ADAMTS-5: a difficult teenager turning 20

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Running title: ADAMTS-5 in health and disease

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SUMMARY

A Disintegrin And Metalloproteinase with ThromboSpondin Motif (ADAMTS)-5 was identified in 1999 as one of the enzymes responsible for cleaving aggrecan, the major proteoglycan in articular cartilage. Studies *in vitro*, *ex vivo* and *in vivo* have validated ADAMTS-5 as a target in osteoarthritis (OA), a disease characterised by extensive degradation of aggrecan. For this reason, it attracted the interest of many research groups aiming to develop a therapeutic treatment for OA patients. However, ADAMTS-5 proteoglycanase activity is not only involved in the dysregulated aggrecan proteolysis which occurs in OA, but also in the physiological turnover of other related proteoglycans. In particular, versican, a major ADAMTS-5 substrate, plays an important structural role in heart and blood vessels and its proteolytic processing by ADAMTS-5 must be tightly regulated. On the occasion of the 20th anniversary of ADAMTS-5 discovery, I will review the evidence for its detrimental role in OA, as well as its physiological turnover of cardiovascular proteoglycans. Moreover, I will highlight other potential functions of this enzyme. Finally, I discuss challenges and emerging trends in ADAMTS-5 research.

Keywords: ADAMTS, proteoglycans, aggrecan, versican, osteoarthritis, cardiovascular disease

1. INTRODUCTION

Twenty years ago, a team of scientists at the pharmaceutical company Dupont unveiled the identity of the elusive enzymes responsible for cleaving aggrecan, a major proteoglycan in the articular cartilage.[1,2] This 'aggrecanase activity' has been the subject of intensive research efforts during the past two decades. By mid-80s it became apparent from post-traumatic animal models of osteoarthritis (OA) that aggrecan degradation is one of the major changes affecting extracellular matrix (ECM) function.[3,4] Later, cartilage proteoglycan fragments were detected in the synovial fluid of patients with knee injury.[5] In 1992, Sandy et al.[6] identified a major cleavage event, occurring at the Glu392↓Ala393 bond, was responsible for releasing aggrecan fragments in the synovial fluid of OA patients. This initiated a worldwide hunt for the 'aggrecanase' that ended only 7 years later. Aggrecanase activity

was found to be a distinct enzymatic feature of two members of A Disintegrin and Metalloproteinase with Thrombospondin Motifs (ADAMTS) family of metalloproteinases, aggrecanase-1 (ADAMTS-4) and aggrecanase-2 (ADAMTS-5, which was originally named ADAMTS-11).[1,2,7] Since then, multiple lines of evidence have established ADAMTS-5 as a major aggrecanase in humans and mice. Therapeutic treatment of degenerative joint diseases appeared to be around the corner. However, twenty years of frantic investigations have revealed a complexity in ADAMTS-5 biology that has made the development of a therapeutic ADAMTS-5 inhibitor more difficult to achieve. The child was a difficult one. Here on the occasion of its 20th birthday, I will attempt to summarise the teenage years of ADAMTS-5 and our current knowledge of its place in the ECM field.

2. THE FIRST DECADE: ADAMTS-5 AND OA

2.1. Setting the stage: OA, cartilage destruction and aggrecan

OA is the most common joint disorder, affecting a significant proportion of the human population.[8] The pain related to this pathology is the leading cause of impaired mobility in the elderly population in the USA.[9] A major hallmark of OA is the destruction of articular cartilage.[10] The function of articular cartilage is to absorb the stress created within the joint during movement and distribute it to the underlying bone. Articular cartilage is composed of chondrocytes embedded in an ECM that is rich in type II collagen fibrils and aggrecan. The composition of human articular cartilage is 65-80% water, 10-30% collagen, 10-15% proteoglycans and <2 % chondrocytes.[11] Collagen is the main structural component of cartilage and provides tensile strength to the tissue whereas aggrecan provides flexibility, viscoelasticity and compressibility. These functions are mediated by the negatively-charged glycosaminoglycans (GAG) chains attached to its core which ultimately are responsible for the high osmotic pressure in cartilage (3.4-3.6 atmospheres).[12] GAGs attached to aggrecan protein core are chondroitin sulphate (CS) and keratan sulphate (KS) chains, which cluster into so-called CS-1 and CS-2 domains and a KS-rich region (**Figure 1**).[13,14] To give an idea of the predominance in mass of the GAGs over the protein core, the latter only corresponds to ~10-15% of the weight of the molecule (220 kDa) and bears as many as 50-100 CS chains and up to 60 KS chains.[16-18] The osmotic pressure in cartilage is mainly due to the negative charges on aggrecan

