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Lipocalin 2 and Matrix-Metalloproteinase-9 in Alzheimer's Disease

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Alzheimer's Disease

Alzheimer's disease (AD) is among the most common neurodegenerative disorders. Its incidence increases with age and its characteristic clinical symptoms are progressive and lead to a gradual decline in cognitive function, due to neuronal death and synaptic loss[1]. In the early stage of AD, patients suffer from memory dysfunction. The progression of the disease to the parietal lobe might result in dysphasia and dyspraxia. After further progression and disorders of the cortical and subcortical regions, patients lose the ability to move and communicate[2].

From a macroscopic point of view, AD is connected with a change in the brain's morphology, which is expressed by a decrease in brain weight due to cerebral atrophy. The highest decline in weight (41 %) is observed in the temporal lobe, followed by the parietal (30 %) and the frontal lobe (14 %)[2].

The hallmark of this neurodegenerative disorder is the extracellular accumulation of amyloid β (Aβ) in the brain, resulting in plaques[3,4]. This aggregation is either due to an up-regulation in Aβ production or a decrease in its degradation[1]. Aβ oligomers can cause impaired synaptic functions, thus changing neuronal activities. Subsequently, glial cells release neurotoxic mediators which result in neuronal damage[1]. However, correlations between the amount and distribution of Aβ deposition and the degree of clinical dementia are rather weak. Aβ accumulation does not only occur in AD patients but can also be present in healthy individuals[2]. It was shown more recently that instead of the amount of Aβ deposition, the solubility of Aβ might play a bigger role in connection with the clinical expression of the disease. Murphy *et al.* revealed that Aβ from patients with MCI is more soluble upon extraction with diverse buffers compared to Aβ from healthy controls.[5,6].

Specific forms of AD can also be caused by genetic mutations. Mutations in the Aβ precursor protein (APP) gene on chromosome 21, as well as mutations in presenilin-1 or -2 (PS1 or PS2) on chromosome 14 or 1, which play a role in processing the APP are associated with familial early onset forms of AD[7]. Apolipoprotein E (ApoE) is responsible for the breakdown of Aβ oligomers. The presence of the *APOE*4 allele increases the risk of late-onset AD[8]. 60 % of all the people with two *APOE*4 alleles will develop AD by the age of 85[9]. ApoE ε2 and ε3 are shown to have a protective[10] or neutral effect in relation to AD. In addition to impairing Aβ clearance, Apo ε4 is thought to promote Aβ deposition and cleavage of ApoE ε4 leads to neurotoxic fragments which are involved in tau pathology in AD[1].

The intracellular accumulation of neurofibrillary tangles (NFT) of tau in the brain is another common characteristic of AD. Tau is primarily present in the cytosol, but can also be found connected to the cell membrane, mainly in axons. There are six isoforms of tau known to be expressed in the human brain, varying in the presence of either three or four repeats of 31 or 33 amino acids (3R tau, 4R tau) and zero, one or two inserts at the N-terminus (0N, 1N, 2N), depending on the mRNA splicing. 4R tau is thought to be involved in the formation of NFTs[11].

Usually, tau is necessary in the cytoskeleton by playing a role in the assembly and stabilization of microtubules. The interaction between tau and the microtubules is regulated by phosphorylation. In certain neurodegenerative diseases some regions, such as the microtubule-binding region in tau become hyperphosphorylated. Upon this phosphorylation the effective binding of tau to microtubules is inhibited[12]. Subsequently, microtubules become unstable and the unbound, hyperphosphorylated tau proteins form aggregations, which over time develop into NFTs. NFT accumulation then impairs neuronal crosstalk due to a disrupted microtubule network and dysregulated axonal transport[13].

The correlation between NFTs and the cognitive impairment in AD seems to be higher than the correlation between plaques and AD[14,15]. Nevertheless, NFTs can also be present in healthy brains. Transgenic mouse studies showed that NFTs do not inevitably cause neuronal dysfunction and are not necessarily AD specific[16]. Some studies also ascribe a protective function to tau, as they suggest that neurons compensate oxidative stress with excessive tau phosphorylation[12].

The Aβ Precursor Protein Processing

Aβ is derived from amyloid precursor protein (APP), a transmembrane glycoprotein, which is produced in neurons. An overexpression study in mice showed that APP is favorable for cell growth[17]. Furthermore, rodent embryos treated with APP RNAi revealed the importance of APP in neuronal precursor cell migration during the development of the brain[18].

APP can be processed in two pathways. Alpha secretases (a disintegrin and metalloproteinase domain-containing protein (ADAM) family members, β-secretase 2 (BACE2)) cleave APP in a nonamyloidogenic way, liberating soluble AAPα (sAAPα), which is important for neuronal plasticity and memory function[19]. The cleavage is followed by processing the 83 amino acid (aa) domain, which is left in the membrane. This step is done by γ-secretase with PS1 and PS2 in their catalytic subunit[20].

