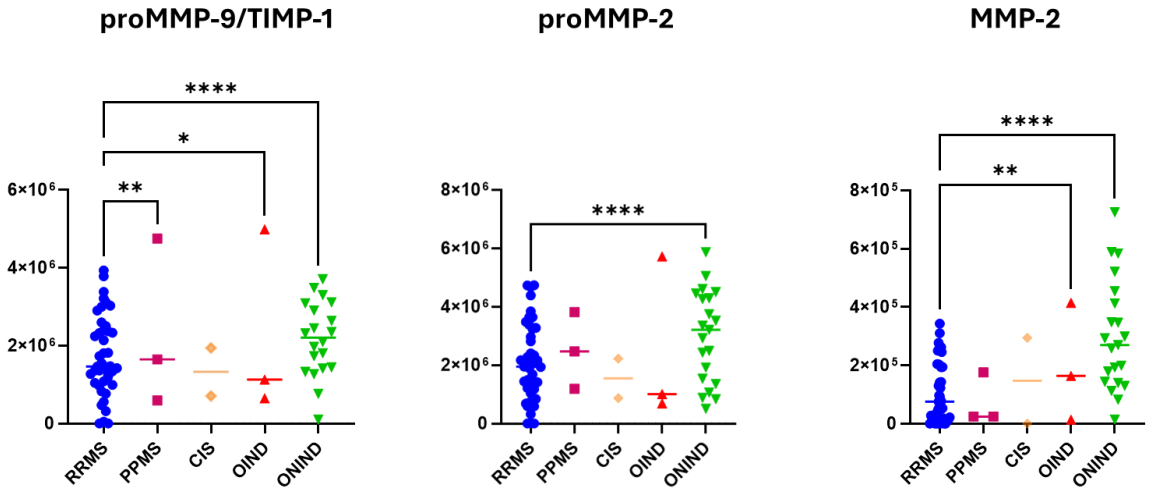

Figure 51 **MMPs expression levels in CSF of MS patients stratified by clinical subgroups.** Gelatinolytic activity in CSF samples from patients with RRMS (n=43), PPMS (n=3) and CIS (n=2), compared to controls OIND (n=3) and OIND (n=21). The graphs show the distribution of proMMP-9/TIMP-1 complex, proMMP-2 and active MMP-2 across clinical categories. Increased variability is observed between RRMS and OIND groups. * p=0.0136; ** $p < 0.001$; *** $p < 0.0001$.

To further investigate the role of MMPs in relation to disease activity, additional stratification of patients with multiple sclerosis according to clinical phase was performed, distinguishing RRMS patients between subjects in relapse and remission (Figure 52). This approach allowed us to assess whether the expression of gelatinases in cerebrospinal fluid was modulated by the active state of the disease or reflected a more stable profile independent of clinical phase.

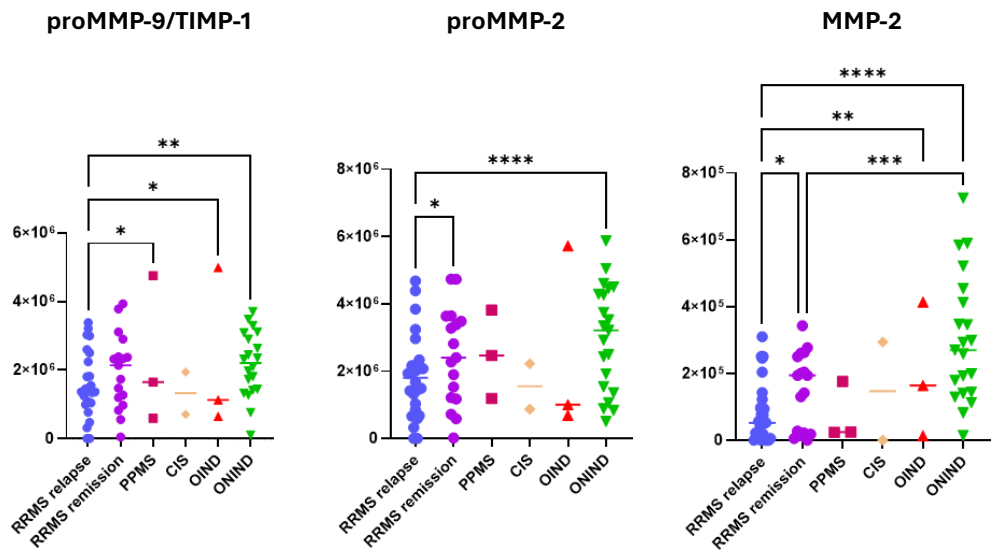

Figure 52 **MMPs expression levels in MS patients stratified according to relapse and remission phases.** Gelatinolytic activity in CSF samples from patients with RRMS in relapse phase (n=26) and remission (n=17), PPMS (n=3) and CIS (n=2), compared to controls OIND (n=3) and OIND (n=21). The graphs show the distribution of proMMP-9/TIMP-1 complex, proMMP-2, and active MMP-2 across clinical categories. Increased variability is observed between RRMS and OIND groups. * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$.

From this stratification, it was interesting to verify how the lower expression of MMPs was associated with the acute phase of the disease. Indeed, MMP expression levels were lower in patients in the relapse phase than in those in the remission phase, with particular significance for MMP-2 in both inactive (p=0.0472) and active (p=0.0479) forms. Interestingly, MMP levels during remission phases are similar to those detected in CSFs of patients with noninflammatory diseases. RRMS patients in the acute phase of the disease, on the other hand, again present lower than patients with ONIND (proMMP-9/TIMP1 p=0.0099; proMMP-2 and MMP-2 $p <$ 0.0001). The unexpected finding of decreased MMP levels during the acute relapse phase compared to the remission phase can be explained by considering a possible temporal desynchronization between the clinical inflammatory peak and the enzymatic response. An alternative hypothesis is that during the remission phase of RRMS, MMPs released in the CSF should be involved in repair mechanisms, tissue remodelling, or chronic BBB dysfunction.

2.2.3.3 Detection of Gelatinase Isoforms in CSF

To detect possible isoforms differentially expressed between analysed samples/groups, a 2D-IPG zymography was performed on n=12 CSF samples (n=6 from MS patients and n=6 from controls). A typical 2D gelatin zymography is reported in Figure 53. The lytic spots correspond to proMMP-2 (three different isoforms) and activated MMP-2 (one isoform). The expected isoelectric point (p*I*) of proMMP-2 is 5.26, while the p*I* of activated MMP-2 is 5.02. Further investigations are necessary to obtain the lytic bands of other MMPs and highlight these isoforms' possible functional roles.

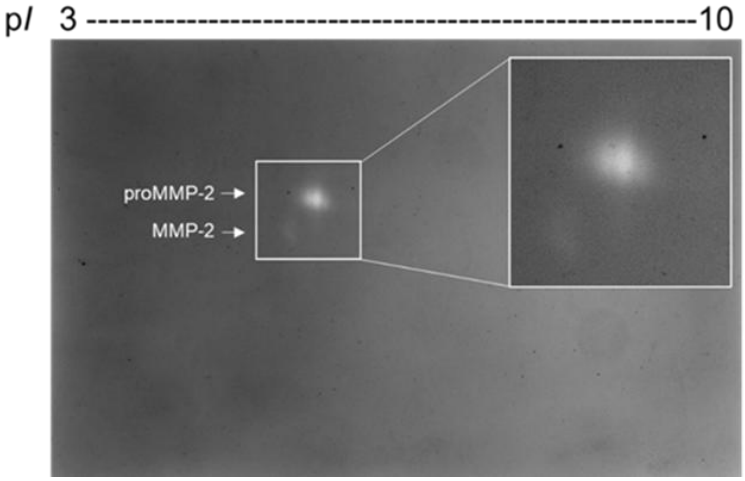

Figure 53 **Two-dimensional (2D-IPG) gelatin zymography of a CSF sample.** The zymogram shows three different isoforms for proMMP-2 and a spot for MMP-2.

2.2.3.4 Gelatinase Active Forms in CSF-derived EVs

Extracellular vesicles were isolated from a CSF sample and analysed by zymography to reveal differences with their source fluids ("SUR" in Figure 54). A significant amount of proMMP-2 and its active form was detected within the CSF-derived EVs. To a minor extent, the activated MMP-9 is present, but not the TIMP-1/ProMMP-9 complex, which is highly expressed in CSFs. The results encourage the hypothesis of a possible enrichment of the active forms of both MMP-2 and MMP-9 within EVs. In contrast, the pro-enzymatic forms were detected only in the CSFs. These findings add complexity to the role explicated by MMPs in MS pathogenesis and deserve further investigation.

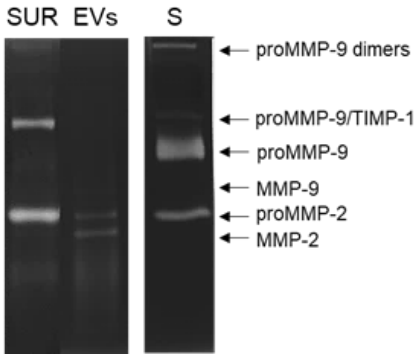

Figure 54 **CSF-derived Extracellular Vesicles present both proMMP-2 and its active form, as well as the activated MMP-9**. Instead, the proMMP-9/TIMP-1 complex is absent. "SUR" stands for the surfactant after ultracentrifugation; "S" stands for a serum sample of a non-pathological individual used as MMP expression control.

2.3 Peripheral Compartment: Serum and Plasma Analysis

Diagnosis and follow-up of multiple sclerosis are mainly based on clinical assessment, magnetic resonance imaging (MRI), and CSF analysis. Although CSF oligoclonal bands are a golden standard in the diagnostic criteria for MS, lumbar puncture is still an invasive procedure for patients. Hence, there is an increasing need for the identification of stable biomarkers in more accessible biological fluids, such as those derived from blood. Peripheral biomarker discovery can result in early detection and facilitate monitoring of the disease. Serum and plasma are certainly the most suitable matrices for routine clinical use and guarantee greater reproducibility of measurements.

MMP matrix metalloproteinase analysis, in particular MMP-2 and MMP-9, could be an advance in the discovery of new biomarkers. This section investigates gelatinase activity in serum and plasma of MS patients compared to other neurological diseases, studying their relationship with the systemic inflammatory status and MS disease clinical stage. This approach aimed to understand peripheral aspects of the disease beyond the CNS compartment and might lead to the development of less invasive diagnostic tools.