GAGs, which attract counter ions, such as Na^+ and, consequently, due to the Donnan effect, induces water into the tissue.[12] The protein core of aggrecan comprises globular domains at the N- and C-terminus (named G1 and G3, respectively), similar to other large aggregating proteoglycans, but additionally contains an internal G2 domain (**Figure 1**). The region between G2 and G3 contains the GAG-attachment sites, whereas the region between G1 and G2 (called interglobular domain, IGD) comprises 150 amino acids. The aggrecan G1 domain interacts with link protein, a glycoprotein, and hyaluronan (HA), an anionic, nonsulfated GAG, to form larger molecular weight aggregates. These contains up to 100 aggrecan molecules per molecule of HA.[19]

Earlier studies have shown that aggrecan, collagen and HA constantly undergo turnover in healthy cartilage.[20,21] Under physiological conditions, cartilage homeostasis is maintained by a balance between the synthesis and degradation of aggrecan and collagen, but in OA and other joint disorders this equilibrium shifts towards catabolism. Proteases involved in the catabolism of collagen and aggrecan are secreted in response to inductive stimuli (such as inflammatory cytokines, mechanical stress and injury) both by chondrocytes and synoviocytes.[10] Synoviocytes comprise cells such as fibroblasts and macrophages which are present in the synovium, the thin membrane around the joint that secretes the lubricating synovial fluid.

Aggrecan degradation represents an early stage of cartilage destruction, preceding degradation of the major fibrillar type II collagen. In bovine articular cartilage explants stimulated with interleukin-1 (IL-1), collagen release could be detected only after 14 days of culture, when most of the aggrecan had already been depleted from the ECM.[22] Moreover, whereas aggrecan degradation can be compensated for by new synthesis and is completely reversible upon anabolic stimuli, collagen degradation is an irreversible process.[23,24] Remarkably, a collagenase-selective inhibitor (Ro-32-3555/Trocade/Cipemastat), developed by Roche, did not prevent progression of joint damage in patients with rheumatoid arthritis[25] despite the favourable preclinical data[26] and pharmacokinetics.[27] Since it has been shown that collagen fibrils interact with the KS domain of multiple aggrecan molecules,[28] it has been proposed that aggrecan protects the collagen fibrillar network from proteolytic attack by collagenases due to steric and charge hindrance by the long negatively-charged GAG chains attached to its core.[29] To support this, live explants that were depleted of aggrecan GAGs

by pre-treatment with chondroitinase ABC showed release of collagen in as little as 24 h after IL-1 stimulation.[30] Cleavage of aggrecan within the IGD domain would then release the protective C-terminal portion of the molecule, thus exposing the collagen fibril to collagenolytic activity. Aggrecan fragments bearing the GAG-rich domains are in fact detected in the synovial fluid of both normal and OA patients, following diffusion from the cartilage.[31,32] A major cleavage site in the human aggrecan IGD was identified as the Glu373↓Ala374 bond.[6] This corresponds to the Glu392↓Ala393 bond in the modern nomenclature (Uniprot ID P16112 for human aggrecan). The reason for the 19-residue discrepancy between the classic nomenclature and the Uniprot database numbering is that the former starts from the natural N-terminus of protein (first identified as 20VEVS in porcine aggrecan, corresponding to 20VETS in human).[33] In this review, I will follow the Uniprot numbering so that researchers not familiar with the field can easily track the cleavage sites.

Cleavage within the IGD releases most of the molecule from its anchorage to HA and results in loss of mechanical properties. On the other hand, cleavage sites towards the C-terminus of the CS-rich domains leave most of the molecule still functional within the tissue[34] and for this reason may represent homeostatic turn-over of aggrecan.[35,36] Although members of other families of proteases such as matrix metalloproteinases (MMPs) and cysteine proteases such as cathepsins can cleave aggrecan at different cleavage sites in the IGD and CS-rich domains (reviewed in [15]), the 'aggrecanase' cleavage site at Glu392↓Ala393 was shown to play a fundamental role in cartilage degradation. In mouse models of arthritis, cleavage fragments generated by aggrecanases at this site appear before those generated by MMPs in the IGD (cleavage at N360↓F361), but they disappear with the progression of cartilage damage.[37] Perhaps the most compelling evidence comes from mice bearing a mutated 'aggrecanase' site (393ALG→393NVYS) that makes them resistant to aggrecanase activity in the IGD, without affecting other cleavage sites.[38] These mice are protected from aggrecan loss and cartilage erosion in surgical and inflammatory models of OA and show increased cartilage repair.[38] Importantly, they do no show cartilage or skeletal anomalies.