Instead of being cleaved by α-secretase, APP can be processed by β-secretase (BACE1), causing the release of sAAPβ and leaving behind a 99 aa membrane bound fragment (CTFβ). sAPPβ is a ligand for death receptor 6 (DR 6). It can induce apoptosis by activating caspase 3 and 6[21]. CTFβ is further processed by γ-secretase and Aβ is created. Depending on the exact cleavage site, different forms of the neurotoxic Aβ exist (e.g. $A\beta_{1\text{-}40}$ or $A\beta_{1\text{-}42}$)[22,23,24]. The investigation of postmortem AD brain samples found an increase in γ- and a decrease in α-secretase, underlining the significance of the latter pathway in neurodegeneration[25].

The function of Aβ varies from regulating the release of synaptic vesicles[26] to controlling neuronal activity via a negative feedback regulation[27]. It acts as a ligand for receptors and can be transported across the blood brain barrier (BBB) and in between tissues[28,29]. Another study

revealed a natural antimicrobial role of Aβ, thereby suggesting that this peptide also plays a role in the innate immune response[30]. Thus, Aβ might have an influence on neuroinflammation which is widely observed in Alzheimer's disease.

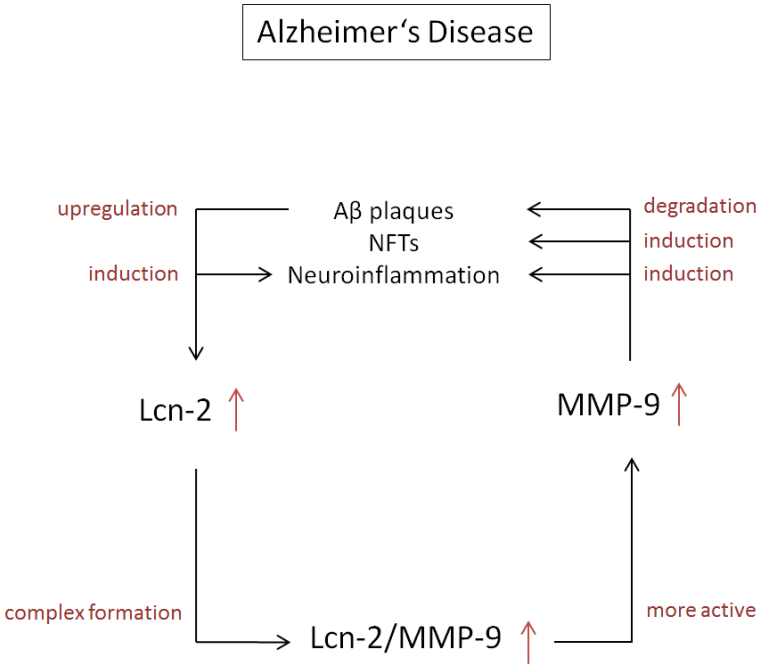

Figure 1: **The interplay of Lcn-2 and MMP-9 in AD.** Lcn-2 is upregulated by Aβ and it might cause neuroinflammation which increases Lcn-2 expression even further. MMP-9 is able to process Aβ. If present in complexation with Lcn-2, MMP-9 is protected from degradation, thus it is more active. In addition to being neuroprotective by degrading Aβ, MMP-9 might be involved in AD by being favorable for NFT formation and by contributing to neuroinflammation by activating growth factors and other proinflammatory cytokines.

Neuroinflammation

A controlled immune response in the brain involves neuroinflammation and it is a necessary process in order to protect the brain from infection, toxic metabolites and other harmful stimuli. Microglial cells are the main mediators of inflammation in the brain. Astrocytes and neurons play a role as well, by releasing and reacting to cytokines in order to protect the brain. However, chronic neuroinflammation due an overreaction in immune response and continuous stimuli with proinflammatory cytokines is thought to contribute to neurodegenerative diseases such as AD.

The presence of neuroinflammation in AD could be observed by investigating target genes, which are involved in inflammation. *TREM2* and *CD33* are immune receptor encoding genes, which are associated with AD[31]. CD33 is a sialic acid-binding immunoglobin-like lectin that plays a role in the innate immunity in the brain. Both genes were shown to be upregulated in microglial cells in postmortem AD brain samples[32]. In addition, the upregulation of inflammatory cytokines was observed in Aβ plaque areas. IL-1 was shown to be increased. IL-1 drives the activation of astrocytes and the induction of acute phase protein expression in these cells, as well

as the complement component C3, indicating the presence of inflammation. Moreover, TNF-α was shown to be upregulated in AD serum and Microglia which were exposed to Aβ overexpressed this pro-inflammatory cytokine[33]. Together these findings support the assumption of neuroinflammation in AD.

