The multifaced role of HtrA1 in the development of joint and skeletal disorders

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Abstract

HtrA1 (High temperature requirement A1) family proteins include four members, widely conserved from prokaryotes to eukaryotes, named HtrA1, HtrA2, HtrA3 and HtrA4. HtrA1 is a serine protease involved in a variety of biological functions regulating many signaling pathways degrading specific components and playing key roles in many human diseases such as neurodegenerative disorders, pregnancy complications and cancer.

Due to its role in the breakdown of many ExtraCellular Matrix (ECM) components of articular cartilage such as fibronectin, decorin and aggrecan, HtrA1 encouraged many researches on studying its role in several skeletal diseases (SDs). These studies were further inspired by the fact that HtrA1 is able to regulate the signaling of one of the most important cytokines involved in SDs, the TGFβ-1.
This review aims to summarize the data currently available on the role of HtrA1 in skeletal diseases such as Osteoporosis, Rheumatoid Arthritis, Osteoarthritis and Intervertebral disc degeneration (IDD). In fact, the use of HtrA1 as a marker of frailty in geriatric medicine would allow identifying older individuals at risk of developing skeletal disorders, evaluating an appropriate intervention to improve quality care in these people avoiding or improving age-related SDs in the elderly population.

Keywords: HtrA1; Osteoporosis; Rheumatoid Arthritis; Osteoarthritis; Intervertebral disc degeneration; Musculoskeletal disorders

1. Introduction

The High-Temperature Requirement A (HTRA) serine protease belongs to a serine proteases family protein widely conserved in both prokaryotes and eukaryotes and firstly identified in *E. coli* [1, 2]. These proteins function as heat shock-induced serine proteases and have an additional chaperone activity. Four human homologues have been identified, i.e. HtrA1, HtrA2, HtrA3 and HtrA4 [3, 4].

As shown in Figure 1, HtrA1 family proteins share several structural similarities including at least one protease domain (PD) and one C-terminal PDZ domain (except HtrA3 short form: HtrA3-S). The catalytic site of PD domain is composed by histidine (His), arginine (Asp) and serine (Ser) residues while the PDZ domain is composed by ZO-1 (*zonula occludens* 1), PSD-95 (mammalian postsynaptic density of 95 kDa) and DLG (Drosophila discs large tumor suppressor) domains [5].

The N-terminal domains of HtrA1, 3 and 4 are characterized by a signal peptide (SP), an insulin-like growth factor binding protein (IGFBP) domain and a Kazal-type serine protease inhibitor (KI) domain [6] while the HtrA2 homologous has a transmembrane anchor (TM) depending on cellular localization and function. Moreover, due to alternative splicing, two HtrA3 isoforms have been found: HtrA3-S of 39 kDa and a long form of 49 kDa (HtrA3-L) [7]. These proteolytic enzymes have a pivotal role in many signaling pathways by degrading specific components and playing key roles in many human diseases such as cancer [8], arthritis [9], age-related macular degeneration [10], neurodegenerative disorders [11], endometriosis [12] and pregnancy complications [2].

The role of HtrA1 as a secreted protease has prompted investigations into identifying the primary targets of HtrA1-dependent proteolysis such as ECM substrates, fibronectin, decorin, aggrecan, and elastin [13, 14], and different growth factors, as transforming growth factor- β (TGF-β) [15].

Many researches have been encouraged on studying the role of HtrA1 in several skeletal diseases (SDs) due to its role in cell differentiation processes [13, 16-19] and in the remodelling of the ECM [20-22].

It has been suggested that HtrA1, produced and secreted by both osteoblasts and osteoclasts [13, 16], may be able to regulate bone formation by acting on TGF-β and BMP pathways [15] and by degrading BMP2, BMP4 and TGF-β1 [23].

Contrasting results regarding the role of HtrA1 in bone homeostatis have been found in both animal and cellular models. In fact, HtrA1 knockout mice showed an higher bone density and increased trabecular bone mass [24, 25] suggesting a negative role for HtrA1 in bone formation. Contrarly, some in vitro studies showed a positive role of HtrA1 in bone formation [18, 26]. Moreover, it has been demonstrated that HtrA1 is upregulated during osteogenesis favoring the formation of mineralized matrix [18] probably upregulating TGF-β1 signaling [26]. In addition, it has been also shown that HtrA1 plays also a key role in bone resorbision by favoring osteoclastogenesis degrading Osteoprotegerin (OPG), an inhibitor of osteoclastogenesis [16]. Interestingly, HtrA1 is also expressed in chondrocytes suggesting a

possible role of this protease in regulating homeostasis of articular cartilage. In fact, it has been shown that HtrA1 expression is increased in chondrocytes exposed to inflammatory citokynes such as TGF-β1 contributing to cartilage degeneration characteristic of articular diseases such as osteoarthritis and rheumatoid arthritis [27, 28]. So, it can be suggested that alterations in HtrA1 expression may impair the proper regulation of TGF-β family proteins signaling leading to SDs onset. Many data support that HtrA1 expression is very low in healty tissues but it rapidly increases in inflammaty conditions such as osteoarthritis and rheumatoid arthritis worsening the outcome of these pathologies [29-33].

Osteoarthritis, rheumatoid arthritis and osteoporosis are SDs that affect the locomotor system typically characterized by pain and limited mobility persisting lifelong [34]. Moreover, SDs are commonly related to aging, becoming the most common cause of morbidity and mortality in elderly individuals [35].
Due to abilty of HtrA1 to modulate the signaling of TGF-β family proteins directly (interacting with BMP2, BMP4 and TGF-β1 [23]) or indirectly (cleaving their receptors [25]), HtrA1 can play a key role in SDs onset. HtrA1 may indirectly worsen SDs by cleaving fibronectin in the ECM generating fibronectin fragments that increase the expression of MMP-1,-3,-13 by binding to the integrins on the cellular membranes [36].
The aim of this review is therefore to improve current knowledge regarding the involvement of HtrA1 in human diseases in order to evaluate HtrA1 as a marker and/or potential therapeutic target of SDs.