2.3.1 Gelatinolytic Activity in Serum of MS Patients

Of the same subset of patients described above (Table 3), serum samples were analysed to assess whether the proteolytic activity of gelatinases could be associated with MS progression.

The analysis was conducted in serum samples from n=27 MS patients (Figure 55a) and n=17 ONDs (Figure 55b). The results showed a predominant expression of the inactive form of proMMP-9 (92 kDa), overall higher than proMMP-2 (72 kDa) levels. Notably, as expected, dimers of proMMP-9 (200 kDa) are present, as well as the complex proMMP-9/TIMP-1 (130 kDa). Active forms of MMP-2 and MMP-9 are absent.

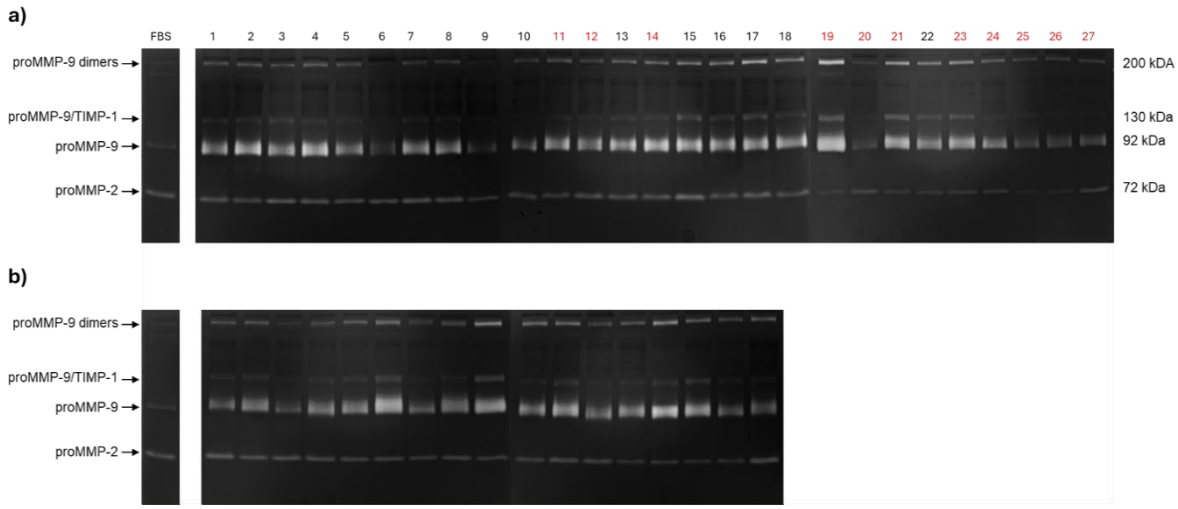

Figure 55 **Gelatin zymograms of serum samples in MS and OND patients.** Zymograms show MMP lytic activity in n=27 MS patients (a) and n=17 NC (b) cohorts. Qualitatively, proMMP-9 dimers, proMMP-9, proMMP-2 and proMMP-9/TIMP-1 were detected. The active forms of MMP-2 and -9 were absent. Experiments were performed in triplicate. ImageJ was used to quantify the densitometric bands corresponding to the MMP's gelatinolytic activity.

The higher expression of proMMP-9 compared to proMMP-2 could depend first on the different cellular origin of the two proteins. Whereas MMP-9 is massively produced by circulating immune cells, such as neutrophils and macrophages, even under subclinical conditions, proMMP-2 is mainly produced by stromal cells under constitutive conditions. Indeed, it has been shown that many MMPs, including MMP-9, are constitutively expressed by peripheral blood mononuclear cells in patients with multiple sclerosis (Bar-Or et al., 2003). In neutrophils, MMP-9 is synthesised during the late maturation process in the bone marrow and then stored in specific granules and gelatinases until needed. Furthermore, the higher levels of MMP-9 in serum result from release by neutrophils during the coagulation process due to processing of the serum sample in the tube (Gerlach et al., 2007).

The densitometric bands were quantified to compare the expression levels of MMPs in sera between MS and OND groups. A significantly lower expression of MMPs (p<0.05) was also found in patients with sclerosis compared to controls (Figure 56).

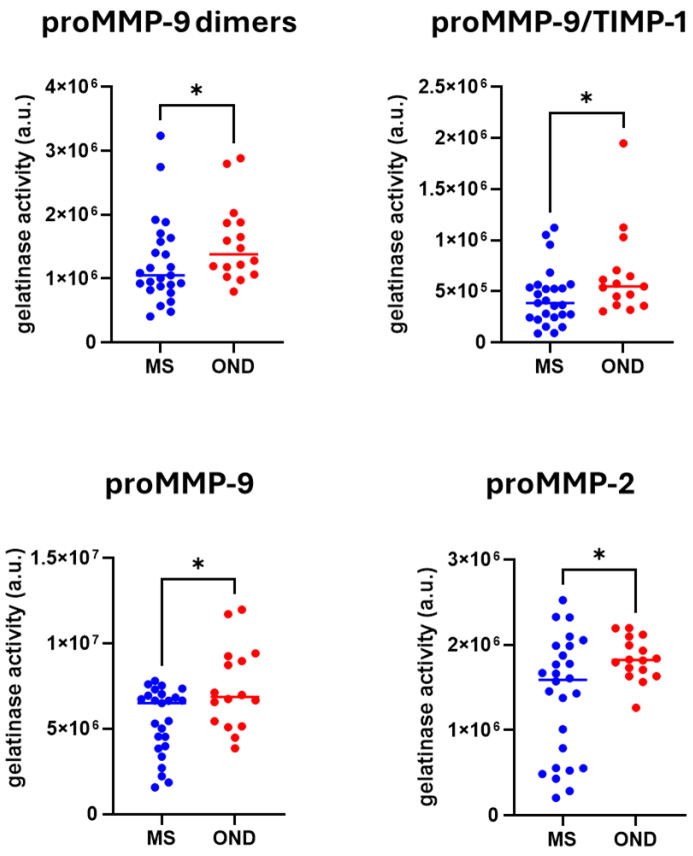

Figure 56 **Gelatinase activity in sera of MS patients compared to controls.** Scatter plots showing the expression levels of MMPs evaluated by gelatin zymography in n=44 serum samples analysed from n=27 patients with multiple sclerosis (MS) and n=17 neurological controls (NC). The lines indicate median values of the triplicates. The densitometric analysis, performed by measuring the intensity levels of each band and the corresponding area, is referred to as volume. Statistical analysis was performed by applying the Mann–Whitney *U*-nonparametric test. Graphics were obtained by GraphPad software. * $p<0.05$.

The lower gelatinolytic activity in serum could be related to a different nature of the inflammatory response compared to other neurological conditions. Therefore, a possible correlation between MMPs activity and the systemic inflammatory status of patients with sclerosis compared to controls was investigated.

2.3.1.1 Associations with the Inflammatory Status

Serum CRP protein levels were detected in n=10 MS patients and n=12 controls. The densitometric comparison between the two groups showed a lower expression of CRP in MS patients compared to OND subjects, although not significantly (Figure 57c), suggesting the

presence of a lower systemic inflammation in MS patients compared to patients with other neurological diseases, including non-inflammatory ones.

To assess a possible association between serum MMP gelatinolytic activity and the inflammatory status, Pearson correlation analysis was performed between the expression of MMPs, assessed by zymography (Figure 57a), and CRP levels (Figure 57b). Interestingly, a negative correlation between MMPs and CRP expression levels was found. In particular, proMMP-2 was significantly (p=0.0376) negatively correlated with CRP levels in MS patients (Figure 57d, e).

Table 5 **Pearson Correlation between MMPs and CRP in serum of MS and controls**

	MS				OND			
CRP vs	**proMMP-9 dimers**	**proMMP-9/ TIMP-1**	**proMMP-9**	**proMMP-2**	**proMMP-9 dimers**	**proMMP-9/ TIMP-1**	**proMMP-9**	**proMMP-2**
Pearson r	-0.4051	0.05297	-0.4611	-0.7355	-0.009512	-0.09091	-0.3812	-0.5388
P (two-tailed)	0.3195	0.9009	0.2502	0.0376	0.9792	0.8028	0.2772	0.1081
P value summary	ns	ns	ns	*	ns	ns	ns	ns

MMPs are multifaceted enzymes, and it has been widely discussed how their intervention in numerous processes provides an additional level of regulation in cellular processes, including inflammation. Indeed, while MMPs are often associated with a pro-inflammatory role, especially tissue-specific, at the systemic level, it has been shown that they may have an anti-inflammatory function (Fingleton, 2017). This duality also characterises the MMP-2, which exhibits anti-inflammatory properties by processing pro-inflammatory mediators such as MCP-3 into receptor antagonists (McQuibban et al., 2000). Indeed, MMP-2 can degrade the alarmin S100A9 to attenuate DAMP-mediated inflammatory signalling and modulate cytokine activation dynamics through the cleavage of pro-IL-1β (Parks et al., 2004; Schönbeck et al., 1998). More interestingly, the same inverse correlation between proMMP-2 and CRP has already been assessed in sera of long-living individuals (Cancemi et al., 2020). Thus, the anti-inflammatory activity of MMP-2 can explain the negative associations with CRP in the peripheral compartment.