Inflammation of the CNS can act in two ways. On the one hand activated microglia have a protective function by clearing the brain from Aβ plaques. On the other hand, these beneficial effects are counteracted by e.g. the production of free radicals, inflammation-related production of Aβ, thus driving neurodegeneration. TNF-α, one of the predominant proinflammatory cytokines in neuroinflammation has also been shown to have both, neuroprotective and neurotoxic properties. It activates signaling cascades via the interaction with TNF receptor 1 or 2 (TNFR1, TNFR2). Stimulation of TNFR1 results in the induction of proinflammatory genes, whereas activation of TNFR2 leads to neuroprotection[34]. The close relation between neuroprotection and neurotoxicity observed in AD-related neuroinflammation marks the complexity of this neurodegenerative disease. It furthermore underlines the importance of comprehending the process in order to gain deeper insights in the pathology of AD. One aspect in understanding the development of the disease is the comprehension of the order of the observed mechanisms to be able to distinguish between cause and consequence and to allow therapeutic intervention.

One way of gaining deeper insight in the regulation of neuroinflammation in AD is the investigation of the transcription profile of rodent cortical neurons upon TNF-α[35]. A gene microarray revealed the up-regulation of lipocalin-2 (Lcn-2) via activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway[36]. NF-κB was first identified in 1968 as a transcriptional enhancer of the κB light chain gene locus in activated B cells. The activated form of NF-κB regulates the transcription of about 150 target genes, including the pro-inflammatory cytokine TNF-α[37,38]. It has been shown that NF-κB is also involved in memory and synaptic transmission in mice[39]. Therefore, a dysfunction in NF-κB signaling might contribute to the development of Alzheimer's disease.

Lipocalin-2

Lipocalins belong to an evolutionary conserved family of more than 20 members, which are characterized by the ability to bind and transport small hydrophobic molecules in a binding pocked made of an eight-stranded antiparallel β-sheet structure[40]. More recently, further functions like odor recognition, cell homeostasis or the carrying of pheromones and hormones have been ascribed to lipocalins. Lipocalins are known to be involved in inflammatory diseases and cancer[40,41]; Lcn-2 for example, has been established as a biomarker in several cancers[42].

Lipocalin-2 is a 25 kDa protein. It was first identified in cytoplasmic granules of human neutrophils[43]. It was found to be covalently bound to the gelatinase matrix metalloproteinase-9

(MMP-9). MMP-9 is secreted by neutrophils to remodel the extracellular matrix (ECM). The association of Lcn-2 and MMP-9 explains its original naming: neutrophil gelatinase-associated lipocalin (NGAL); other names of the protein are: 24p3, siderocalin and uterocalin. Instead of being bound to MMP-9, Lcn-2 also exists in a monomeric or homodimeric form free from the proteinase[43]. It acts as an acute phase protein and its expression is induced upon proinflammatory stimulation. The protein is involved in the innate immune response. During an infection, bacteria take up iron by producing siderophores which first bind host iron and are then transported into the infectious agent. Lcn-2 acts as a siderophore chelator and it sequesters free iron, thus preventing bacteria to take up iron during an infection. In vitro, *LCN2* expression was mainly shown to be induced in macrophages upon lipopolysaccharide (LPS) stimulation of toll-like receptor 4 (TLR-4). Moreover, Lcn-2-deficient mice are more susceptible to bacterial infection[44], showing the importance of Lcn-2 in the innate immunity.

Furthermore, the upregulation of Lcn-2 has been connected to cancer, diabetes and renal diseases, where it is used as a biomarker[42]. Only recently, Lcn-2 was shown to also be contribute to dysfunctions of the CNS[45].

Lipocalin-2 in Alzheimer's Disease

In addition to being involved in the body's immune response against bacteria, Lcn-2 was also shown to be expressed in the brain. The protein is produced and secreted mainly by astrocytes in a proinflammatory environment[46,47]. Lcn-2 expression was shown to be involved in inducing astrocyte and microglia apoptosis in the brain, via the upregulation and translocation of the proapoptotic marker BIM to the mitochondrium[48]. In glial cells Lcn-2 was also observed to regulate cell morphology and motility and also polarization[49]. Furthermore, it has been linked to neuronal cell death[50]. Other than playing a role in the regulation of these cellular processes, Lcn-2 dysregulation is associated with CNS disorders like experimental autoimmune encephalomyelitis, intracerebral hemorrhage, spinal cord injury, neuropathic, ischemic stroke and neurodegenerative diseases[45].