2. HtrA1 in skeletal diseases

2.1 Osteoporosis

Osteoporosis is a skeletal disorder characterized by decreased density (mass/volume) of normally mineralized bone that leads to decreased mechanical strength thus making the skeleton more likely to fracture [37]. Differently from osteoporosis, the term osteopenia is used to define bones weaker than normal but with a bone density not as low as in osteoporosis [38]. If untreated, osteopenia can progress into osteoporosis.
The crosstalk between osteoblasts and osteoclasts is an essential requirement for the balancing of bone remodeling: the alteration of coordinated activities between osteoclasts and osteoblasts contributes to bone diseases [39]. Discovering novel factors that mediate these interactions become increasingly important to understand the molecular mechanisms underlying bone homeostasis.
Wang and colleagues, by genotyping patients affected by Cushing disease (CD), a rare disease that causes metabolic syndrome, cardiovascular disease, and osteoporosis due to hypercortisolism, found a correlation among Secreted phosphoprotein 1 (SPP1), collagen type Iα1 chain (COL1A1), 5'-nucleotidase ecto (NT5E), angiopoietin 1 (ANGPT1), HtrA1 and the onset of osteoporosis in these

patients. In particular, they suggested that the pituitary adenoma secretes altered concentrations of the above-mentioned five proteins tagged to skeletal targets leading to osteoporosis [40].

Tiaden and colleagues showed that HtrA1 was significantly increased in mesenchymal stem cells isolated from human bone marrow (hBMSCs), mouse embryonic stem cells, and mouse adipose-derived stromal cells (mASCs) when osteogenesis was induced *in vitro*. Moreover, recombinant HtrA1 could enhance osteogenesis of hBMSCs suggesting an important role of this protein in bone fracture repair by promoting new bone formation [18].

In addition, Wu and colleagues [19] showed that HtrA1 was highly up-regulated in Receptor Activator of Nuclear factor Kappa-B Ligand (RANKL)-induced osteoclastogenesis, inhibiting osteoblast differentiation and attenuating the activation of Smad1/5/8, ERK1/2, p38 and the gene expression of alkaline phosphatase (ALP), Runt-related transcription factor 2 (RUNX2), osteocalcin (OCN) and osteopontin (OPN) induced by bone morphogenetic protein-2 (BMP2) in pre-osteoblasts. These data suggested a role of HtrA1, produced by both osteoclasts and osteoblasts, in regulating bone remodeling processes by inhibiting osteoblast differentiation presumably preventing the activation of BMP2 receptor via HtrA1 direct binding to BMP2 [23].

Moreover, it has been shown that HtrA1 expression was increased in differentiating 2T3 osteoblasts before mineralization but was subsequently down-regulated in fully mineralized 2T3 cultures. The PD and the PDZ domains are essential for the inhibitory effect of HtrA1 on mineralization because they are involved in the cleavage of specific ECM proteins such as decorin, fibronectin, and matrix Gla protein. This suggests a key role of HtrA1 in regulating ECM calcification via degrading these specific substrates [13].

In addition, a recent study showed that HtrA1 can regulate osteoclast differentiation by degrading Osteoprotegerin (OPG), a decoy receptor of RANKL, that inhibits osteoclastogenesis blocking RANKL signaling [16]. This suggested a potential role of HtrA1 as a target in the treatment of osteoporosis due to its action on osteoclasts in favouring osteoclastogenesis.

As known, angiogenesis plays a key role in bone remodeling since blood vessels supply nutrients, oxygen, hormones, and cytokines; not physiological processes, oxidative stress, and drugs can impair endothelial function affecting bone formation processes [41-43]. In addition, it has been shown that bone cells can modulate endothelial cells producing angiogenic factors such as TGF-β family proteins, highlighting an interplay between these cell types [44].

In this regard, as above reported HtrA1 can regulate TGF-β signaling presumably degrading their receptors or the ECM proteins that positively modulate TGF-β signaling [23] and consequently can modulate angiogenesis by TGF-β family members [45].

Thus, it is also possible that the locally produced HtrA1 by osteoclasts and osteoblasts may contribute to the homeostasis of cartilage subchondral bone interface. In addition, given that HtrA1 is widely expressed in many tissues including the brain, heart, and ovary, it remains to be determined whether an excess in the production/secretion of HtrA1 protein by other cell types in disease conditions might contribute to the bone and joint pathologies.

In summary (see Table 1), HtrA1 may modulate bone development and repair regulating osteoclast and osteoblast-mediated bone remodeling, and thus may serve as a potential therapeutic target for the treatment of bone disorders such as osteopenia and osteoporosis.

2.2 Rheumatoid Arthritis

Rheumatoid arthritis (RA), is an autoimmune and inflammatory disease that causes inflammation with pain and swelling that commonly affects joints in the hands, wrists, and knees leading to the damage of the joint tissue [46]. The inflammatory cytokines produced during this process (i.e. IL-1β and TNF-α) stimulate the production of matrix metalloproteinases (MMPs) that can degrade the components of the articular cartilage ECM [47]

HtrA1 can degrade cartilage by a direct cleaving of the ECM components and stimulating the production of matrix metalloproteinase 1 (MMP1) and 3 (MMP3) in synovial fibroblasts [29], therefore an impaired expression of HtrA1 may be involved in the pathogenesis of arthritic diseases.

Toll-like receptor 4 (TLR4) activation by Lipopolysaccharides (LPS) significantly increased HtrA1 through the NF-kB pathway, while Interferon γ (IFN- γ) significantly inhibited both HtrA1 basal and LPS-induced expressions by p38 MAPK/STAT1 pathway in fibroblasts and macrophages in collagen-induced arthritis (CIA) mouse model [48]. These data proved that HtrA1 expression can be modulated by different pathways playing a role in the onset of arthritis.

Moreover, the inhibitory effect of IFN-γ on HtrA1 expression was also shown in human synovial cells obtained from patients affected by RA. These data were further confirmed in IFN-γ and IFN-γR1 knockout mice, where HtrA1 expression was increased in the joint tissues of the CIA mouse model. Interestingly, the use of neutralization antibodies against HtrA1 significantly ameliorated CIA frequency and severity in IFN-γ- and IFN-γR1-deficient mice. [49]. These studies suggest an important role of HtrA1 in the pathogenesis of RA by using antibodies against HtrA1 as a potential treatment in this pathology and highlight a protective effect of IFN-γ in the developing RA.

The inhibitory effect of IFN- γ on HtrA1 expression in joint tissues may be one of the reasons for the protective effect of IFN-γ against RA in mice and humans [50, 51]. In particular, STAT1 binds directly to HtrA1 promoter downregulating HtrA1 transcription in p38 MAPK/STAT1 pathway mediated by

IFN-γ [49], identifying STAT1 as an important transcriptional inhibitor of HtrA1 expression and as a key molecule in the RA development.