2.2. ADAMTS-5 as a target in OA

In 1999, the two enzymes responsible for cleavage at the Glu392↓Ala393 bond were purified from bovine cartilage tissues.[1,2] It was found that their N-terminal peptide sequences shared significant homology with a murine enzyme described two years before as the founding member of the A Disintegrin And Metalloproteinase with ThromboSpondin motifs (ADAMTS) family of metalloproteinases, ADAMTS-1.[39] For this reason, the two aggrecanases were named ADAMTS-4 (aggrecanase-1) and ADAMTS-5 (aggrecanase-2), respectively[1, 2,7] (The gene cloned in the original report and named ADAMTS-11[2] was found to match with an EST previously named ADAMTS-5[7]). The three proteases were found to be secreted extracellular proteins and share a similar domain organisation, consisting of a signal peptide, a prodomain, a metalloproteinase catalytic domain, followed by non-catalytic ancillary domains such as a disintegrin-like domain, a thrombospondin-type I motif domain, a cysteine-rich, domain, a spacer domain, and a various number of thrombospondin-type I motifs in the C-terminus (2 for ADAMTS-1, 1 for ADAMTS-5 and none for ADAMTS-4). The metalloproteinase domain contains the sequence H**E**IGHLLGLSHD, in which the three underlined histidine residues coordinate a zinc atom and the glutamate residue (in bold) exerts a catalytic role. This motif is followed C-terminally by a conserved methionine residue which constitutes a tight turn ("Met-turn") acting as a constraint in the topology of active site.[40] The C-terminal ancillary domains are essential for catalytic activity against native substrates such as aggrecan, since they contain residues (called exosites) that mediate recognition and binding of substrates.[41] Later, other ADAMTS family members such as ADAMTS-1,[42] -8,[43] -9, -16 and -18[44] were shown to cleave at the 'aggrecanase site' Glu392↓Ala393, although this was achieved using non physiological enzyme to substrate ratios (up to 1:0.3 for ADAMTS-8[43]). With the notable exceptions of ADAMTS-4 and -9, the expression of all other aggrecanases have been found to increase either in the synovium or in the cartilage of OA patients as compared to controls (for a review, see ref 45). So what made ADAMTS-5 the favoured target for OA therapies? Four lines of evidence support this conclusion.

2.2.1. ADAMTS-5 is the most potent aggrecanase *in vitro*

Semi-quantitative studies using western blot analysis have established that purified human recombinant ADAMTS-5 is a ~30-fold more potent aggrecanase at the Glu392↓Ala393 bond than ADAMTS-4. Moreover, it is a ~20-fold more potent in the CS2 domain (Glu1679↓Gly1680 bond,

Figure 1).[46] To give an idea of the extreme potency of this enzyme, as little as 2 pM or 1 nM of active ADAMTS-5 is sufficient to show robust aggrecan cleavage within the CS-2 (Glu1953↓Ala1954 bond) and the aggrecanase site, respectively, following 2 h digestion at 37°C.[41,47] It is important to highlight that the 1000-fold figure for the higher aggrecanase activity of ADAMTS-5 compared with ADAMTS-4,[48] still sometimes found in the literature, was determined using batches of ADAMTS-4 contaminated with heparin, which has been shown to inhibit aggrecanase activity.[46] By comparison, approximately 300 nM ADAMTS-1 is required to generate cleavage at the Glu392↓Ala393 bond (concentration calculated from ref 42), suggesting a ~150,000-fold lower activity than ADAMTS-5.* Cofactors such as fibulin-1 have been shown to enhance the activity of both ADAMTS-1 and -5.[49,50] However, while it is possible that cofactors may enhance the activity of one or more aggrecanases *in vivo*, it is very unlikely that they will change the relative potency of these enzymes.