Moreover, induced Lcn-2 levels were observed in postmortem analysis of hippocampus and entorhinal cortex in AD patients compared to age-matched controls. Lcn-2 gene and protein expression in primary cortical neurons, astrocytes and microglia is increased in the presence of TNF-α via the stimulation of TNFR1 and is dependent on NF-κB. This TNF-α-mediated activation of TNFR1 also caused higher Lcn-2 secretion of the respective cells[35]. These findings lead to the assumption that Lcn-2 expression is induced after TNF-α stimulation in neuroinflammation. Aβ production and accumulation, which was shown to have many similarities with antimicrobial peptides, could then be a subsequent step in the brain's innate immune response activated by the inflammation[30].

Another study provided evidence that Lcn-2 is produced and secreted upon the accumulation of Aβ by cells of the CNS. It was shown that primary mouse astrocytes and CP epithelial cells, which were incubated with Aβ, had an increased Lcn-2 expression and secretion. This upregulation was not observed in microglial cells and neurons, which were isolated from rat and mouse brains[51]. Nevertheless, Aβ was shown to have an effect on microglia by polarizing them into a M1 proinflammatory phenotype[49]. Incubation of astrocytes with Aβ also caused increased cell death. This effect was abrogated in respective cells from Lcn-$2^{-/-}$ mice meaning the loss of Lcn-2 results in protection against Aβ-toxicity. Moreover, the presence of Aβ caused an upregulation of proinflammatory genes in primary astrocytes. Interleukin-6 expression was significantly induced in Lcn-$2^{+/+}$ astrocytes. This upregulation could not be observed in Lcn-$2^{-/-}$ astrocytes; instead, the cells seemed be anti-inflammatory. In this respect the lack of inflammation in Lcn-$2^{-/-}$ could also be neuroprotective. Interestingly, comparison of Lcn-2 expression in IL-$6^{+/+}$ and IL-$6^{-/-}$ cells showed a decrease in the latter cells. Therefore, Lcn-2 seems to be necessary to induce the expression of proinflammatory genes in these cells and the other way round. This interdependency emphasizes the complexity of the underlying mechanism[51].

Lcn-2 is known to play an important role in the iron mechanism in the body's immune reaction against bacteria. Iron is known to accumulate in the brain, preferentially the hippocampus and cortex during normal ageing and in AD it supports the accumulation of Aβ[52,53]. Mesquita *et. al* observed an upregulation of the iron metabolism related genes heme oxygenase-1 (*HMOX1*) and ferritin heavy chain (*FTH*), a subunit of ferritin, which stores iron, upon co-culture of rodent primary astrocytes or CP epithelial cells with Aβ. This upregulation could not be observed in Lcn-$2^{-/-}$ astrocytes, thus implying that Lcn-2 plays an essential role in the regulation of *HMOX1* and *FTH* expression[51]. In addition to mediating iron metabolism inside the cell, secreted Lcn-2 can have an impact on the accumulation of Aβ through influencing extracellular iron[52].

Treatment of neurons with Aβ did not cause alterations in Lcn-2 expression. Nevertheless, 24p3r, a Lcn-2 surface receptor was shown to be upregulated on neurons upon Aβ stimulation. This increase indicates that neurons might still be able to respond to higher Lcn-2 levels in AD[51].

Furthermore, Naude *et. al* observed lower Lcn-2 levels in CSF of patients with mild cognitive impairment and AD. Lcn-2 serum levels were shown to be unchanged[35]. This is important in respect to diagnosing AD or subsequent treatment, with Lcn-2 being a potential biomarker. However, this has to be noticed with caution, as another study showed on upregulation of Lcn-2 in the serum of patients suffering from mild cognitive impairment[54].

In general these studies show that Lcn-2 plays a significant role in regulating factors, which drive cognitive impairment and memory loss in AD. Lcn-2 expression is hereby associated with Aβ accumulation and a proinflammatory environment. To be able to understand the function of Lcn-2 in neurodegeneration and to possibly establish this protein as a biomarker, more studies are necessary. One approach could be the investigation of Lcn-2 in connection with MMP-9, as it was shown that these molecules also exist covalently bound to each other.

Matrix Metalloproteinase-9

Matrix metalloproteinases (MMPs) are endopeptidases, which are mainly involved in the breakdown of extracellular matrix (ECM) proteins. These enzymes depend on Zn^{2+} and Ca^{2+} and belong to the family of metzincins, among which are also ADAMs. MMPs have a 3-histidine zinc-binding catalytic (HEXXHXXGXXH) domain in common. A conserved methionine, which follows this histidine-rich domain offers another zinc-binding site and is thus important during catalysis[55]. Due to their ability to interact with the ECM, MMPs play a role in e.g. embryonic development, wound healing, angiogenesis and apoptosis[56].