Although the function of TLR ligands and IFN-γ in RA is well delineated, they have to be considered two factors among many other factors. It was reported that cytokines associated with T helper 17 cells (Th17), such as IL-17, IL-21, IL-22, and IL- 23, were involved in the pathogenesis of many human diseases, including RA [52]. In addition, IL-6, and IL-12 caused significantly higher HtrA1 production in Mouse Embryonic Fibroblasts (MEFs) and macrophages [49]. Thus, proinflammatory cytokines such as IL-21, IL-6, and IL-12 might contribute to the RA pathogenesis, at least in part, via the HtrA1-dependent pathway.

Polygonum orientale L. stems and leaves extracts have been shown to have anti-inflammatory and analgesic effects [53]. Interestingly, *Polygonum orientale* L. extracts significantly ameliorated symptoms of RA reducing paw swelling volume, arthritis score, synovial hyperplasia, inflammatory cell infiltration, cartilage surface erosion, and joint degeneration. The concentrations of Prostaglandin E2 (PGE2), Tumor necrosis factor α (TNF-α), and IL-1β were also significantly decreased. Moreover, it has been shown that flavonoids extracted from this plant formed strong protein-ligand complexes with HtrA1 (especially), Janus kinase 1 (JAK1), Cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), and Prostaglandin E2 (PGE2) protein. These compounds, inhibiting the inflammatory function of the above-mentioned proteins, could ameliorate RA symptoms and reduce tissue damaging characterizing this disease [54]. A hypothesis of HtrA1 role in RA maintenance is depicted in Figure 2A and a summary of the studies about the role of HtrA1 in RA is reported in Table 1.

2.3 Osteoarthritis

Osteoarthritis (OA) is a multifactorial inflammatory disorder associated with many risk factors such as aging, obesity, joint misalignment, and acute injury. It is one of the most common chronic diseases worldwide and is characterized by loss of mobility, joint pain, and deformity that can lead to a progressive loss of joint functionality [55].

Interestingly, the levels of HtrA1 mRNA were 7-fold more elevated in cartilage from patients with OA compared with non-arthritic controls [30]. This may be due to the fact that in OA cartilage both hypomethylation of the HtrA1 gene and overexpression of HtrA1 protein were found, strongly suggesting an epigenetic regulation of HtrA1 expression in this pathology [56].

Polur and colleagues found increased HtrA1 mRNA expression in the knee and temporomandibular joints (TMJ) of type IX collagen-deficient, type XI collagen-haploinsufficient mice or with a surgically induced OA [32], suggesting a potential involvement of HtrA1 in the initial phases of the OA development. Moreover, it has been shown that resting chondrocytes start to differentiate and

express HtrA1 leading to increased HtrA1 levels in the joint cartilage after inducing OA in mice. HtrA1 can degrade cartilage components, such as aggrecan, fibromodulin, decorin, and soluble type II collagen suggesting a potential role of HtrA1 in promoting degeneration of cartilage [33]. Increased expression of HtrA1 was also found in knee articular cartilage and TMJ of collagen type II α 1 chain (COL2A1) mutant mice by immunohistochemical analysis. Consequently, HtrA1 upregulation preceded fibrillation and fissuring of the articular surfaces [57, 58].

Chen and colleagues found that the degradation of articular or condylar cartilage was significantly delayed in $HtrA1^{-/-}$ and $COL11A1^{+/-}$mice, and $HtrA1^{-/-}$ mice after the Destabilization of the Medial Meniscus (DMM) on the knee or its Partial DiscEctomy (PDE). Moreover, they found that the amount of collagen type VI was significantly higher in the articular cartilage of $HtrA1^{-/-}$ and $COL11A1^{+/-}$ mice, compared with $COL11A1^{+/-}$ mice suggesting that genetic removal of HtrA1 could delay articular or condylar cartilage degradation in mice [21]. These data proved that HtrA1 is involved in cartilage remodelling/degradation in normal conditions.

Xu and colleagues found an increased expression of TGF-β1, pSmad2/3 and HtrA1 in the articular chondrocyte of knee joints in the mouse models of OA. They also found an increased expression of pSmad2/3 and HtrA1 co-localized in the articular chondrocyte of the knee joints. In addition, the level of HtrA1 mRNA was increased in human and mouse articular chondrocytes when treated with TGF-β1 compared with the untreated ones, suggesting a key role of canonical TGF-β1 signaling in the induction of HtrA1 in the articular chondrocytes of OA mouse models [28].

Moreover, Long and colleagues, by using two models of OA mice, chondrodysplasia mutation ($cho^{-/+}$) (a COL11A1 mutation) and disproportionate micromelia mutation ($Dmm^{-/+}$) (a COL2A1 mutation), found and increased expression of TGF-β1, pSmad2, and HtrA1 in the chondrocytes of both mutant condylar cartilages. They also found hypocellularity and chondrocyte clustering, with surface fissuring in both mutants, demonstrating the involvement of these molecules in the degradative pathway of OA [27]. In addition, by using immunohistochemical analysis of temporomandibular joint (TMJ) of Receptor for Advanced Glycation End products (RAGE) knock out (KO) mice with surgically induced OA, Matias and colleagues found statistically lower levels of MMP-13 and HtrA1 but higher TGF-β1 compared to WT mice demonstrating a potential role of RAGE in modulating HtrA1 in mouse TMJ OA [59].

Articular cartilage vesicles (ACVs) are extracellular organelles of 50–150 nm containing RNA and proteins found in normal articular cartilage [60]. Interestingly, Rosenthal and colleagues, isolating ACVs from normal and OA human knee cartilages, found decreased levels of matrix proteoglycans and increased levels of TGF-β–inducible gene H3 (βig-H3), Developmental Endothelial Locus-1

(DEL-1), vitronectin and HtrA1 compared to normal ACVs suggesting that HtrA1 is also present in these extracellular organelles [61].

In addition, mouse tissues with late OA after destabilization surgery were positive for HtrA1 and negative for TGF-β1 suggesting an important role of HtrA1 in lowering TGF-β1 expression with the progression of OA, likely inhibiting the control of the TGF-β1-mediated inflammation occurring during the early OA and worsening the disease [31].