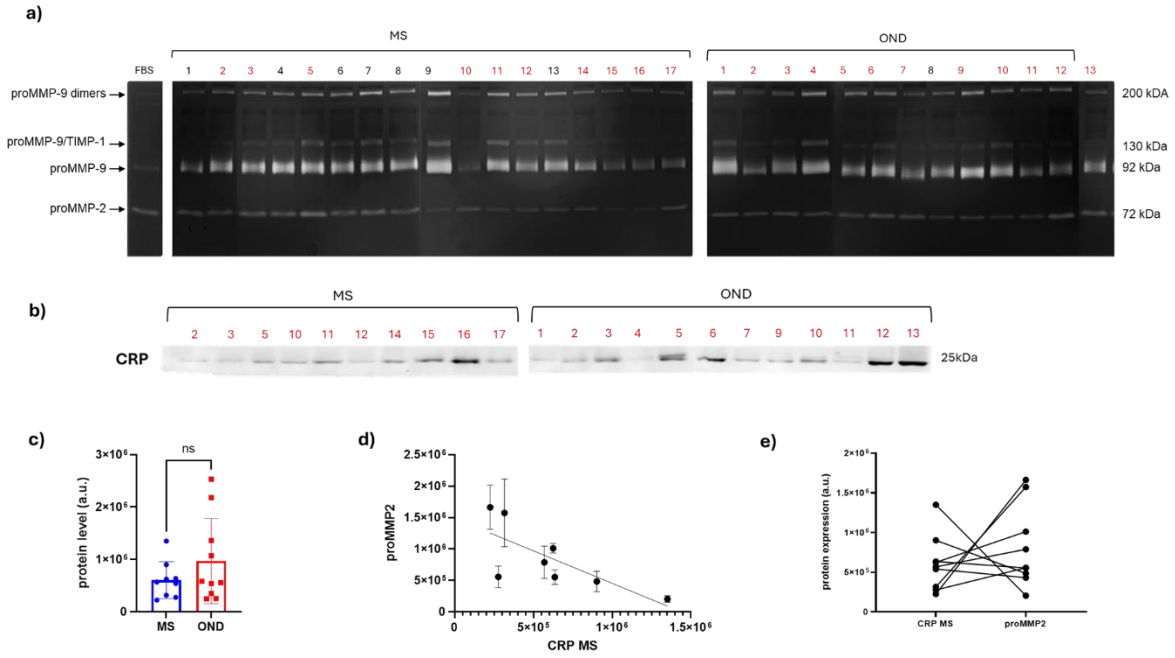

Figure 57 **Correlation between MMPs and inflammatory status in the serum of MS patients.** Gelatin zymography (a) and CRP immunoblotting (b) performed on serum samples from MS (n=10) and OND (n=12) patients. (c) Quantification of CRP expression shows lower levels in MS compared to OND. Statistical analysis was performed using the Mann–Whitney *U*- nonparametric test. (d) and (e) Significant positive correlation between CRP levels and proMMP-2 expression in MS patients was identified by linear regression analysis (p= 00087). Graphics were obtained by GraphPad software.

2.3.1.2 Clinical correlations

To further investigate the differences in gelatinolytic activity, patients were stratified according to clinical disease phases. All multiple sclerosis patients whose sera were available were at the RRMS stage (n=26), while individuals in the OND group were subdivided into OIND (n=7) and ONIND (n=9). This stratification aimed to evaluate whether the observed variability in MMP expression among MS patients was linked to disease stage or inflammatory status. In particular, the analysis wanted to determine whether the reduced gelatinolytic activity detected in the serum was associated with clinical disease progression.

Even in the peripheral compartment, in the serum of RRMS patients, MMP showed a lower activity compared to non-inflammatory controls. Both proMMP-9 dimers and proMMP-9/TIMP-1have significantly lower levels than OINDs; the monomeric proMMP-9 form is also statistically lower expressed, although the difference is less marked. These results may indicate a further level of inhibition that the protein undergoes peripherally, given the high degree of regulation of the MMP-9 systemically. Interestingly, however, in serum, there is no significant

statistical difference in the gelatinolytic activity of MMP-2 in its inactive form (Figure 58).

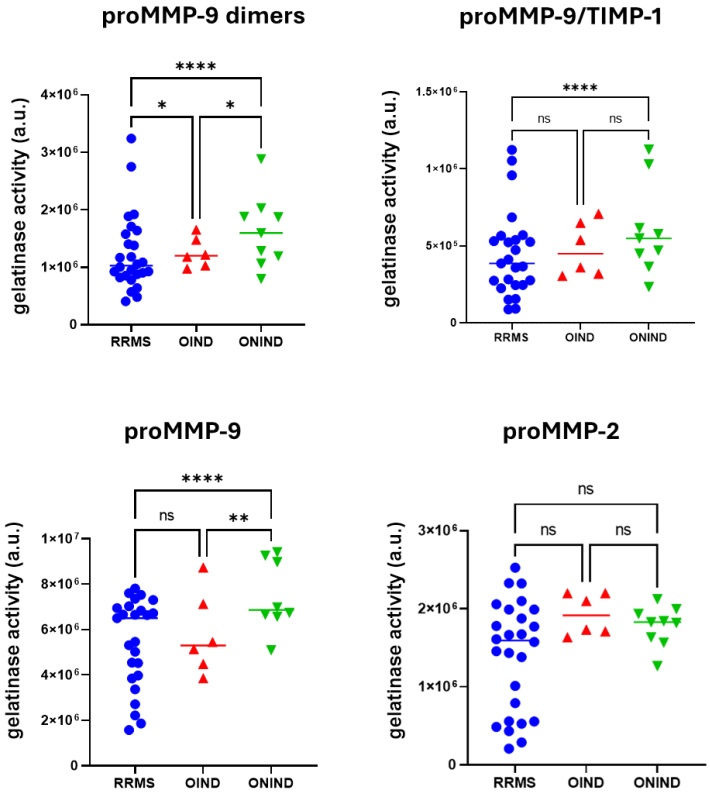

Figure 58 **MMPs expression levels in sera of MS patients stratified by clinical subgroups.** Gelatinolytic activity in CSF samples from patients with RRMS (n=26) compared to controls OIND (n=7) and OIND (n=9). The graphs show the distribution of proMMP-9 dimers, proMMP-9/TIMP-1 complex, proMMP-9, and proMMP-2 across clinical categories. Increased variability is observed between RRMS and OIND groups. * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$.

To gain deeper insight into the role of MMPs in relation to disease activity, patients with multiple sclerosis were further stratified based on clinical phase, specifically distinguishing RRMS patients experiencing relapse from those in remission (Figure 59).

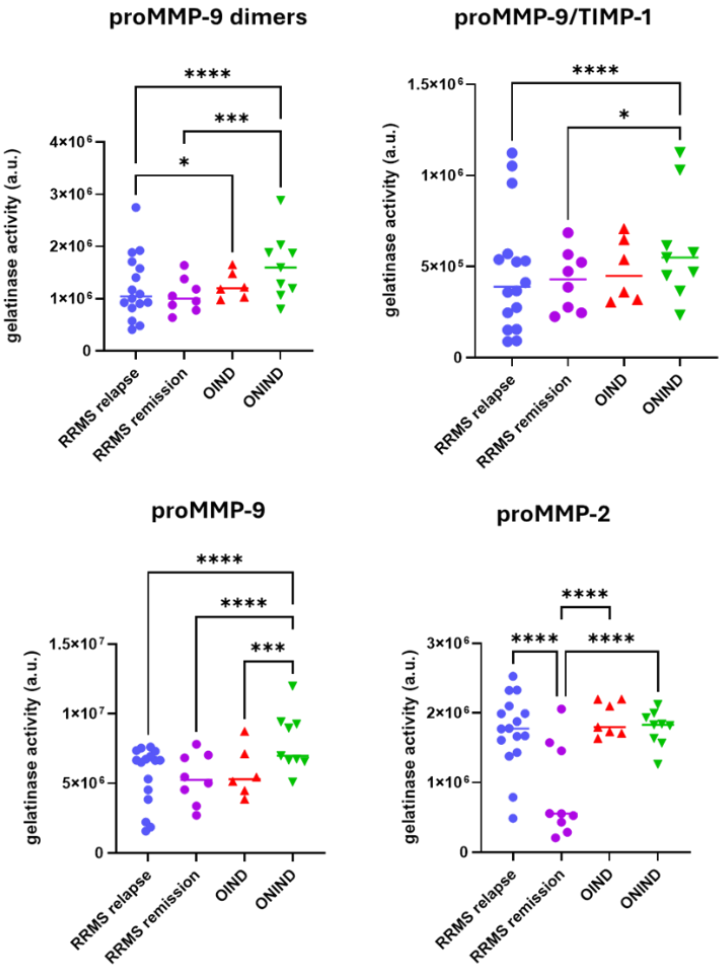

Figure 59 **MMPs serum levels in MS patients stratified according to relapse and remission phases.** Gelatinolytic activity in serum samples from patients with RRMS in relapse phase (n=16) and in remission (n=9), compared to controls OIND (n=7) and OIND (n=9). The graphs show the distribution of proMMP-9 dimers, proMMP-9/TIMP-1 complex, proMMP-9, and proMMP-2 across clinical categories. Increased variability is observed between RRMS and OIND groups. * $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$.

Remarkably, MMP-9 complexes and monomeric forms do not vary between MS relapse and stable patients, still maintaining significantly lower expression compared to OINDs. In contrast, an interesting variation concerns proMMP-2 expression in RRMS relapse and remission groups: patients in the acute phase of the disease have higher levels of MMP-2 activity than patients in remission. Moreover, proMMP-2 levels during relapse are significantly comparable in both inflammatory and non-inflammatory neurological disorders.

2.3.2 Comparative Analysis of Gelatinase Expression in Serum vs Plasma

Comparative zymographic analyses between serum and plasma are essential in biomarker

studies, as the choice of matrix can profoundly affect protein levels, stability, and detectability. Therefore, in a separate cohort consisting of multiple sclerosis patients (n=31), serum (n=29) and plasma samples (n=31) were analysed (Figure 60). Notably, both sample types were available for n=29 patients, allowing a direct comparison between the two matrices.