Growth factors, cytokines and physical stress, but also cell-matrix and cell-cell interactions are able to induce MMP gene expression. The proteolytic function of MMPs is regulated by inhibitors such as tissue inhibitor of metalloproteinases (TIMPs) or α-macroglobulins[56].

Depending on their structure, MMPs are further divided into 6 subgroups. MMP-2 and MMP-9 are gelatinases. They are associated with the degradation of molecules in the basal lamina of the ECM. These MMPs enable neurogenesis, as well as angiogenesis. In addition, these gelatinases are involved in tissue injury and repair and participate in inducing cell death. Stromelysins (MMP-3, MMP-10, MMP-11) are responsible for ECM proteins such as proteoglycans, laminin, elastin, fibronectin and collagen type III, IV and V. MMP-1, -8 and -13 are collagenases which regulate collagen type I, II, III, V, VII, VIII and X, as well as fibronectin degradation in bone and cartilage. Moreover, MMP-7 is a matrilysine with collagen IV, glycoproteins and gelatin as its substrate. The breakdown of cell surface markers, as well as the activation of other MMPs is regulated by various enzymes, including membrane-type MMPs (MT-1 to -6-MMP). Some other uncategorized MMPs (MMP12, 19, 20, 23, 27, 28) are known to be involved in e.g. the degradation of amelagenin, aggrecan and elastin[57].

MMP-9 is, as well as all other MMPs, secreted as an inactive zymogen, which has to be processed in order to be activated. In addition to MT-MMPs, several enzymes, such as trypsin, chymotrypsin, the serine protease kallikrein, and cathepsin G are able to cleave the inhibiting pro-domain from MMPs. Moreover, chemical compounds containing mercury can interact with pro-MMP-9, thereby triggering MMP-9 auto-activation. *In vitro* activation of MMP-9 with kallikrein revealed a cleavage site between Arg87 and Phe88 in the N-terminal area, generating an active 82 kDa MMP-9[41].

MMP-9 has been shown to interact with TIMP-1 in lung fibroblasts and exists in three other forms, free from TIMP. The MMP-9 monomer has a size of 92 kDa in its inactive form. It also exists as a 220 kDa dimer and a 135 kDa protein associated with Lcn-2. The ability of MMP-9 to covalently bind Lcn-2 makes it an interesting enzyme to investigate in regard to AD[43]. Moreover, MMP-9 was shown to be up-regulated upon the UV light-mediated activation of protein kinase JNK-2. When human skin is exposed to ultraviolet B MAP kinase pathways are activated in the

human body, which cause an up-regulation of the transcription factor AP-1 and finally transcription of MMP-9. As most other MMPs, MMP-9 is secreted as a pro-enzyme. It is activated via proteolytic cleavage of the propeptide at the N-terminus by MMP-3. The active MMP-9 (82 kDa) consists of three fibronection type II domains, which are surrounded by catalytic domains. A hemopexin-like domain, which is able to interact with TIMP (mainly TIMP-1[58]) is linked to the catalytic part of the enzyme[56,57]. As the name suggests, MMP-9 or gelatinase B is mainly involved in digesting denatured collagen, gelatin. The fibronectin type II domains are also able to bind ECM protein and subsequently degrade them[59].

Because of their involvement in tissue remodeling, MMPs are associated with ECM-related pathologies, such as fibrosis, but also with cancer, cardiovascular disease and diseases of the CNS[56,58].

Matrix Metalloproteinse-9 in Alzheimer's disease

In the brain, MMP-9 is mainly released by neurons and microglial cells, but its expression was also found in astrocytes[60,61]. In healthy conditions the enzyme is only present in low concentrations in the hippocampus, cerebellum and cerebral cortex[62]. An up-regulation of MMP-9 was observed with aging. MMP-9 is thought to be involved in processing Aβ[63]. This correlation could explain its up-regulation with age, as Aβ was also shown to be more abundant in older people[2]. Another function of MMP-9 in the brain is controlling memory and learning processes by regulating the synaptic plasticity of neurons. MMP-9 was for example shown to be activated in the hippocampus and the prefrontal cortex in rats which completed the Morris water maze task[64]. The knockout of MMP-9 in mice resulted in shorter dendrites and a lower complexity of neurons, thus suggesting that MMP-9 plays a role in the maintaining neuron morphology.

Furthermore, MMP-9 was mentioned to play a role in neurogenesis, angiogenesis, myelinogenesis, axonal growth and apoptosis[57,58]. As reviewed by Vafadari *et al.* MMP-9 has many distinct targets in the brain: growth factors, as well as their precursors (e.g. mature nerve growth factor, pro-form of brain-derived neurotrophic factor), cell surface receptors and cell adhesion molecules[58].