Since OA is an irreversible disease that causes the degradation of the articular cartilage, innovative treatments have been studied to improve chondrocyte stimulation and cartilage restoration. Autologous chondrocyte implantation (ACI) aims to restore the articular cartilage, inducting hyaline-like cartilage formation [62]. Ollitrault and colleagues [17] studied an interesting combination of treatments that were able to improve the human chondrocyte phenotype before ACI. In fact, they found that targeting type I collagen and/or HtrA1 serine protease with transfected siRNA led to an increase of type II collagen and aggrecan, two markers of restored hyaline cartilage, with an improvement in the chondrocyte differentiation by using *nude* mice and human articular chondrocytes (HACs). This study proved a key role of HtrA1 in chondrocyte differentiation and hyaline cartilage, suggesting HtrA1 as a target to improve ACI efficiency.

Although aging is one of the most common risk factors associated with the development of OA, early-onset OA can develop in young people secondary to dysplasia of the hip (DDH) [63]. Hernandez and colleagues showed that chondrocytes of patients affected by hip dysplasia highly express HtrA1 suggesting a correlation between high levels of HtrA1 in DDH and early-onset OA, which play a key role in damaging joint cartilage. So, prompt therapeutic treatments lowering HtrA1 may improve or avoid cartilage damage in DDH patients blocking the possible occurrence of early-onset OA [64].

Interestingly, topical application of Wogonin - a compound extracted from the plant *Scutellaria baicalensis* with anti-inflammatory and anti-oxidative properties - applied as a cream in a surgical mouse model of OA, ameliorating OA symptoms and attenuating the severity. Moreover, immunohistochemical analysis showed a significant decrease in the expression of HtrA1, TGF-β1, matrix metalloprotease 13 (MMP13) and NF-κB proteins in wogonin-treated mice [65]. Moreover, Losartan, an angiotensin II receptor antagonist, has been revealed to attenuate the progression of OA in the synovial TMJ and promote joint repair reducing fibrocartilage formation [66, 67]. Losartan treatment of OA chondrocytes or intraarticular injection of 10 mg/mL Losartan into the knee joint led to PPARγ activation reducing levels of IL-6, IL-1β, TNF-α, COX-2, TGF-β1, MMP13, HtrA1, and iNOS, along with reduced pSMAD2 and pSMAD3 activation *in vivo* and *in vitro* [68]. The beneficial effects of Losartan were further proved by using an OA mouse model (COL11A1 mutation): results showed a diminished degeneration of TMJ condylar and knee joint articular cartilage after Losartan

treatments. Interestingly, a decreased expression of HtrA1 was also found *in vitro* and *in vivo* demonstrating the utility of Losartan to improve treatment of OA inhibiting HtrA1 expression and articular cartilage damage [66].

Marine origin collagen hydrolysates such as Promerim® were proven to be clinically effective for treating joint stiffness associated with OA [69]. Interestingly, Bourdon and colleagues showed that Promerim® was able to increase protein synthesis of collagen types I and II and decrease HtrA1, MMP1, MMP3, Adamts5, and Cox2 using equine articular chondrocytes (eACs) cultured as organoids. Moreover, the effect of Promerim® was enhanced under inflammatory conditions (in presence of IL-1β) [70]. The same research group found decreased expression of HtrA1 and MMP1 by using human articular chondrocytes (HACs) as organoids in the presence of Promerim® with or without IL-1β to mimic an OA environment. Moreover, treatment with Promerim® increased the neo-synthesis of type I collagen and/or type II collagen isoforms [71]. These last two studies demonstrated that these substances could be considered beneficial dietary supplements to relieve OA symptoms or delay OA progression. A hypothesis of HtrA1 function in OA is shown in Figure 2A and a summary of the studies about the role of HtrA1 in OA is reported in Table 1.

2.4 Intervertebral Disc Degeneration

Intervertebral disc (IVD) degeneration (IDD) is an age-related disorder due to the loss of disk height, increased disk bulging and subsequent pressure on nerve roots that lead to chronic low back pain (LBP) [72]. The leading cause of IDD is the alteration of matrix turnover due to the increased production of matrix-degrading enzymes that lead to the degeneration of IVDs [73]. In fact, IDD begins in the nucleus pulposus (NP) when the number of IVD cells decreases and they lose functionality leading to the gradual degradation of ECM components and fibrosis of NP [74].

Li and colleagues found that the expression level of HtrA1 increased with decreasing mineral bone density using MRI T2 values. Moreover, they highlighted a linear correlation between HtrA1 levels and the T2 value, suggesting a strong correlation between HtrA1 and the degree of IDD. This indicates that the degradation of ECM proteins (e.g., aggrecan and type II collagen) may be associated with an increased expression of HtrA1, suggesting its possible involvement in IDD pathological progress [75].

Urano and colleagues showed an association of the functional rs11200638 single nucleotide polymorphism (SNP) in the HtrA1 gene with radiographic features of IDD in 513 Japanese postmenopausal women, founding that the subjects without the G allele (AA) had a significantly higher spinal disc space narrowing score than the subjects with at least one G allele (GG + GA). This study suggests that a genetic variation at the HtrA1 gene promoter locus may be associated with IDD occurrence [76].

On contrary, Tiaden and colleagues in a different cohort of study composed of 39 patients undergoing spinal surgery for symptomatic degenerative disc disease, did not find any correlation with the appearance of rs11200638 SNP in the promoter region of the HtrA1 gene. However, HtrA1 mRNA and protein expression were significantly increased in degenerated disc tissues. Moreover, the treatment of IVD cells with recombinant HtrA1 increased MMP1, MMP3 and MMP13 production through a mechanism MEK-dependent. Additionally, HtrA1-induced fibronectin proteolysis led to the formation of various sized fragments which caused a significant increase in MMP1, MMP3 and MMP13 expression when added to IVD cells. These results support the hypothesis of a possible role of HtrA1 in promoting IVD degeneration through the proteolytic cleavage of fibronectin [36].

In addition to the previous mentioned ECM proteins, a key role in IVD degeneration is played by Chondroadherin (CHAD), a member of the leucine-rich repeat family, already involved in some idiopathic scoliosis [77]: when CHAD is cleaved by proteases, its fragments rise linearly with the increasing of the severity of disc degeneration. Interestingly, ADAMTS (4 and 5), cathepsins (K, B, L) and MMP (-3, -7, -12, and -13) were incapable to cleave CHAD, unlike HtrA1, suggesting a key role of HtrA1 in IDD onset [20].