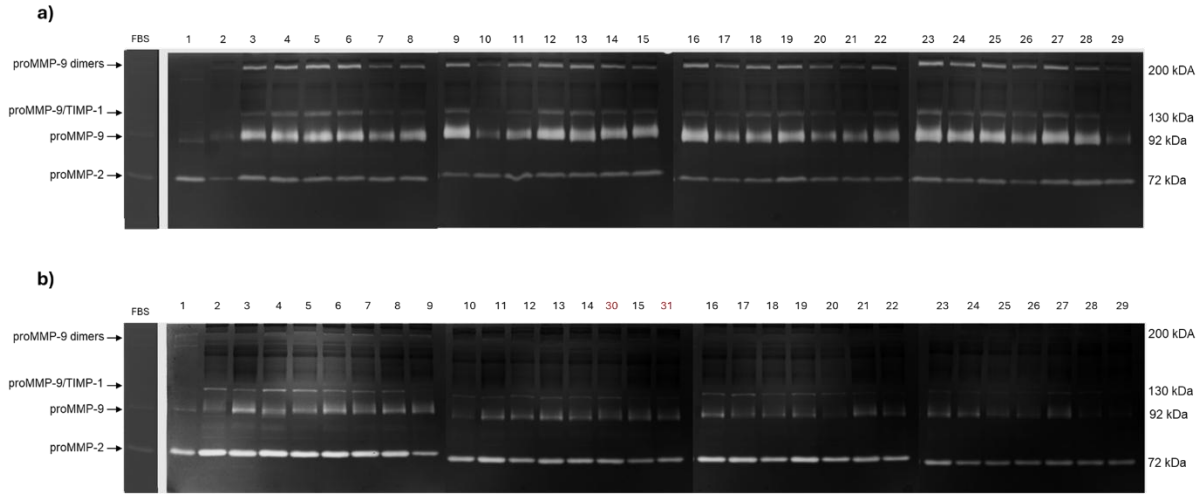

Figure 60 **Zymographic analysis of serum and plasma samples in MS patients**. Gelatin zymography of (a) n=29 serum and (b) n=31 plasma samples from MS patients showing gelatinolytic activity of MMP-2 and MMP-9. ProMMP-9 dimers (200 kDa) are observed only in serum; MMP-9 in complex (proMMP-9/TIMP-1, 130 kDa) and monomeric inactive form (proMMP-9, 92 kDa), as well as proMMP-2 (92 kDa), were detected in both biological matrices. Experiments were performed in triplicate. *ImageJ* software was used to quantify the densitometric bands corresponding to the MMP gelatinolytic activity.

Comparative analysis of gelatinolytic activity in serum and plasma showed a clear discrepancy between the two matrices. MMP-2, while generally less abundant, showed a substantially overlapping expression pattern, with an overall uniform distribution among the samples. In contrast, the expression of MMP-9 was significantly higher in serum ($p<0.0001$), both in monomeric and complexed form, while the dimeric form is almost completely absent compared with plasma. In addition, experimental data reveal less interindividual variability in plasma samples than in serum samples (Figure 61).

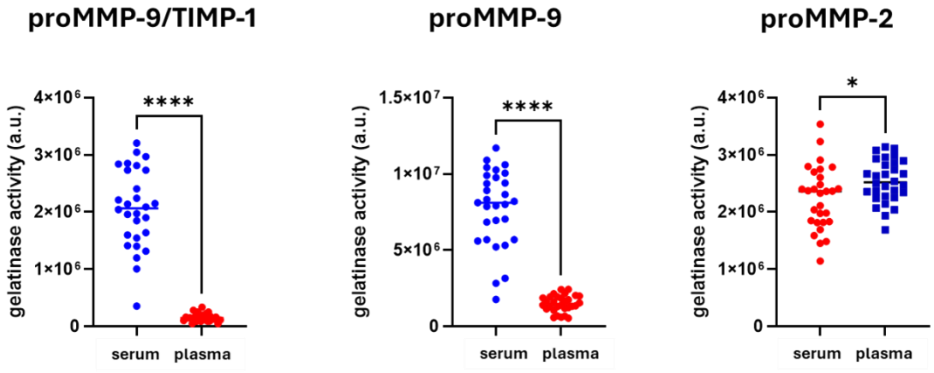

Figure 61 **MMPs expression levels in sera and plasma of MS patients.** Gelatinolytic activity in serum and plasma samples from patients with MS (n=29). The graphs show the distribution of proMMP-9/TIMP-1 complex, proMMP-9, and proMMP-2 across the two different matrices. Increased variability is observed in both complex and monomeric proMMP-9 expression, higher in serum than in plasma. MMP-2 showed overlapping expression levels between serum and plasma samples. * p=0,0360; *** $p < 0.0001$.

The discrepancy between the expression of MMP-2 and MMP-9 in the two types of samples is particularly relevant because it suggests that MMP-2 levels are not affected by the coagulation process, unlike MMP-9. This phenomenon can be explained by considering the cellular origin of the two metalloproteinases: MMP-2 is mainly produced by non-circulating stromal cells, including fibroblasts, endothelial cells, and vascular smooth muscle cells, which do not actively participate in the coagulation process (Howard et al., 2012; Risinger et al., 2006). Consequently, the stability of MMP-2 levels between serum and plasma further strengthens the significance of the results obtained in previous analyses, in which differences in pro-MMP-2 expression were observed among clinical subtypes of patients. The absence of preanalytical influences related to the sampling matrix for MMP-2 thus provides greater strength to the biological and clinical interpretation of these results, supporting the hypothesis that the observed variations are intrinsic to the pathology.

In contrast, MMP-9 is released in large amounts by activated monocytes, neutrophils, and platelets during coagulation. Indeed, monocytes represent a major source of MMP-9 in peripheral blood, further contributing to the explanation for the large increase observed in serum samples (Bar-Or et al., 2003). Furthermore, the study by Gerlach et al. (2007) also showed that MMP-9 levels were significantly higher in serum than in plasma, consolidating the results shown.

3 Analysis of Primary Fibroblasts from Dupuytren's Disease Patients

This study is part of a research project conducted by Miss Mira Pecheva, PhD student at the University of East Anglia (UEA) in Norwich, under the supervision of Dr. Rose K. Davidson and Prof. Ian Clark. The main project aimed at evaluating the effects of an inhibitory antibody targeting MMP-14 in myofibroblasts derived from Dupuytren's disease patients.

In this study, the purpose was to elucidate the functional role of the rs1042704 polymorphism in the *MMP14* gene in regulating MMP-2 activity and in modulating the cellular response to anti-MMP-14 antibody treatment, according to the patient's genotype. To achieve this goal, the experimental strategy adopted was based on the use of primary myofibroblasts isolated from surgically resected nodule tissue and genotyped for the SNP. A subset of n=12 patients was selected to represent the three genotypic groups associated with the *MMP14* rs1042704 polymorphism (GG, GA, and AA), with n=4 individuals per genotype. Myofibroblasts derived from patients with a known genotype for rs1042704 were analysed: homozygous GG, heterozygous GA, and homozygous AA.

The SNP results in a non-synonymous substitution where Asp_{273} (MT1-D_{273}) is substituted with Asn_{273} (MT1-N_{273}) within the catalytic domain. The alternative allele (A) encodes for asparagine (N) at position 273, while the ancestral allele (G) encodes for aspartate (D). Consequently, in homozygous (GG), *MMP14* codes exclusively for the functional form MT1-D_{273}; heterozygous (GA) express both MT1-D_{273} and MT1-N_{273} variants, and homozygous (AA), coding only for the mutated form MT1-N_{273}. Therefore, it was analysed whether this SNP variant entails functional consequences that can be correlated with the Dupuytren's disease phenotype. This approach allowed a direct study of how the allelic variant affects the regulatory activity of MMP-14 on the proteolytic activity of MMP-2 and its susceptibility to pharmacological inhibition.

3.1 Evaluation of Technical Replicate Variability

The initial steps in the experimental analysis focused on assessing the consistency and reproducibility of technical replicates to ensure the reliability of subsequent data interpretation. To this end, three patients were selected, each representing one of the three genotypes for the rs1042704 polymorphism (GG, GA, AA), and for each, culture supernatants from three parallel technical replicates were analysed via zymography (Figure 62). The results revealed a great

degree of congruence in the gelatinolytic activity profiles. The absence of significant differences among the replicates confirmed reproducibility and consistency of experimental conditions in order to attribute any variation to technical or methodological artifacts. This step was necessary to enhance the robustness of genotype- and treatment-based analyses.

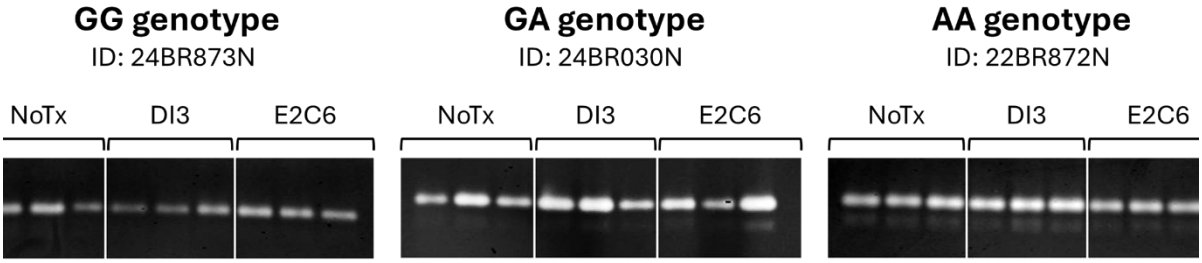

Figure 62 **Evaluation of technical replicate consistency in DD-derived fibroblast cultures from three genotyped patients.** Zymographic analysis of culture media from three Dupuytren's disease patients, each representing one of the three rs1042704 MMP14 genotypes (GG, GA, AA), treated in triplicate. For each patient, fibroblast supernatants were collected after treatment and subjected to gelatin zymography to assess gelatinolytic activity.

3.2 MMP Profiles in Distinct MMP-14 SNP Genotypes

To investigate the potential functional impact of the SNP variant for the MMP14 gene on the proteolytic activity of MMP-2 in myofibroblasts derived from Dupuytren's disease patients, zymography experiments were conducted on untreated cell supernatant (Figure 63). The results qualitatively showed a marked difference between the genotypes, with myofibroblasts homozygous for the A allele (genotype AA) presenting significantly higher activity than heterozygotes (GA) and G homozygotes (GG). Notably, the MMP-2 active form is seen exclusively in AA patients (Figure 63a). However, the quantitative analysis of the lytic bands did not show statistically significant differences between the groups, mainly due to the high intra-group variability observed in AA patients, emphasising the need to increase the sample size (Figure 63b).