In contrast to rather low MMP-9 concentrations in a healthy brain, MMP-9 was shown to be upregulated upon pathological stimuli and its dysregulation is connected to epilepsy, schizophrenia, autism, brain tumors and neurodegenerative diseases such as AD. Hereby, MMP-9 seems to be involved in inflammatory processes that accompany these pathologies[58].

As mentioned before, AD and neuroinflammation are associated processes, which are thought to influence each other. MMPs play a major role in inflammatory reactions throughout the body and MMP-9 has been shown to play a prominent role in neuroinflammation. It is able to activate pro-inflammatory cytokines and it can cleave β-dystroglycan, thus causing the disruption of the BBB.

Subsequently, leukocytes might migrate into the brain where they can further increase inflammation and secrete more MMP-9, thus further promoting neurodegeneration[58]. Moreover, an up-regulation of MMP-9 was observed in plasma levels of AD patients[65]

The role of MMP-9 in Aβ pathology

In AD animal models, MMP-9 expression and secretion has been shown to be increased in astrocytes which surround Aβ plaques[66]. The enhanced secretion of MMP-9 in the presence of Aβ might be regulated by integrin receptors. Both, integrin receptors, as well as their ECM ligands were shown to be increased in Aβ plaques. Furthermore, Aβ itself was established as an integrin ($\alpha_3\beta_1$ and $\alpha_2\beta_1$) ligand *in vitro*[67].

MMP-9 inhibition caused an increase in Aβ, thereby indicating an important role of MMP-9 in the Aβ pathology[68]. Both findings support the idea that MMP-9 might be involved in the cleavage of Aβ, thus it is hypothesized that the sporadic form of AD might be caused by an impaired Aβ clearance due to a dysregulation of MMP-9[68]. In this case MMP-9 acts in a neuroprotective way, by cleaving the neurotoxic $A\beta_{42}$. In addition, Talamagas *et al.* confirmed that MMP-9 is also involved in processing APP. Overexpression of MMP-9 in HEK cells led to a higher secretion of sAPPα and reduced Aβ, hence showing that MMP-9 plays a role in the nonamyloidogenic processing of APP. Further investigation showed that the inhibition of MMP-9 abrogated the effect of enhanced sAPPα and co-localization of MMP-9 and APP695 was observed on the cell surface. These findings indicate that MMP-9, which has a very similar structure to ADAM α-secretases might have α-secretase activity itself and directly act on the APP[67]. Nevertheless, it cannot be completely excluded that MMP-9 does not cleave APP, but only activates other α-secretases, in a mechanism that might resemble the activation of ADAM10 by meprin-β[69].

In general, this information suggests, that MMP-9 acts in a neuroprotective way, either by cleaving Aβ or by increasing the neurotrophic sAPPα, thereby also passively lowering Aβ levels. Neuroinflammation could be considered a negative side effect of constant up-regulation of MMP-9 due to increased Aβ in AD.

Before being activated, MMP-9 exists as an inactive pro-enzyme, which was shown to co-localize with possible activators. It was suggested by Talamagas *et al.* that secreted pro-MMP-9 might interact with APP in a way to keep it close to the cell surface, to enable its activation by other proteolytic enzymes. Active MMP-9 might subsequently cleave APP, thus releasing sAPPα. This hypothesis was supported by a co-immunoprecipitation study which revealed co-existence of inactive 92 kDa pro-MMP-9 and APP[67]. MMP-3 is one of the enzymes, which is able to activate MMP-9. MMP-3 has been shown to be up-regulated by astrocytes and neurons upon exposure to Aβ and in brain regions with senile plaques a co-localization of MMP-9 and MMP-3 was observed. This indicates that the activation of MMP-9 by MMP-3 also plays an important

role. This regulation should be further studied, as it might be responsible for the dysregulation of MMP-9 in neurodegenerative diseases[13].

In addition to MMP-9, there are other enzymes which are involved in the proteolytic cleavage of Aβ, all exhibiting different cleavage sites: neprilysin, insulin-degrading enzyme, endothelin- and angiotensin-converting enzyme, plasminogen activators and MMP-2.

MMP-9 in tauopathies

The aggregation of tau in neurofibrillary tangles in the brain is one of the hallmarks of AD. Various proteinases cleave the tau protein, thereby creating pro-aggregatory tau fragments, which assemble to NFTs. It has been shown that specific tau fragments, such as tau_{391} and tau_{421} appear in NFTs more frequently, emphasizing the fact that individual proteinases might play a more important role in tauopathy than others. MMP-9 has been identified as a tau proteinase by Nübling *et al*. It was shown to specifically cleave tau in regions close to the C- and N-terminus, creating fragments, which are favorable for NFT formation[13]. Therefore, another negative side effect of MMP-9 might be its involvement in creating NFTs. This idea is supported by a study which observed an increase of MMP-9 in the hippocampus of postmortem AD brain samples around these tangles[62,70].