2.2.2. ADAMTS-5 ablation or inhibition protects mice from joint degeneration

The importance of ADAMTS-5 in facilitating the progression of cartilage loss has been outlined by *in vivo* models of OA (**Table 1**). In these models, specific *Adamts* knockout mice are challenged either mechanically, by knee surgery (destabilisation of medial meniscus, DMM), or by injection of methylated bovine serum albumin into the joint (antigen-induced arthritis, AIA) and their phenotype is compared with that of wild-type littermates.

Initially, the phenotype of *Adamts4* and *Adamts1* knockout mice was analysed. These mice did not exhibit any significant protection from cartilage aggrecan loss in the DMM or the AIA models, respectively,[51,52] suggesting that the contribution of these proteases to joint pathology in mice is negligible. Mechanistically, the articular cartilage from these mice showed significant cleavage of aggrecan at the at the Glu392↓Ala393 bond.[51,52] On the other hand, mice with a targeted deletion of the catalytic domain of ADAMTS-5 were protected from cartilage loss both in the DMM[53] and AIA[54] models, with minimal cleavage of aggrecan at the Glu392↓Ala393 bond. Moreover, in these mice a significant cleavage in the CS-2 domain was observed, suggesting an involvement of ADAMTS-5 in

* In contrast with active-site titrations for ADAMTS-5, Rodriguez-Manzaneque et al.,[42] used optical density of purified ADAMTS-1 to estimate concentrations. However, generally the difference between the two concentrations is no more than 2/10- fold for ADAMTS-5 (my unpublished results), making the relative difference between ADAMTS-5 and ADAMTS-1 still impressive.

pathological aggrecan proteolysis rather than physiological turnover.[54,55] *Adamts5* knockout mice also show a significant reduction in the thickness of the subchondral plate and less epiphyseal trabecular bone following DMM compared with wild-type mice,[56] suggesting that the protective effects of ADAMTS-5 ablation are extended to the bone, where ADAMTS-5 is also normally expressed.[57,58] Importantly, *Adamts5* knockout mice do not develop mechanically-induced pain sensitisation (allodynia), a major cause of distress in OA patients, up to 8 weeks post DMM.[59] In an OA model induced by the combination of Transforming Growth Factor (TGF)-β injection and enforced uphill treadmill running (TTR), *Adamts5* knockout mice were protected from joint fibrosis and cartilage erosion and showed increased aggrecan deposition,[60] but their tendons showed a reduced maximum tensile stress as a result of disrupted collagen organisation and may be more susceptible to rupture.[61,62] *Adamts4/Adamts5* double knockout mice were also protected from developing OA and physiologically normal.[63] In the absence of both ADAMTS-4 and -5, aggrecan cleavage still occurs in the CS-2 domain,[64] an activity probably involved in the homeostatic turnover of the molecule.

Another approach to investigate the role of ADAMTS-5 *in vivo* is by analysing the effect of small interfering RNAs (siRNA). An advantage is that in this case the effect can be tissue-limited and short-lived, therefore mimicking more closely therapeutic administration. Intra-articular injection of *Adamts5* siRNA was protective in DMM models and the combined injection of an MMP-13 (collagenase-3) siRNA showed a synergistic protective effect on cartilage integrity.[65, 66]

The role of ADAMTS-5 in the pathology of murine OA was further corroborated by studies which employed monoclonal antibodies (mAbs). Systemic treatment with anti-ADAMTS-5 mAbs either before or after DMM protected mice from OA progression[67] and mechanical allodynia,[68] thus phenocopying the findings from *Adamts5* knockout mice. Similarly, an anti-ADAMTS-5 mAb was found to be protective in a murine model of spontaneous OA.[69] Importantly, an anti-ADAMTS-4 mAb was not effective as a prophylactic treatment.[67]

Collectively, these data point to a pivotal role exerted *in vivo* by ADAMTS-5 in mouse cartilage breakdown during the progression of OA. However, ADAMTS-5 seems to exert a role also in bone and tendon biology and a future ADAMTS-5-based OA therapy should take also this into consideration.