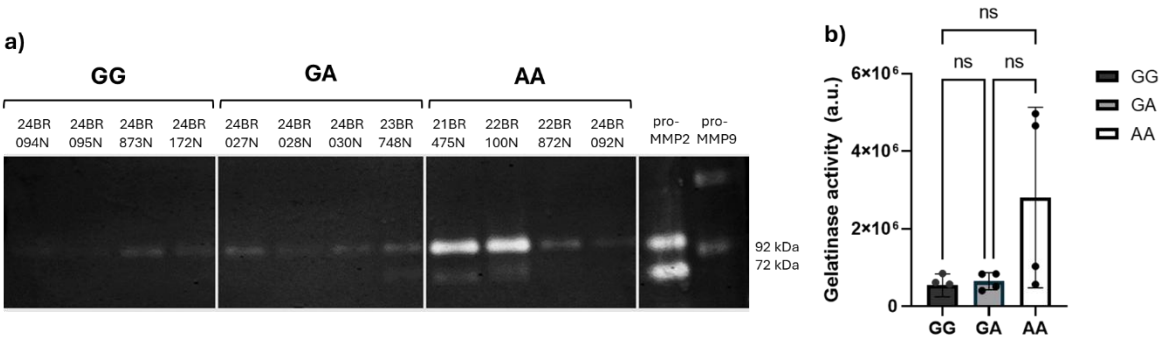

Figure 63 **Evaluation of proMMP-2 activation in DD-derived fibroblasts.** (a) zymographic analysis and (b) quantification of gelatinolytic activity in primary myofibroblasts isolated from Dupuytren's disease patients with known rs1042704 *MMP14* genotypes: homozygous (GG), heterozygous (GA), and homozygous (AA). ProMMP-9 activity was not detected. Recombinant proMMP-2 (9 ng) and proMMP-9 (0.03675 ng) were used as molecular controls to verify band identity and migration. ns, not significant.

This finding appears unexpected, considering that the A allele coding for the mutated form MT1-N_{273} has been shown to be associated with lower MMP-2 activity analysed by zymography in transfected fibroblast-like COS7 cells expressing the MT1-N_{273} form (Itoh et al., 2021). In the study by Itoh et al. (2021), gelatinolytic activity was evaluated in primary cells derived from surgically resected tissue of patients with Dupuytren's disease, treated with type I collagen (100 µg/ml) to stimulate proMMP-2 activation. The results showed a reduced activation of proMMP-2 in myofibroblasts with GA and AA genotype compared to GG, although the differences did not reach statistical significance. Notably, under unstimulated conditions, Itoh et al. reported a marked qualitative variability in gelatinolytic activity between genotypes and between patients, consistent with the differences observed in our analysis. Thus, these findings support the notion that the activating capacity of MMP-14 on MMP-2 may be impaired by the presence of SNP rs1042704, although the degree of impairment appears to vary substantially across individuals, such that further investigation on a larger sample is required.

To determine whether the observed differences in proMMP-2 activity among genotypes were associated with changes in protein expression levels, immunoblotting analyses were performed on cell lysates from the same patient samples. The results revealed that the gelatinolytic activity differences were not mirrored by MMP-2 protein levels, which remained comparable across GG, GA, and AA genotypes. Surprisingly, MMP-14 expression was also unaffected by genotype, despite the functional alterations associated with the rs1042704 variant. Notably, MMP-9 was undetectable in all samples analysed, suggesting it is not significantly expressed in this cellular context under basal conditions (Figure 64).

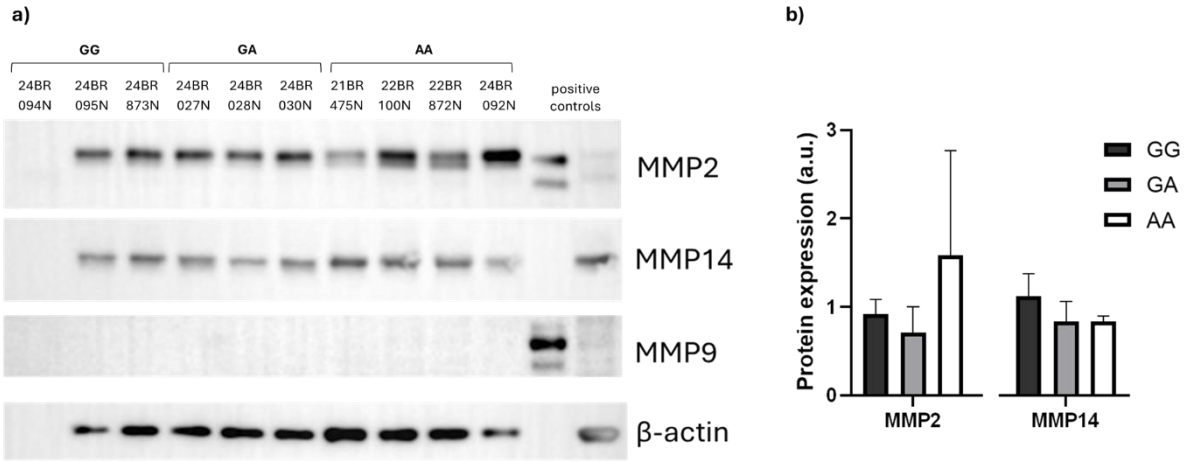

Figure 64 **MMP expression level profiles across SNP *MMP14* genotypes in DD myofibroblasts.** (a) Western blot analysis of MMP-2, MMP-14, and MMP-9 protein levels in primary myofibroblasts derived from Dupuytren's disease patients with known rs1042704 SNP genotypes (GG, GA, AA). (b) Quantification of MMP-2 and MMP-14 protein expression levels, normalised to β-actin expression to account for differences in protein loading.

3.3 Effect of Anti-MMP-14 Antibody Treatment Across SNP Genotypes

To assess whether the effect of MMP-14 inhibition is *MMP14* SNP genotype-dependent, DD-derived myofibroblasts were treated with an inhibitory anti-MMP-14 antibody (E_2C_6) or its isotype control (DI_3). Following the above, AA genotype cells exhibited the highest basal proMMP-2 activity (NoTx cells). Unexpectedly, zymography revealed that antibody treatment increased proMMP-2 activity in all genotypes. In addition, no significant differences between the antibody and its inactive isotype were noted (Figure 65). Quantitation of lytic bands indicates that proMMP-2 activity enhancement after antibody treatment is more pronounced and significantly different across genotypes. Specifically, it is significantly greater in myofibroblasts with the AA genotype than in those with GG and GA genotypes (Figure 60b), suggesting a genotype-specific difference in response to MMP-14 inhibition.

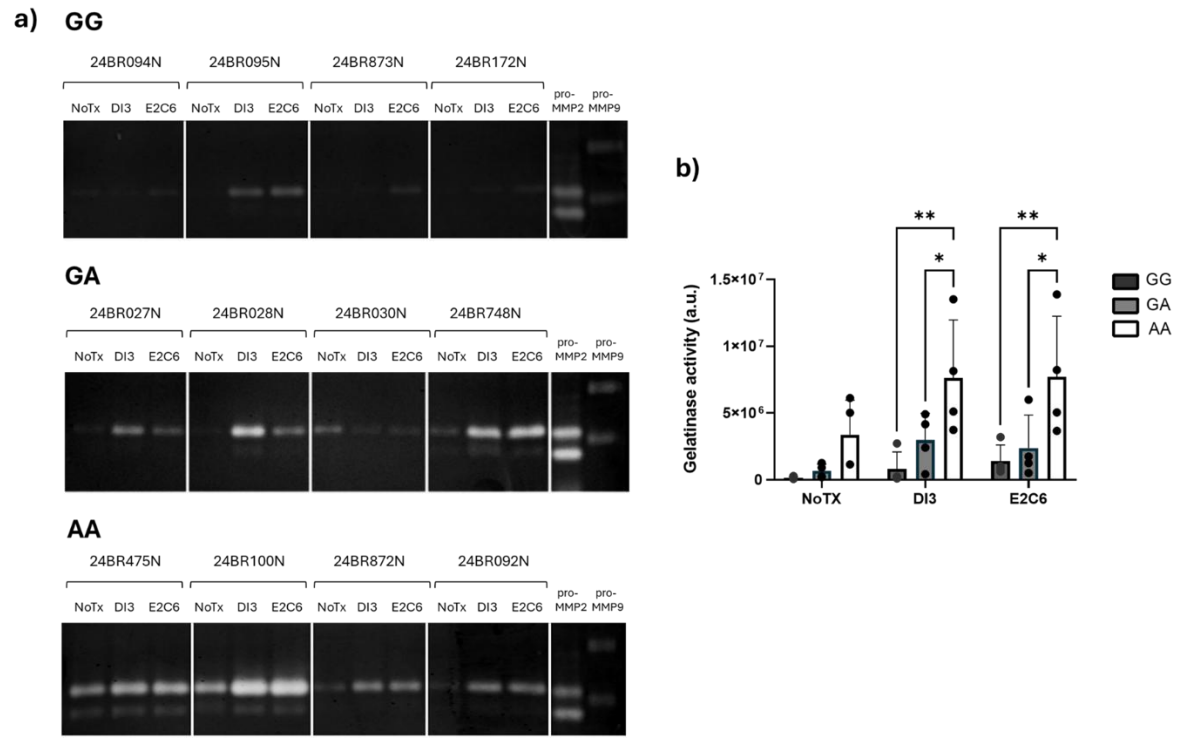

Figure 65 **Effect of anti-MMP-14 antibody treatment on proMMP-2 activity across *MMP14* SNP genotypes.** (a) Gelatin zymography and (a) quantification of proMMP-2 activity in primary myofibroblasts derived from DD patients according to rs1042704 genotypes (GG, GA, AA). Cells were treated with either an anti-MMP-14 inhibitory antibody (E_2C_6) or an isotype control (DI_3). ProMMP-9 activity was not detected. Recombinant proMMP-2 (9 ng) and proMMP-9 (0.03675 ng) were used as molecular controls to verify band identity and migration. * $p < 0.05$; ** $p < 0.01$.

To test whether the increase in gelatinolytic activity observed following treatment with the anti-MMP-14 antibody was accompanied by changes in protein expression levels, a western blot analysis was performed on cell lysates derived from myofibroblasts of patients with Dupuytren's disease, with a known genotype for the rs1042704 polymorphism of the *MMP14* gene (GG, GA, AA) (Figure 66). The cells were treated with the MMP-14 inhibitor antibody (E_2C_6) or the control isotype (DI_3), and the expression of the MMP-2 and MMP-14 proteins was assessed. The values obtained were normalised to the expression of β-actin, which was used as a loading control.

The results show that, while there was a noted increase in gelatinolytic activity as assayed by zymography, there was a trend toward decreased expression of the proteins MMP-2 and MMP-14 after antibody treatment. However, this effect did not reach statistical significance, and no significant differences were noted when comparing the anti-MMP-14 antibody effect with

isotype control (Figure 66 b, c). Therefore, the expression of MMP proteins appears to be consistent in different genotypes and treatment groups, suggesting that the treatment has no general effect on protein expression.