Furtwängler and colleagues found a decrease in disc height significantly correlated with glycosaminoglycans (GAGs) changes in NP tissues of bovine IVD injected with active HtrA1 compared to controls, suggesting that an increased HtrA1 expression may contribute to decreasing disc height under pathological conditions [78]. In addition, Li and colleagues, by treating human nucleus pulposus cells (HNPCs) with recombinant HtrA1 alone or in combination with specific inhibitors of p38, ERK, NF-κB, and JNK, proved that HtrA1 was able to induce the expression of ADAMTS-5 in a dose-dependent manner by modulating ERK, NF-κB and JNK pathways [22]. The same group found significant increases in mRNA expression of HtrA1 and MMP1, 3, 9, and 13 in IDD tissues compared with control. Moreover, HNPCs treated with recombinant HtrA1 led to increased expression of MMP1, 3, and 13 mRNAs and proteins. When HNPCs were treated with ERK1/2 and ROCK signaling pathway inhibitors there was a decrease in rHtrA1-induced MMPs production [79]. These two studies clearly showed a key role of HtrA1 in modulating different MMPs by acting on specific pathways suggesting that suppression of HtrA1 or a local inhibition of ERK/NF-κB/JNK and/or Rho-associated Kinase (ROCK) signaling pathways may be a useful therapeutic strategy for the treatment of IDD.

Increased HtrA1 levels were also found in the Annulus Fibrosus (AF) of IDD patients suggesting a role of HtrA1 in the impairment of this structure [80]. A hypothesis of HtrA1 function in IDD is depicted in Figure 2B and a summary of the studies about the role of HtrA1 in IDD is reported in Table 1.

3. Conclusion and further remarks

Frailty is a geriatric syndrome that results from a multi-system impairment associated with increased vulnerability to stressors and increased risk of major adverse health outcomes, including disability, falls, institutionalization, hospitalization, and mortality [81, 82]. Due to the overall weakness and limited motor performance characterizing frailty syndrome, elder people affected by frailty are subjected to develop an age-related musculoskeletal disease called sarcopenia, a condition characterized by a progressive and generalized loss of muscle mass and strength [83]. Interestingly, a recent study showed an association of HtrA1 with a decreased incidence of sarcopenia, particularly in men, suggesting a potential role of HtrA1 in this disease and a possible function as a biomarker [84].

The age-related inflammation imbalance can be a major driving force for frailty. Interestingly, Lorenzi and colleagues demonstrated an association of plasma levels of HtrA1 with frailty status. In particular, HtrA1 plasma concentration was significantly higher in frail compared to non-frail individuals, regardless of potential confounders. Moreover, they showed a trend of increased HtrA1 levels with a worsening of frailty status suggesting this protein as a potential marker of frailty severity [85].

The up-regulation of HtrA1 in plasma of frail old people can cause an inhibition of TGF-β1, which is involved in the inflammatory process. Indeed, HtrA1 is implicated in the inhibition of TGF-β superfamily proteins signaling by its protease domain as demonstrated in *in vitro* and *in vivo* experiments [23]. TGF-β1 is probably the isoform more deeply involved in frailty among the TGF-β isoforms that are secreted in latent forms and then bioactivated by proteolysis [86]. TGF-β1 was shown to have an important role in the regulation of immune response and inhibition of inflammation [87], being the predominant isoform expressed in the immune system [88].

In addition, an inverse relationship between HtrA1 and TGF-β1 expression patterns was reported in several inflammatory diseases as in frail individuals. Hence, HtrA1, being a multifunctional protein involved in a variety of physiological and pathological processes [23, 89], may reflect the intrinsic complexity of frailty.

HtrA1 could be defined as an inflammatory molecule playing not only a physiological role but also a fundamental role in regulating pathological processes including osteoporosis, osteoarthritis, rheumatoid arthritis and IDD. Due to its serine protease activity, HtrA1 is able to regulate TGFβ-1, one of the most important cytokines involved in many diseases including SDs [15, 90-92] and it plays important actions on this cytokine in bone omeostasis, in fact, modulating TGF-β/BMP and RANKL/OPG signaling, HtrA1 can regulate bone formation and resorption. HtrA1 is also able to cleave many ECM components of articular cartilages such as fibronectin, decorin and aggrecan making these components primary targets of HtrA1-dependent proteolysis [13, 14, 93].

In addition to previously reported studies [94], in the present review we summarised the current literature regarding the multifaceted role of HtrA1 in SDs. In particular, we dicussed its role in bone homeostatis regulating osteoblasts differentiation by controlling integrin-binding sialoprotein (IBSP) turnover, bone morphogenetic protein 5 (BMP5) and sclerostin [18]. Moreover, HtrA1 can induce osteoclasts differentiation attenuating BMP2 [19] and degrading OPG [16]. In addition, increased HtrA1 levels have also been found in synovial fluids of patients affected by rheumatoid arthritis and osteoarthritis [29, 30], probably associated with hypomethylation of its promoter [56]. HtrA1 may also be involved in worsen these pathologies by directly or indirectly degrading articular or condylar cartilage. In fact, an excessive mechanical stress can led to joint inflammation due to an increased release of TGF-β1 and other inflammatory citokynes by chondrocytes. In turn, TGF-β1 can activate SMAD2/3 stimulating HtrA1 expression and secretion, then leading to ECM degradation [27, 28]. TGF-β1 can also be regulated by HtrA1 itself. In fact, in osteoarthritis HtrA1 was only observed in tissues which stained negative for TGF-β1 [31] suggesting a key role of HtrA1 in regulating TGF-β signaling. Figure 3 summarizes the pathways involved in the possible regulation of HtrA1 expression and function.

An important aspect of HtrA1 is its rule as a marker in many cancerous [95-97] and not cancerous diseases [85, 98-100] preventing or allowing an early treatment of these pathologies. This aspect may be very important in the clinical practice of SDs. In fact, HtrA1 could also be used as early marker in diagnosis of SDs allowing an early treatment and then improving SDs outcome. Thus, in geriatric medicine, identifying older individuals at risk of becoming frail could allow an appropriate and early evaluation and intervention to improve quality care in these people avoiding or improving age-related SDs in the elderly population. Differently from other parameters [101], the detection of HtrA1 plasma levels could represent a non-invasive and simple approach to screen frailty in the elderly.