2.2.3. Inhibition of ADAMTS-5 in human chondrocytes significantly reduces aggrecan degradation

The role of aggrecanases in human OA has been extensively investigated using either isolated chondrocytes or cartilage explants. In these models it is important to take into account the role of inflammation in OA. Chondrocytes as well as macrophages and mononuclear cells in the synovial membrane of OA joints exhibit an activated phenotype and play a pivotal role in cartilage degradation by releasing pro-inflammatory cytokines such as interleukin (IL)-1β and tumour necrosis factor (TNF)-α, whose levels are found increased in OA patients.[70,71] For this reason, cytokines are often added in chondrocytes/explants cultures. However, whereas the expression of *ADAMTS4* is upregulated by pro-inflammatory cytokines, that of *ADAMTS5* is constitutive in OA synovium and cartilage (for a review, see ref [72]). Under these inflammatory conditions, human normal cartilage explants and primary chondrocytes have been subjected to siRNA-mediated knockdown of *ADAMTS4* or *ADAMTS5*, which showed that both treatments were equally effective in decreasing aggrecan cleavage.[73] The same effects were observed following siRNA-mediated knockdown of either gene in cartilage of OA patients, but an siRNA against *ADAMTS1* failed to inhibit aggrecan degradation in either normal or OA cartilage.[73] Similar results were observed with inhibitory mAbs. In human OA explants, anti-ADAMTS-5 mAbs were more effective than anti-ADAMTS-4 mAbs in inhibiting aggrecan degradation under unstimulated conditions, whereas in the presence of cytokines the two mAbs showed similar efficacy.[67] A different anti-ADAMTS-5 mAb, 2D3, effectively inhibited aggrecan degradation both in healthy human chondrocytes[47] and human OA cartilage explants[74] under non-inflammatory conditions.

Taken together, these results suggest that ADAMTS-5 plays a major role in human OA pathology under non-inflammatory conditions, whereas in the presence of cytokines both ADAMTS-5 and -4 contribute to aggrecan degradation.

2.2.4. Impairment of ADAMTS-5 endocytosis significantly increases aggrecan degradation

In light of all the evidence for an important role of ADAMTS-5 in OA, it was puzzling to find that ADAMTS-5 mRNA levels do not correlate with aggrecan degradation.[75-77] This suggests that ADAMTS-5 dysregulation in OA may principally result from altered post-translational regulation. In

fact, it has been found that ADAMTS-5 half-life is regulated by endocytosis through the low-density lipoprotein receptor-related protein-1 (LRP-1) receptor.[78] Interestingly, LRP-1 protein levels are severely reduced in OA cartilage and this has been attributed to an increase in the shedding of its ectodomain by proteases such as ADAM-17 and MMP-14.[74,78] To test the effect of an impairment in ADAMTS-5 endocytosis on aggrecan degradation by human chondrocytes, we developed a mAb, 1B7, able to specifically bind to ADAMTS-5 and inhibit its endocytosis with no effect on its aggrecanase activity.[79] Addition of 1B7 to normal chondrocytes under unstimulated conditions increased the levels of endogenous ADAMTS-5 and produced a significant increase in aggrecan degradation, mimicking the effect of impaired ADAMTS-5 uptake by LRP-1. This activity could be specifically inhibited by another anti-ADAMTS-5 mAb, 2D3, suggesting that the observed aggrecanase activity was ADAMTS-5-dependent.[79] These results strongly link aggrecan degradation with a dysregulated post-translational regulation of ADAMTS-5 levels in cartilage.

2.3. Side effects of anti-ADAMTS-5 mAbs

Currently, OA treatment is limited to steroidal and nonsteroidal anti-inflammatory drugs which provide symptomatic relief for pain and inflammation.[80] Physical activity is one of the most widely prescribed non-pharmacological therapies for OA management.[81] So there is an unmet demand for drugs able to reverse, halt or slow disease progression and improve the quality of life in OA patients. The body of work described in the previous section strongly advanced ADAMTS-5 as a viable target for such disease-modifying osteoarthritic drugs. In the previous section, mAbs were introduced as a tool to investigate the potential of an anti-ADAMTS-5 therapeutic therapy. In *Cynomolgus*, a primate which spontaneously develops OA-like symptoms,[82] an anti-ADAMTS-5 mAb, GSK2394002, was indeed able to decrease the levels of circulating aggrecan fragments upon systemic administration.[67] However, this study also exposed cardiovascular side effects associated with ADAMTS-5 inhibition, ranging from increased mean arterial pressure to subendocardial haemorrhage.[83] Such cardiovascular effects were not due to cross-reaction of the mAb[67] and pose significant challenges for clinical development since the OA population may be already affected by cardiovascular disease. In addition, many individuals who are strong candidates for medical treatment but not surgery are young athletes with post-traumatic OA, and it would be wise to be wary of long-term impact in these patients. On the

other hand, this disappointing outcome prompted a reassessment of ADAMTS-5 function in the cardiovasculature.