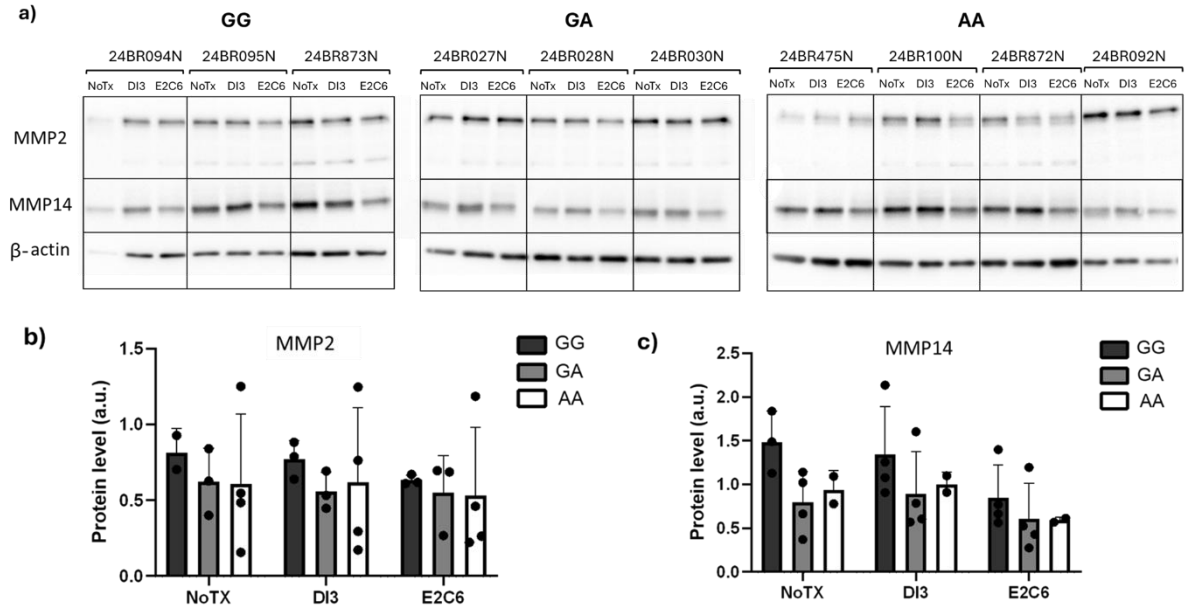

Figure 66 **Analysis of MMP-2 and MMP-14 protein expression following antibody treatment in DD myofibroblasts of different genotypes.** (a) Western blot analysis and (b, c) quantification of MMP-2 and MMP-14 protein levels in primary myofibroblasts derived from DD patients genotyped for *MMP14* rs1042704 SNP (GG, GA, AA). Cells were treated with either MMP-14 inhibitory antibody (E_2C_6) or an isotype control (DI_3). β-actin was used as a loading control, and protein expression levels were normalised accordingly.

These data indicate that increased gelatinolytic activity is not associated with increased protein production, but rather with changes in enzyme activation mechanisms or post-translational regulation. The absence of correlation between expression and activity strengthens the hypothesis that the presence of the rs1042704 variant may influence MMP-14 functionality by altering its interactions with substrates or cofactors, such as TIMP-2, in a genotype-dependent manner.

4 Comparative Integration Across Biological Systems

In this section, a cross-sectional integration of the data obtained in the three biological models analysed in assisted reproduction, multiple sclerosis, and Dupuytren's fibrosis is proposed. The aim is to systemically compare the activity of MMP-2 and MMP-9 gelatinases in order to identify recurrent and/or divergent patterns, highlighting both conserved mechanisms and regulatory differences associated with the different pathophysiological microenvironments.

Considering aspects such as compartmentalisation, functionality and application implications, analyses showed a clear distinction between MMP-2 and MMP-9 in terms of activity and expression, which are summarized in Table 6.

Table 6 **Comparative profiling of MMP-2 and MMP-9 across biological systems**

MMP activity/expression		Reproduction		Multiple sclerosis				Dupuytren's disease	
		FF	FF-EVs	CSF	CSF-EVs	serum	plasma	myofibroblast secretion	myofibroblast lysates
MMP-2	forms °	pro-	pro- and active	pro- and active	pro- and active	pro-	pro-	pro- and active	pro-
	</> MMP9 °°	>	>	>	>	<	>	>	>
	CRP correlation	n.s.	n.s.	positively	n.a.	negatively	n.a.	n.a.	n.a.
	age correlation	negatively	negatively	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	other clinical notes	-	-	< in relapse	-	> in relapse	-	> in AA genotype	= in genotypes
MMP-9	forms °	pro-	pro-	pro- and TIMP-1	n.d.	dimer, pro-, TIMP-1	pro- and TIMP-1	n.d.	n.d.
	</> MMP2 °°	<	<	<	<	>	<	-	-
	CRP correlation	positively	n.s.	n.s.	n.a.	n.s.	n.a.	-	-
	age correlation	negatively	negatively	n.a.	n.a.	n.a.	n.a.	-	-
	other clinical notes	-	-	< in MS	-	< in MS	-	-	-

°forms: pro- = proenzyme; dimer = proMMP-9 dimers; TIMP-1 = proMMP-9/TIMP-1 complex. °° </> MMP-2/MMP-9 = compartmental comparison between the two gelatinases. n.d. = not detected; n.s. = not significant; n.a. = not assessed

MMP-2 in its zymogenic form is constitutively expressed in all the compartments analysed. Indeed, it was stably detected in FF, CSF, serum, and plasma as well as in conditioned media and cell lysates from DD myofibroblasts. From a functional point of view, MMP-2 is a constitutive enzyme expressed by granulosa cells, astrocytes, and fibroblasts (Curry & Osteen, 2003; Howard et al., 2012; Ogier et al., 2006) and is therefore characterised by relatively stable inter-individual expression. However, the detection of its active form was closely related to specific compartmentalisation, especially within EVs in both the reproductive and neurological contexts. This compartmentalisation suggests a conserved mechanism of targeted release, particularly relevant in circumscribed and sensitive microenvironments such as the ovarian follicle and the central nervous system (Geraci et al., 2018; Machtinger et al., 2015; Shimoda & Khokha, 2017), where ECM degradation must be tightly regulated to preserve tissue integrity. Correlation analyses with the clinical parameters across cohorts revealed a compartment divergence in MMP-2 behaviour. In FF, the lack of significant correlation between

the expression of MMP-2 and CRP might suggest a weak association with inflammation localised in the follicle environment. On the contrary, in the CSF microenvironment, MMP-2 showed a positive correlation with CRP levels, suggesting possible local activation associated with neuroinflammation. However, at the peripheral level, this correlation was inverted: in the serum of MS patients, MMP-2 correlated negatively with CRP, indicating a distinct regulation between central and systemic compartments. This dichotomy in the neurological disease was also reflected in MMP-2 correlated to clinical status: in CSF MMP-s levels were reduced during relapse compared to remission, while in serum, an increase was observed during the acute phase. Although this apparent contradiction, it may instead represent a tissue-specific regulatory mechanism, reinforcing the value of MMP-2 as a differential biomarker across biological systems. On the other hand, MMP-2 gelatinolytic activity in DD was influenced by the rs1042704 genotype and regulated by the expression of MMP-14. This encourages a context-specific activation mechanism, related also to further genetic and environmental factors. Thus, MMP-2 may also represent a potential therapeutic target besides a pathophysiological marker.

MMP-9 exhibits a much more variable behaviour and distribution than MMP-2 across the models. Its expression was generally low in confined compartments such as FF and CSF and absent in *in vitro* cell models. This finding is consistent with the immune origin of MMP-9, which is mainly produced by cells such as neutrophils and macrophages in response to inflammatory stimuli (Murphy et al., 1980; Opdenakker et al., 2001). This dependence on pro-inflammatory signals also explains its absence in cell lysates or conditioned media from Dupuytren myofibroblasts cultured without immune stimulation. When present, MMP-9 was mainly detectable in its pro-form, while the active form was rarely observed. Zymography allowed the identification of complexed forms of MMP-9, such as dimeric proMMP-9 and that bound to the physiological inhibitor TIMP-1. In FF, only the zymogenic form of MMP-9 was found, correlating positively with CRP levels and negatively with age, indicating a regulation influenced by individual and subclinical factors. In CSF and serum of MS patients, MMP-9 was also detected in other complexed forms but not in the active form. Surprisingly, MMP-9 in MS was significantly less expressed compared to patients with other non-inflammatory diseases, without correlation to CRP levels. The consistent detection of proMMP-9/TIMP-1 complex in CSF suggests that the regulatory role of TIMP-1 may be determinant in MMP-9 control within the CNS (Fainardi et al., 2006). Even at the peripheral level, the presence of the proMMP-9/TIMP-1 complex was constant, indicating the importance of the balance between gelatinase and inhibitors at the systemic level as well (Fainardi et al., 2006). Investigating the expression

of the regulator TIMP-1 could be a cue for further understanding the regulatory processes of MMP-9. In the serum of MS patients, MMP-9 was abundantly expressed, both in proenzyme and complexed or dimeric form. Interestingly, the dimeric form of proMMP-9 was detected only in serum but not in plasma or closed compartments such as CSF or FF. This pattern advises that the phenomenon may be due to coagulation-induced neutrophil degranulation processes during sample preparation (Gerlach et al., 2007). Due to its high variability, linked to both biological and technical factors, MMP-9 might be less suited as a direct biomarker. Nevertheless, a deeper investigation into the regulatory mechanisms of its expression and activation could open up new perspectives for its use in systemic monitoring.

To conclude, the studies conducted and illustrated in this thesis confirm that MMPs are multifaceted enzymes, whose expression and regulation vary significantly both between different biological systems and within the same pathophysiological context. The multisystem analysis of physiological and pathological conditions showed that MMP behaviour is strongly context-dependent, tissue-specific, and closely related to health status. The results also showed a high inter-individual variability influenced by various factors such as age or subclinical inflammation. Although a uniform interpretation is hard to achieve, this regulatory and functional complexity underlines the importance of a contextual approach, aimed at investigating the specificities of the cellular microenvironment, tissue origin and pathological framework in which these proteases operate. Further studies are necessary to uncover the molecular mechanisms involved in MMP regulation.