Interestingly, Wogonin, Losartan and Collagen hydrolysates such as Promerim® were shown to be promising compounds in the treatment of OA by lowering HtrA1 expression and inflammatory cytokines such as TGF-β1, IL-6, IL-1β, TNF-α ameliorating OA symptoms or delaying OA progression reducing articular cartilage damage [65, 66, 68, 70, 71]. Moreover, natural flavonoids extract from *Polygonum orientale* L. was able to form strong protein-ligand complexes with HtrA1 potentially inhibiting its function ameliorating RA symptoms and reducing tissue damage [54]. Further beneficial effects on RA were highlighted from the use of neutralization antibodies against HtrA1 significantly reducing RA symptoms and severity [49].

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Table 1. Studies of HtrA1 protein in joint and skeletal disorders

MODEL STUDIED	PATHOLOGY	RESULTS	REFERENCE
Cushing disease (CD) patients	Osteoporosis	Secreted phosphoprotein 1 (SPP1), collagen type I α 1 chain (COL1A1), 5'-nucleotidase ecto (NT5E), angiopoietin 1 (ANGPT1) and HtrA1 genes have been found dysregulated in CD patients.	[40]
human bone marrow-derived MSCs (hBMSCs), mouse embryonic stem cells (mASCs), mouse adipose-derived stromal cells (mASCs)	Osteoporosis	HtrA1 increased in hBMSCs and mASCs when osteogenesis was induced *in vitro*. Recombinant HtrA1 could enhance osteogenesis of hBMSCs	[18]
Primary mouse bone marrow macrophages (BMM), mouse bone marrow stromal cell line (KusaO)	Osteoporosis	Increased HtrA1 in RANKL-induced osteoclastogenesis inhibiting osteoblast differentiation and attenuating BMP2-induced activation of Smad1/5/8, ERK1/2, p38 and the gene expression of ALP, Runx2, OCN and OPN in pre-osteoblasts	[19]
2T3 osteoblast cell line	Osteoporosis	HtrA1 expression was increased in differentiating 2T3 osteoblasts before mineralization and subsequently down-regulated in fully mineralized 2T3 cultures. HtrA1 cleaved decorin, fibronectin, and matrix Gla protein by its protease domain (PD) and the PDZ domain.	[13]
Primary osteoblastic cells from mice and bone marrow macrophages (BMM)	Osteoporosis	HtrA1 degrades OPG regulating osteoclasts differentiation	[16]
IFN-γ and IFN-γR1 knockout mice, mouse embryonic fibroblasts (MEFs), mouse macrophages and human synovial cells from patients	Rheumatoid Arthritis	IFN-γ inhibited the basal and LPS-induced HtrA1 expression in fibroblasts and macrophages through activation of p38 MAPK/STAT1 pathway. Increased HtrA1 expression in the joint tissues. IL-21 increased HtrA1 expression in MEFs but not in macrophages, whereas IL-6 and IL-12 increased HtrA1 expression in macrophages but not in MEFs. Neutralizing antibodies against HtrA1 reversed the enhanced CIA frequency and severity in IFN-γ and IFN-γR1 knockout mice.	[49]
Human Synovial Fibroblasts (HSF) from patients	Rheumatoid Arthritis	Elevated synovial HtrA1 levels in fluids obtained from patients. Treatment of synovial fibroblasts with HtrA1 or HtrA1-generated fibronectin fragments increased MMP1 and MMP3.	[29]

C57BL/6 mice, mouse macrophages and Mouse embryonic fibroblasts (MEFs)	Rheumatoid Arthritis	LPS activates TLR4 increasing HtrA1 expression in fibroblasts and macrophages through the NF-kB pathway	[48]
RA rat model	Rheumatoid Arthritis	*Polygonum orientale* L. extracts significantly ameliorate symptoms of rheumatoid arthritis reducing paw swelling volume, arthritis score, and concentrations of PGE2, TNF-α, and IL-1β. Flavonoids contained in the plant extracts inhibit HtrA1, JAK1, COX-2, iNOS, and PGE2 protein target receptors	[54]
Receptor for Advanced Glycation End-product (RAGE) knockout mice	Osteoarthritis	HtrA1 positive staining was only observed in tissues which stained negative for TGF-β1. Tissues with moderate cartilage degradation, early OA, stained positive for TGF-β1, while no TGF-β1 staining was present in specimen with late OA where HtrA1 was present	[31]
Human articular chondrocytes (HACs) and *nude* mice	Osteoarthritis	Silencing type I collagen and/or HtrA1 led to an increase of type II collagen and aggrecan, improving chondrocyte differentiation	[17]
Cartilage from patients	Osteoarthritis	Elevated HtrA1 mRNA in cartilage from patients with osteoarthritis compared with nonarthritic controls.	[30]
IX collagen-deficient, type XI collagen-insufficient and surgically induced OA mice	Osteoarthritis	Increased HtrA1 mRNA expression in the knee and temporomandibular joints at early stages of the disease	[32]
OA induced in BALB/c mice by anti-collagen antibodies and LPS	Osteoarthritis	Increased HtrA1 expression in joint cartilage of OA induced mice.	[33]
Patients with OA secondary to DHH	Osteoarthritis	Chondrocytes of DHH patients with OA showed higher expression of HtrA1 compared to DHH patients without OA	[64]
Mouse models of osteoarthritis	Osteoarthritis	Degradation of articular or condylar cartilage was significantly delayed in $HtrA1^{-/-}$ and $COL11A1^{+/-}$ mice and $HtrA1^{-/-}$ mice. Increased expression of collagen type VI in the articular cartilage of $HtrA1^{-/-}$ and $COL11A1^{+/-}$ mice, compared with $COL11A1^{+/-}$ mice	[21]
COL2A1 mutant mice	Osteoarthritis	Increased expression of HtrA1 in knee articular cartilage and knee articular cartilage and TMJ of COL2A1 mutant mice	[57, 58]
surgically induced OA mice	Osteoarthritis	Decreased protein expression of HtrA1, TGF-β1, matrix MMP13 and NF-κB in wogonin-treated mice	[65]
equine articular chondrocytes (eACs)	Osteoarthritis	Promerim increased protein synthesis of collagen types I and II, while decreasing HtrA1, MMP1, MMP3, Adamts5, and Cox2.	[70]
human articular chondrocytes (HACs)	Osteoarthritis	Decreased expression of HtrA1 and MMP1 in HACs treated with Promerim with or without IL-1β	[71]
Human primary chondrocytes and Mouse models of osteoarthritis	Osteoarthritis	Increased expression of TGF-β1, pSmad2/3 and HtrA1 in the articular chondrocyte of knee joints of OA mice. Increased levels of HtrA1 mRNA in human and mouse articular chondrocytes treated with TGF-β1	[28]
COL11A1 and COL2A1 mutant mice	Osteoarthritis	Increased expression of TGF-β1, pSmad2, and HtrA1 in the chondrocytes of both mutant condylar cartilages	[27]