Table 1: Lcn-2 and MMP-9 in AD

Healthy Brain	**Alzheimer's Disease**
Lcn-2 - secreted by astrocytes[46,47] - astrocyte/microglia apoptosis[48] - regulate microglial morphology/polarization[49] - "anti-bacterial"[44]	**Lcn-2** - induced levels in hippocampus, entorhinal cortex[35] - increased secretion upon Aβ accumulation[49] - increase in proinflammatory cytokines upon Lcn-2[51] - neuroinflammation via TNFR1[35] - involved in iron metabolism → Aβ accumulation[52] - decrease of Lcn-2 in CSF[35]
MMP-9 - released by neurons and microglia - upregulated during aging - involved in Aβ degradation - regulate synaptic plasticity of neurons[64] - involved in neurogenesis, angiogenesis, myelinogenesis, axonal growth, apoptosis[57,58]	**MMP-9** - increased secretion by Aβ surrounding astrocytes[66] - MMP-9 inhibition causes an increase in Aβ - possible α-secretase activity[67] - activation of proinflammatory cytokines → neuroinflammation[71,72]

- activation of growth factors[58]	- tau proteinase favors NFT formation[13]
Lcn-2/MMP-9 - complex with Lcn-2 protects MMP-9 from degradation[41]	**Lcn-2/MMP-9** - Hypothesis: increase in Lcn-2 and MMP-9 → higher complex formation → Neuroinflammation, NFTs

The interplay of Lipocalin 2 and MMP-9 in Alzheimer's Disease

Lcn-2 was first found to be bound to the pro-active form of human neutrophil gelatinase (MMP-9). Purification of this proteinase resulted in a 135 kDa protein, a complex of the 92 kDa pro-MMP-9 and the 25 kDa Lcn-2[43]. Lcn-2 binds to the PEX domain of MMP-9 (aa residue: 518-704) which is located at its N-terminus[42]. This binding has been shown to regulate MMP-9 activity, thus both proteins might indirectly influence their gene expression. As a difference in both Lcn-2 and MMP-9 expression was observed in AD, the interplay of these targets might play an important role in the pathology.

A study investigated the influence of the covalent binding of Lcn-2 to MMP-9 and found that it causes an upregulation in the activation of MMP-9 by $HgCl^{2-}$ and the serine protease plasma kallikrein. Lcn-2 itself has been shown to be capable of activating pro-MMP-9. The co-incubation of kallikrein, $HgCl^{2-}$ with pro-MMP-9 revealed a stronger activating effect on the proteinase. Interestingly, it was shown that Lcn-2 glycosylation is not necessary for its activating properties[41].

Moreover, Lcn-2 is able to protect the proteinase from proteolytic degradation in a dose dependent manner[41]. These findings suggest that Lcn-2 regulates the activity of MMP-9, thereby also influencing Aβ turnover and NFT formation in AD. The protection from degradation proposes that Lcn-2 stays attached to MMP-9 upon activation and does not negatively influence its function. Indeed, it has been shown, that MMP-9 is able to degrade ECM with and without Lcn-2 in complex[41]. Moreover, a study showed that Lcn-2 caused an up-regulation of MMP-9 via the PI3K/AKT/NF-κB signaling pathway. If this could be confirmed, interfering with this pathway would be a probable treatment possibility[73].

In the brain of an AD patient this interaction could influence MMP-9 activity. An up-regulation of MMP-9 was observed upon an increase in Aβ. MMP-9 was shown to be able to proteolytic ally process Aβ, thus counteracting Aβ-induced impairment of the brain function. In order to maintain MMP-9 activation and protect the enzyme from degradation, Lcn-2 expression might be induced due to an increased need for active MMP-9. This might be a necessary process to prevent brain damage and it might also be ongoing in a healthy brain. However, ongoing Lcn-2 and MMP-9 up-regulation due to either a dysregulation of the underlying mechanism or due to an increase in Aβ deposition could lead to neuroinflammation and mark the beginning of AD development. The elevated activation of MMP-9 might then also have a higher deposition of NFT as a consequence, while Aβ degradation would probably be up-regulated. This could also explain