CONCLUSIONS

This PhD thesis offers new insights into the multifunctional role of matrix metalloproteinases in three different pathophysiological systems, related to reproductive physiology and pathological conditions such as neuroinflammation and fibrosis. Through a multisystem approach and by applying proteomic and functional analyses, important results were achieved to validate MMPs as functional biomarkers and therapeutic targets. The main findings related to the three major thematic areas explored in this thesis are outlined and discussed below.

1 In Reproduction Physiology: Follicle Microenvironment

In the context of reproductive physiology, analysis of follicular fluid from women undergoing assisted reproductive techniques revealed distinct expression profiles of gelatinases MMP-2 and MMP-9. In particular, MMP-2 was consistently present in its inactive form in all samples analysed, suggesting a constitutive function in follicular extracellular matrix remodelling. However, the detection of MMP-2 active form within FF-EVs suggests a mechanism of compartmentalisation of gelatinase, potentially related to a localised and targeted tissue remodelling. On the other hand, MMP-9 showed highly variable expression between subjects, correlating positively with C-reactive protein expression levels, used an indicator of systemic inflammation. This observation reflects the fact that MMP-9 is regulated at multiple levels within the follicle and might be influenced by local inflammatory response, being a possible modulator under conditions of follicular stress.

A relevant element emerged when comparing the age of the patients: both gelatinases, MMP-2 and MMP-9 in the FF showed a decrease in activity with increasing age, indicating a possible age-dependent function of MMPs in the follicular microenvironment. Stratifying the patients into age groups, this trend also occurs in FF-EVs in the younger groups, whereas it is nullified in patients over 40 years. Thus, ovarian ageing could also be reflected in the effectiveness of communication via EVs in the follicle, especially in relation to the targeted and regulated regulation of compartmentalised gelatinases. Moreover, consistent with the literature, analysis of systemic inflammation levels by CRP in patients showed a positive correlation with increasing age. Ovarian ageing, therefore, is not limited to quantitative loss of oocyte reserve, but involves qualitative changes in the follicular microenvironment involving tissue remodelling, inflammatory regulation, and cell-matrix communication, affecting oocyte quality and endometrial receptivity.

It has been observed that follicular fluid from women aged between 25 and 38 years can stimulate endometrial receptivity, evaluated as increased migratory capacity of endometrial epithelial cells in *in vitro* models of receptive and non-receptive tissue. Treatment with the fluid induced a pronounced and prolonged increase in migratory capacity in the non-receptive endometrial model, characterised by a polarised epithelial structure compared to the receptive model, highlighting how FF can transiently induce the same loss of polarity that necessarily occurs during the embryo implantation window. At the molecular level, this resulted in a reduction in the expression of E-cadherin, an adhesion protein responsible for cell adhesion, confirming the activation of processes similar to the epithelial-mesenchymal transition. Both the pro-migratory effect and the decrease in E-CAD expression were found to be age-dependent, being significantly more evident in samples from the younger group of women. This confirms that the molecular quality of FF is favoured in younger patients, reflecting an optimal functional state in the follicular microenvironment. For these reasons, a more in-depth characterisation of the molecular content of FF and FF-EVs through proteomic and transcriptomic approaches could pave the way for new biotechnological applications in reproductive medicine, offering tools to improve endometrial receptivity and implantation success in ART treatments.

Taken together, these findings imply not only quantitative alterations (e.g, oocyte number) but also qualitative and molecular alterations in the follicular microenvironment and endometrial receptivity with aging. The evaluation of MMPs and the functional characterization of FF-EVs could therefore represent a new approach to the discovery of predictive biomarkers for ART treatment success and to the personalized interventions applicable to the techniques.

2 In Neurological Disease: Multiple Sclerosis

A multisystemic approach was also applied to the analysis of biological fluids derived from patients diagnosed with multiple sclerosis. High-resolution proteomic analysis revealed a differential expression profile in MS patients compared to other neurological disorders. Beyond confirming the tissue specificity of the identified proteins, enrichment analyses revealed that these proteins are predominantly associated with biological processes and pathways related to the inflammatory profile of the disease and the dysregulation of the BBB. Among the putative diagnostic markers, IGFBP-2, a binding protein that regulates the growth factor IGF-2, emerged. Since the dysregulation of IGF-2 may be decisive in the pathogenesis of MS, the

overexpression of IGFBP-2, confirmed by ELISA testing, may be promising in the search for specific biomarkers to improve the diagnosis and prognosis of the disease. This insight was encouraged by the application of machine learning models, which highlighted IGFBP-2 as a robust predictor of MS, improving discrimination compared to controls.

Alongside, gelatinolytic activity in the central compartment was assessed by analysing the CSF of patients with multiple sclerosis using zymography. Unexpectedly, overall gelatinase activity was reduced in MS patients compared to neurological controls, with a statistically significant decrease in MMP-2 in its active form. Stratifying patients according to clinical course, it was observed that in the relapsing-remitting MS patients, both gelatinases MMP-2 and MMP-9, in all their forms (active, inactive, and complexed), had significantly lower levels than individuals with non-inflammatory neurological diseases. Furthermore, in RRMS patients, gelatinase levels were further reduced during the relapse phase compared to the remission phase. Finally, CRP expression in CSF as an indicator of neuronal inflammation was explored, also revealing lower levels overall in MS patients. However, a positive correlation was observed between proMMP-2 and CRP, suggesting that in MS patients, even a modest central inflammatory response may influence gelatinase regulation.

The expression profile of gelatinases was also analysed in the peripheral compartment by studying serum and plasma. Also in this context, the activity of both gelatinases (MMP-2 and MMP-9) was generally significantly reduced in patients with multiple sclerosis compared to neurological controls, confirming a trend consistent with that observed in the central compartment. An interesting finding emerged following stratification by clinical stage and disease status, as serum proMMP-2 showed significantly higher activity during the acute phases of the disease than during remission phases. This opposite trend compared to that observed in CSF suggests that peripheral regulation of MMP-2 responds to different regulations, potentially related to the acute systemic phase of the disease. This divergence in the behaviour of serum MMP-2 could represent a useful prognostic indicator and a tool for timely monitoring of the disease, especially in cases of mismatch between clinical symptoms and radiological findings. The possibility of identifying a sensitive marker in an easily accessible and less invasive matrix, such as serum, would enable faster and more replicable follow-up, improving clinical management and early intervention in the event of recurrence.

Further studies are needed to investigate MMP expression in plasma in relation to clinical data from patients with multiple sclerosis. Unlike serum, plasma preserves coagulation factors and

minimises MMP-9 activation due to coagulation-induced degranulation. Extracellular vesicles have been isolated from plasma samples, and their functional characterisation represents an important future prospect after this PhD project. Proteomic and transcriptomic analyses of plasma-derived EVs may contribute to the identification of peripheral biomarkers useful for monitoring and understanding the disease.

3 In Fibrosis: Dupuytren's Disease

This last study explored the functional impact of the rs1042704 polymorphism in the *MMP14* gene on MMP-2 proteolytic activity and MMP-14 inhibition response in primary myofibroblasts of Dupuytren's disease patients. Zymography assays revealed that myofibroblasts homozygous for the variant allele (AA) have the highest basal proMMP-2 activity, contrasting with that described in the literature for the MT1-N273 mutant. This may be related to the inherent biological heterogeneity of primary patient cells, indicating that it is necessary to expand the study group. Therapy with an anti-MMP-14 inhibitory antibody caused a genotype-specific enhancement of gelatinolytic activity that was evident to a greater extent in AA fibroblasts. No changes in the levels of MMP-2 or MMP-14 protein expression were noted, suggesting that enzymatic activity is controlled post-translationally, probably through a modulation of the interaction with cofactors like TIMP-2.

Cumulatively, the findings confirm the hypothesis that the rs1042704 polymorphism affects MMP-14 activity and pharmacological inhibition sensitivity of cells, opening new perspectives for the utilization of individualized treatment approaches to treat localized fibrosis according to the patient's genotype. Further proteomic analyses currently underway will contribute significantly to the interpretation of the results, allowing us to characterise the changes in the fibroblast secretome induced by treatment and offering a broader view of the molecular mechanisms underlying the genotype-dependent response.

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ACADEMIC AND RESEARCH ACTIVITIES

Conferences/workshop attendance

1. 2nd PhD Workshop of Technologies and Science for Human Health, organised by University of Palermo, STEBICEF Department, 23-25 October 2023, Ed. 18.

2. 68° Convegno GEI - SIBSC 2023", Gruppo Embriologico Italiano - Società Italiana Biologia dello Sviluppo e della Cellula (GEI - SIBSC), 5 – 8th June 2023, Oliveri (ME).

3. First STeBiCeF Young Researcher Workshop, Poster Session, organised by University of Palermo, on 12 January 2023, at Ed. 16.

4. 69° Convegno GEI - SIBSC 2024", Gruppo Embriologico Italiano - Società Italiana Biologia dello Sviluppo e della Cellula (GEI - SIBSC), 11 - 14th June 2024, Napoli (NA).

5. CNR/Royal Society-funded meeting, organised by Norwich Medical School, University of East Anglia, Glasgow, 21-23 July 2024.

6. C4Neuro - La rete del CNR per le Neuroscienze, Consiglio Nazionale delle Ricerche (CNR), 24-25 ottobre 2024, Palermo (PA).

7. Third Workshop Technologies and Science for Human Health, organised by University of Palermo, STEBICEF Department, 28-30 October 2024, Ed. 17.

School attendance (Summer, Winter, etc.)

1. XVII. Biotechnet Summer School on Advanced Biotechnology; Palermo; 3-6 September 2023

Oral Presentation at Conferences/workshop

1. Zymographic Analysis of Matrix Metalloproteinases (MMP-2 and MMP-9) in Cerebrospinal Fluid and Sera from Patients with Multiple Sclerosis
   First STeBiCeF Young Researcher Workshop, STeBiCeF Department, University of Palermo, Palermo (PA), 12 January 2023.