		associated with hypocellularity and chondrocyte clustering with surface fissuring	
RAGE KO mice with surgically induced OA	Osteoarthritis	Low expression of MMP13 and HtrA1 but higher TGF-β1 in mouse TMJ OA compared to WT	[59]
Articular cartilage vesicles (ACVs) from normal and OA tissues	Osteoarthritis	Decreased levels of matrix proteoglycans and increased levels of βig-H3, DEL1, vitronectin and HtrA1 in OA ACVs compared to normal ACVs	[61]
OA tissues from patients	Osteoarthritis	hypomethylation and overexpression of HtrA1 in osteoarthritic cartilage tissues	[56]
Chondrocytes from OA patients and OA mouse model	Osteoarthritis	Losartan treatment led to PPARγ activation reducing levels of IL-6, IL-1β, TNF-α, COX-2, TGF-β1, MMP13, HtrA1, and iNOS, along with reduced pSmad2 and pSmad3 activation *in vivo* and *in vitro*	[68]
OA mouse model	Osteoarthritis	Losartan treatment led to diminished degeneration of TMJ condylar and knee joint articular cartilage decreasing expression of HtrA1	[66]
Nucleus pulposus from IVD patients	Intervertebral disc degeneration	Expression of HtrA1 linearly correlate with MRI T2 value	[75]
IVD tissue/blood and IVD cells from patients	Intervertebral disc degeneration	HtrA1 mRNA and protein expression increased in IVD tissues. Treatment of IVD cells with recombinant HtrA1 increased MMP1, MMP3 and MMP13 production through a mechanism MEK-dependent.	[36]
Postmenopausal women	Intervertebral disc degeneration	Patients with rs11200638 SNP in the HtrA1 gene had a significantly higher spinal disc space narrowing score.	[76]
IVD tissue from patients	Intervertebral disc degeneration	Recombinant HtrA1 was able to cleave CHAD suggesting an important role of HtrA1 in IDD	[20]
Nucleus pulposus from bovine IVD	Intervertebral disc degeneration	Decreased disc height significantly correlated with GAGs changes in IVD injected with active HtrA1 compared to controls	[78]
Human Nucleus Pulposus Cells (HNPCs) from IDD patients	Intervertebral disc degeneration	Treatment of HNPCs with recombinant HtrA1 was able to induce the expression of ADAMTS-5 in a dose-dependent manner by modulating ERK, NF-κB and JNK pathways	[22]
Human Nucleus Pulposus Cells (HNPCs) from IDD patients	Intervertebral disc degeneration	Treatment of HNPCs with recombinant HtrA1 led to increased expression of MMP1, 3, and 13 mRNAs and proteins by acting on ERK1/2 and ROCK signaling pathways.	[79]
IDD tissues	Intervertebral disc degeneration	Increased HtrA1 levels in the annulus fibrosus of IDD patients	[80]

OA: Osteoarthritis; IDD: Intervertebral disc degeneration; IVD: InterVertebral Disc; LPS: Lipopolysaccharide; OPG: Osteoprotegerin; CHAD: Chondroadherin; GAGs: glycosaminoglycans; TMJ: temporomandibular joint

Figure legends

Figure 1. Disposition of human serine protease HTRA family domains. SP= Signal Peptide; IGFBP= domain with homology to Insulin-like Growth Factor Binding Protein; KI =Kazal type

serine protease Inhibitor motif; PD= proteolytic domain; PDZ= PDZ domain; MLS= mitochondrial localization signal; TM= transmembrane sequence; IBM= amino acids of the IAP binding motif.

Figure 2. Schematic illustration of HtrA1 functions on Osteoarthritis, Rheumatoid Arthritis (A) and Intervertebral disc degeneration (B)

Figure 3. Schematic illustration of HtrA1 regulation and function. ILs, MMPs and HtrA1are depicted in the extracellular matrix. Fibronectin is depicted as purple linear fragments. From left to right side, five kinds of receptors are present in the cellular membrane. LPS binding to its receptor increases HtrA1 activating NFkB pathway; TGF-β increases HtrA1 activating SMAD2 pathway; unknown receptors trigger pathways to increase HtrA1; fibronectin fragments binding to integrins α-β increase HtrA1; IFNγ receptor decreases HtrA1 activating STAT1 pathway. Integrins-fibronectin fragments binding increases MMP-1, -3 and -13 that are secreted in the extracellular matrix (dashed arrow). In summary, the HtrA1 secreted in the extracellular matrix (dashed arrow) degrades the fibronectin, the binding of its fragments with the integrins triggers the increase of MMPs.