why the correlation observed between NFT and the disease progression is higher than the correlation between Aβ plaques and AD. Nevertheless, it will have to be taken into account that the presence of Aβ oligomers correlate better with the disease progression[74]. Despite the ability of MMP-9 to process Aβ, this might not be the case for oligomers. Like this Aβ oligomers might activate more MMP-9 via a positive feedback loop. The elevated expression of Lcn-2 could promote neuroinflammation, by directly acting as an activating ligand for the NF-κB signaling pathways, hence causing an up-regulation of proinflammatory cytokines and maybe inducing MMP-9 expression. This hypothesis is supported by a study which observed increased IL-6 expression upon Lcn-2 up-regulation in astrocytes[51]. As shown by Tschesche *et al.* Lcn-2 protects MMP-9 from degradation and also leads to higher MMP-9 activation by forming a complex with the protease in a concentration dependent manner[41]. Moreover, Lcn-2 and TIMP have been shown to bind MMP-9 at the same PEX domain. The increased binding of Lcn-2 to MMP-9 therefore also counteracts MMP-9 inhibition by TIMP[42].

Apart from inducing neuronal death, Lcn-2 has not been shown to directly act against Aβ during AD. Via stabilizing MMP-9 it might have an indirect, protective effect against Aβ. To be able to confirm this hypothesis it would be necessary to investigate whether both proteins are co-localized in patients with AD. Co-precipitation studies or stainings could help to gain more certainty. Additionally it is of interest to understand the expression of MMP-9 and Lcn-2 regulation. It seems reasonable that Lcn-2 and MMP-9 are present in a complex in the brain. However, to be able to discriminate between cause and consequence, the sequence and induction of signaling pathways will have to be studied in greater detail. Lcn-2 and MMP-9 expression have been shown to depend on NF-κB.

The Lcn-2/MMP-9 complex is better studied in various cancers. Increased complex formation was observed in acute myeloid leukemia (AML) cells and a decrease of MMP-9 was found in the serum of AML patients. Whereas high serum levels of MMP-9 are being used as a biomarker in several cancers[42], more information is needed to be able to use Lcn-2/MMP-9 as a prognostic or diagnostic marker in neurodegenerative diseases. The decrease of Lcn-2, which can be observed in the CSF[35] of AD patients, indicates that the expected differences in MMP-9 and Lcn-2 expression levels in serum or CSF could be used as a biomarker for the disease later.

In cancer, MMP-9 plays an important role in cleaving and thereby activating growth factors which can further activate receptors, thus promoting tumor growth[42]. Similar mechanisms are expected to be present in the brain. TNF-α is widely accepted to play a major role in neuroinflammation. It is known to induce Lcn-2 and MMP-9 expression[35,72]. An interesting thought would be that MMP-9 itself might be able to activate TNF-α via proteolytic cleavage of its membrane-integrated pro-form. This step is usually performed by metalloprotease ADAM17[71]. Knockdown studies could reveal whether MMP-9 is capable of also activating TNF-α. This would give the enzyme and even more significant impact in the progression of AD.

Moreover, it has been shown that megalin, a Lcn-2 receptor was also able to recognize MMP-9, free from Lcn-2[75,76]. The interaction between the receptor and MMP-9 was dependent on the hemopexin domain and the o-glycosylated linker domain. Further, the degradation of MMP-9 was observed after the cellular uptake of MMP-9 through megalin[75]. This endocytosis might be a process of negative regulation of the metalloprotease.

Patients with AD have been shown to have an increased megalin expression in neurons. As megalin also recognizes ApoE, it was observed that the megalin-related uptake of ApoE by neurons might be responsible for a higher Aβ accumulation in the cell and therefore cause neuronal cell death[77]. The interplay of megalin an MMP-9 in AD needs more studying. The megalin upregulation in neurons might be beneficial if it is followed by degradation and therefore regulates MMP-9 levels. Nevertheless, current studies only comprise the binding of megalin and MMP-9 in the absence of Lcn-2. For more specific information it would be interesting to compare the affinity of megalin and independent MMP-9, Lcn-2, as well as the MMP-9/Lcn-2 complex to come closer to mimicking in vivo conditions.

Conclusion

MMP-9 and Lcn-2 have been shown to form a complex. The binding of both proteins is known to stabilize MMP-9. With a high probability this mechanism is involved in AD and therefore also marks a point where the disease could be therapeutically intervened. To be able to develop successful drugs, which could for example involve inhibitors of the Lcn-2 or MMP-9 complex formation it is necessary to fully understand the mechanism. It is hypothesized that the upregulation of MMP-9 and Lcn-2 in AD causes higher complex formation, thus creating more functional MMP-9 which contributes to AD by the activation of proinflammatory cytokines and again by further inducing Lcn-2 expression. If this hypothesis would be proved true, the inhibition of Lcn-2 and MMP-9 complex formation might be able to counteract the excessive activation of this specific mechanism and at the same time necessary functions of Lcn-2 and MMP-9 would still remain.

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