2. Matrix Metalloproteinases (MMPs) in Follicular Fluids (FF) and FF-Derived Extracellular Vesicles (EVs) from Patients Undergoing In Vitro Fertilization (IVF) Techniques. 68th Congress of the Italian Embryological Group – Italian Society for Developmental and Cell Biology (GEI–SIBSC), Oliveri (ME), 5–8 June 2023.

3. Matrix metalloproteinases (MMP-2 and MMP-9) in biological fluids of multiple sclerosis patients. 2nd PhD Workshop of Technologies and Science for Human Health, STeBiCeF Department, University of Palermo, Palermo (PA), 23–25 October 2023.

4. New Proteomic Insights Into Multiple Sclerosis From Cerebrospinal Fluid
69th Congress of the Italian Embryological Group – Italian Society for Developmental and Cell Biology (GEI–SIBSC), Naples (NA), 11–14 June 2024.

5. Matrix metalloproteinases in Dupuytren's disease: effects of anti-MMP14 antibodies on gelatinases as potential therapeutic application. CNR/Royal Society-funded meeting, organized by Prof. Linda Troeberg, Norwich Medical School (University of East Anglia, UK), Glasgow, United Kingdom, 21–23 July 2024.

6. Exploring Matrix Metalloproteinases as Diagnostic Biomarkers in Multiple Sclerosis: A Comparative Study of Biological Fluids. C4Neuro – La rete del CNR per le Neuroscienze, National Research Council (CNR), Palermo (PA), 24–25 October 2024.

7. Effects of anti-MMP-14 antibody response in Dupuytren's disease: looking forward to novel therapeutic strategies. Third Workshop Technologies and Science for Human Health, University of Palermo, STeBiCeF Department, Ed. 17, 28–30 October 2024.

Presented Posters at Conferences/workshop

1. F. Vaglica, L. Rizzi, E. Peri, G. Palazzo, F. Geraci, A. Ferro, C. Liuzzo, M. Galletta, S. Caudullo, P. Cancemi. From Waste To Wonder: In Vitro Effect Of Follicular Fluid In Enhancing Endometrial Receptivity. 70th Congress of the Italian Embryological GroupItalian Society of Development and Cell Biology (GEI-SIBSC) - (2025). Modena, 9-13 June 2025.

2. F. Vaglica, M. Buttacavoli, S. D. Scilabra, M. Lo Pinto, E. Peri, C. D'Amico, S. Marino, F. Geraci, G. Salemi, P. Cancemi. New Proteomic Insights Into Multiple Sclerosis From Cerebrospinal Fluid. 69th Congress of the Italian Embryological Group-Italian Society of Development and Cell Biology (GEI-SIBSC) - European Journal of Histochemistry: a journal of functional cytology. ISSN: 1121-760X. Vol. 68 No. s1 (2024). Naples, 11-14 June 2024. https://doi.org/10.4081/ejh.2024.4071

3. C. D'Amico, M. Buttacavoli, S. Marino, E. Peri, F. Vaglica, S. Feo, F. Baldi, P. Cancemi. Deciphering The In Vitro Toxicity Of Biogenerated Silver Nanoparticle In Breast Cancer: A Multiomics Analysis. 69th Congress of the Italian Embryological Group-Italian Society of Development and Cell Biology (GEI-SIBSC) - European Journal of Histochemistry: a journal of functional cytology. ISSN: 1121-760X. Vol. 68 No. s1 (2024). Naples, 11-14 June 2024. https://doi.org/10.4081/ejh.2024.4071

4. E. Peri, M. Buttacavoli, C. D'Amico, S. Marino, F. Vaglica, I. Pucci-Minafra, E. Roz, S. Feo, P. Cancemi. New Insights In The Epithelial-To-Mesenchymal Transition In Breast

Cancer Progression. 69th Congress of the Italian Embryological Group-Italian Society of Development and Cell Biology (GEI-SIBSC) - European Journal of Histochemistry: a journal of functional cytology. ISSN: 1121-760X. Vol. 68 No. s1 (2024). Naples, 11-14 June 2024. https://doi.org/10.4081/ejh.2024.4071

5. F. Vaglica, M. Buttacavoli, F. Geraci, A. Ferro, C. Liuzzo, M. Galletta, S. Caudullo, P. Cancemi. Matrix Metalloproteinases (MMPs) In Follicular Fluids (FF) And FF-Derived Extracellular Vesicles (EVs) from Patients Undergoing To In Vitro Fertilization (IVF) Techniques. 68th Congress of the Italian Embryological Group-Italian Society of Development and Cell Biology (GEI-SIBSC) - European Journal of Histochemistry: a journal of functional cytology. Vol. 67 No. s3 (2023) ISSN 1121-760X. Oliveri, 5-8 June 2023. https://doi.org/10.4081/ejh.2023.3836

6. F. Vaglica, M. Buttacavoli, E. Peri, C. D'Amico, S. Marino, F. Geraci, G. Salemi, P. Cancemi. MMP-2 And MMP-9 Expression Levels In Cerebrospinal Fluid And Derived-Extracellular Vesicles From Patients With Multiple Sclerosis. 68th Congress of the Italian Embryological Group-Italian Society of Development and Cell Biology (GEI-SIBSC) - European Journal of Histochemistry: a journal of functional cytology. Vol. 67 No. s3 (2023) ISSN 1121-760X. Oliveri, 5-8 June 2023. https://doi.org/10.4081/ejh.2023.3836

7. E. Peri, M. Buttacavoli, C. D'Amico, S. Marino, F. Vaglica, I. Pucci-Minafra, E. Roz, S. Feo, P. Cancemi. Exploring The Epithelial-To-Mesenchymal Transition in Breast Cancer by Proteomic and in Silico Investigations. 68th Congress of the Italian Embryological Group-Italian Society of Development and Cell Biology (GEI-SIBSC) - European Journal of Histochemistry: a journal of functional cytology. Vol. 67 No. s3 (2023) ISSN 1121-760X Oliveri, 5-8 June 2023. https://doi.org/10.4081/ejh.2023.3836

8. M. Buttacavoli, F. Vaglica, F. Geraci, G. Salemi, P. Cancemi. Zymographic Analysis of Matrix Metalloproteinases (MMP-2 and MMP-9) in Cerebrospinal Fluid and Sera from Patients with Multiple Sclerosis". First STeBiCeF Young Researcher Workshop. STEBICEF Department, University of Palermo. ISBN: 978-88-942066-1-6. 12 January 2023.

9. F. Caradonna, F. Naselli, I. Cruciata, S. Volpes, P.S. Cardinale, L.A. Capici, P. Cancemi, F. Vaglica, S.D. Scilabra, M. Calligaris, A.P. Carreca, R. Chiarelli, G. Schiera, C.M. Di Liegro, I. Di Liegro. P2.5 Clonal Evolution in Genomically Unstable Rat Astrocyte Cell Lines: Cytogenetic, Epigenetic and Proteomic Study. XVI FISV CONGRESS, 3R: Research, Resilience, Reprise. Reggia di Portici, Naples. 14 – 16 September 2022. ABSTRACT BOOK (pp. 101-101).

Papers (published, submitted)

1. Schiera, G., Cancemi, P., Di Liegro, C. M., Naselli, F., Volpes, S., Cruciata, I., Cardinale, P. S., Vaglica, F., Calligaris, M., Carreca, A. P., Chiarelli, R., Scilabra, S. D., Leone, O.,

Caradonna, F., & Di Liegro, I. (2023). An In Vitro Model of Glioma Development. Genes, 14(5), 990. https://doi.org/10.3390/genes14050990

Other activities:

1. Seminar: Look to the Future - La ricerca Scientifica dell'Università degli Studi di Palermo, organized STEBICEF, Ri.MED, Vivere Ateneo, in 16 e 20 December 2021, at Ed. 17, Aula C, duration of 8 hours.

2. Seminar: Progetto CoRI 2021 - Azione D3 Seminars, organised by Prof. Aurelio Lorico, on 15-16 June 2022, at Via Archirafi, 32, duration of 4 hours.

3. Seminar: Progetto CoRI 2019 - Azione D3 Seminars, organised by Prof.ssa Maria Grazia Zizzo, on 25-27 October 2022, at Aula Vittorelli (Ed. 16, Campus Viale delle Scienze, UniPa). Seminar cycle held by Prof. Jakub Fichna on the topic "Neurotransmitters in health and disease".

4. Training: SEM TRAINING: potenzialità della Microscopia Elettronica ed utilizzo del microscopio a scansione FEI-ThermoFisher Versa 3D, organized by ATeN Center, Prof. Vincenzo La Carrubba, and Dott. Giorgio Nasillo, in 20 June 2022, at Ed. 18 - ATeN Center, duration of 2,5 hours.

5. Seminar: Role of Aurora-A Mitotic Kinase in Breast Cancer: From the Bench to the Bedside, Dipartimento STeBiCeF, Prof. Aldo Di Leonardo (PA del S.S.D. BIO/18), Prof. Antonino B. D'Assoro, 16th March 2023, Aula Vincenzo Mutolo, Ed. 16.

6. The Best Poster Presentation Award at the 68th Congress of GEI–SIBSC, Oliveri, 5–8 June 2023, awarded by GEI–SIBSC (Italian Embryological Group – Italian Society for Developmental and Cell Biology), June 2023.

7. Teaching Tutor, selected through a competitive examination based on qualifications and interview, for 200 hours of tutoring, integrative teaching, preparatory, and remedial activities in the field of Biology (BIO/13) at the STeBiCeF Department, University of Palermo, during the academic years 2022/2023 as PhD student in Technologies and Science for Human Health.

8. Student representative on the PhD Board of the Doctoral Program in Technologies and Science for Human Health, 37th cycle, academic year starting 2021/2022.

9. Member of the Quality Assurance Committee for the PhD Programme in Technologies and Sciences for Human Health (Cycle XXXVII), contributing to the review of ANVUR guidelines and the planning of PhD candidate satisfaction surveys.

10. Six-month research period abroad as a visiting researcher at the laboratory of Dr. Rose K. Davidson, University of East Anglia (UEA), Norwich, United Kingdom. The activity was part of the PhD programme and focused on the study of matrix metalloproteinases

(MMPs) in Dupuytren's disease.

11. Member of the Local Organizing Committee of the Third Workshop Technologies and Science for Human Health, October 2024, Viale delle Scienze, Palermo, in the role of PhD Student Representative for the PhD Program in Technologies and Science for Human Health, University of Palermo, 37th cycle.

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