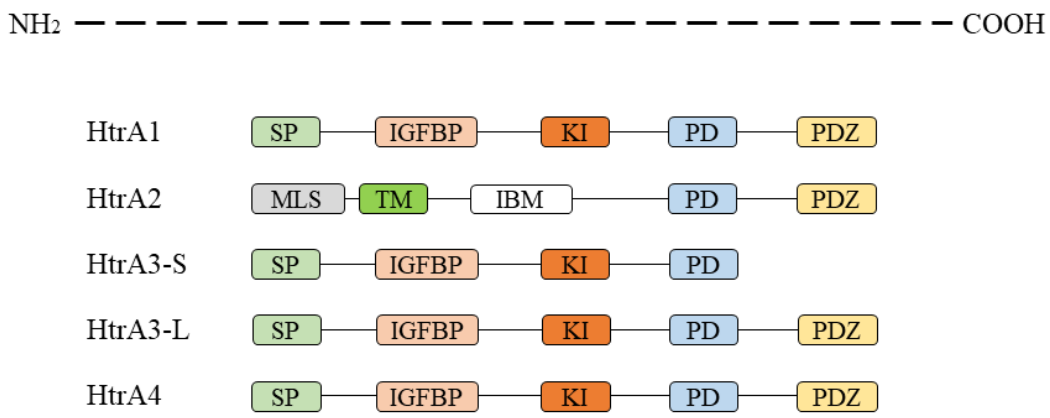


Figure 1

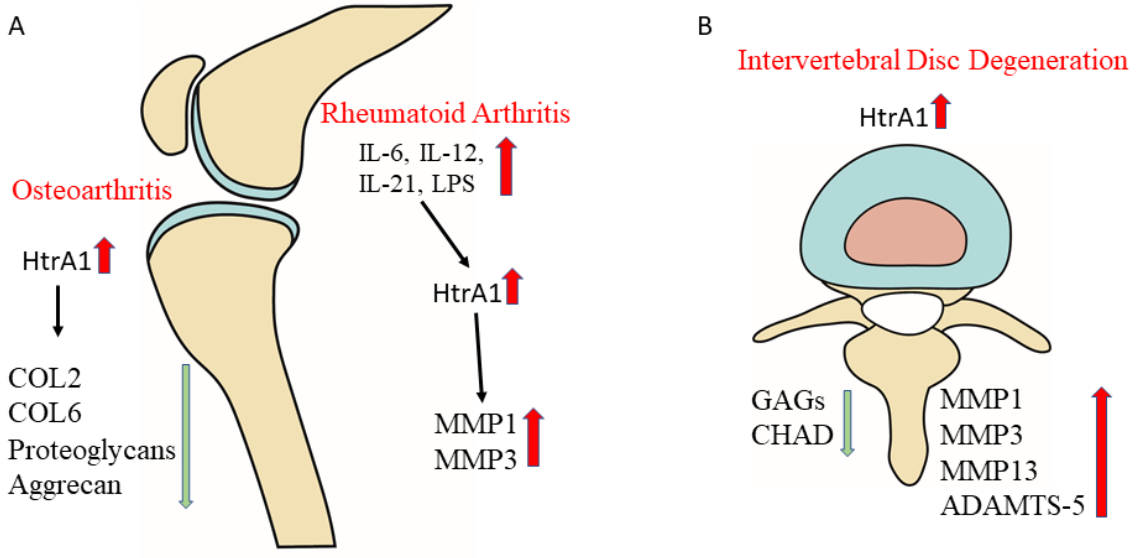
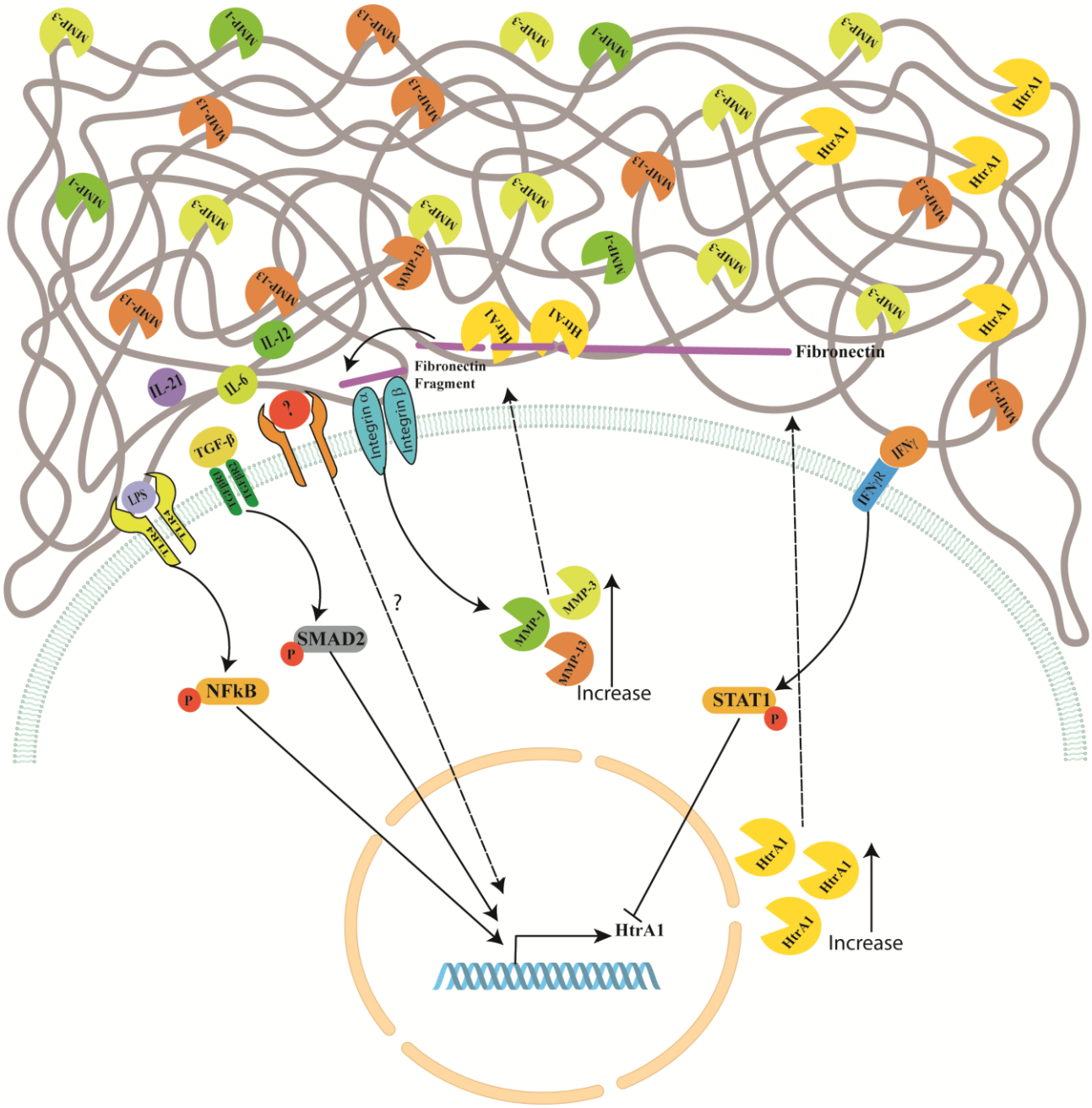


Figure 2


MMP-1
MMP-3
MMP-13
HtrA1
Fibronectin
Fibronectin Fragment
IL-12
IL-21
IL-6
TGF-β
LPS
TLR4
TLR2
TLR1
Integrin α
Integrin β
IFNγ
IFNγR
?
SMAD2
P
NFkB
STAT1
Increase
Increase
HtrA1