3. THE SECOND DECADE: ADAMTS-5 AND CARDIOVASCULAR DISEASE

3.1. ADAMTS-5 and versican in the development of cardiovascular system

In large blood vessels and cardiac valves, versican is the main proteoglycan, although recently also aggrecan has been detected in the aorta.[84-89] Both proteoglycans regulate viscoelasticity and stiffness of large vessels.[88,90,91] Genetic deletion of versican is lethal in mice due to cardiac anomalies,[92] and versican processing is necessary for the remodelling of the cardiac outlet, which will develop into the future aortic and pulmonary arteries of the mature heart.[93] Versican is present in 5 isoforms (V0-V4) arising from alternative splicing of two large exons encoding GAG-attachment domains, named αGAG and βGAG.[91] In adult mice ADAMTS-5 has been shown to be co-expressed with versican in cardiac valves and aorta.[94-96] ADAMTS-5 cleaves versican V1 at Glu441↓Ala442 in the βGAG region[41,97,98] and shares this ability with other cardiovascular proteoglycanases such as ADAMTS-1, -4,[99] -9,[9100] and -15.[101] Perhaps because of this apparent redundancy, researchers in the field desisted from questioning the first reports about the absence of developmental phenotypes in *Adamts5* knockout mice[53,54] (**Table 1**). In fact, later studies demonstrated that *Adamts5* knockout mice show severe anomalies in the pulmonary valve cusps due to decreased versican cleavage and subsequent versican accumulation.[95,102] The pulmonary valves of these mice also exhibit reduced cleavage of aortic aggrecan at the Glu392↓Ala393 site, whereas cleavage in the CS-2 domain still proceeds through other aggrecanases. Mice with a mutant cleavage site at Glu392↓Ala393 also show aortic anomalies, although not as severe as those in *Adamts5* knockout mice,[103] suggesting that ADAMTS-5 activity at other sites (or, alternatively, on other substrates) may compensate for the abrogation of cleavage in the aggrecan IGD. On the other hand, mice over-expressing ADAMTS-4 and -5 show sparse trabeculation in the heart due to excessive degradation of versican.[104]

These data suggest that ADAMTS-5 proteoglycanase activity is required to maintain adequate levels of proteoglycans such as aggrecan and versican, not only during heart development but also in large blood vessels.

3.2. Aneurysms

Increased proteoglycan levels have been detected in ascending aortas from patients with thoracic aortic aneurysm and dissections (TAAD) and mice with Marfan syndrome, particularly in dissected and ruptured aortas.[87,89] The increased proteoglycan content may then lead to tissue swelling and, consequently, alterations in blood flow and peripheral organ perfusion. Aggrecan was found to be increased in TAAD aortas, where the expression of *ADAMTS5* was found to be down-regulated.[87] By contrast, expression of *ADAMTS1* and *ADAMTS4* was found to be normal.[87] Similarly, a transcriptome analysis of aortas with dissections found significant down-regulation of ADAMTS-5 relative to controls.[105] However, a limitation of measuring mRNA levels is that they may not adequately reflect ADAMTS-5 protein levels, since its regulation is mainly post-translational, for example through LRP-1 endocytosis[78] (see Section 2.2.4). Therefore, measuring ADAMTS-5 protein levels in tissues/biological fluids poses a significant challenge. Moreover, there may be differences between human disease and mouse models. When infused with angiotensin II, which induces aortic degeneration, *Adamts5* knockout mice show increased aortic dilation with accumulation of versican and reduced versican cleavage.[85] However, mice with ADAMTS-1 haploinsufficiency also show a predisposition to TAAD in the same model.[106]

3.3. Atherosclerosis

Proteoglycans also play a primary role in the deposition of Low Density Lipoproteins (LDL) in the arterial wall and in the aetiology of atherosclerosis. These LDLs reach the arterial wall when the integrity of the endothelium is compromised.[107] Already in the mid-70's, complexes between LDL and proteoglycans were isolated from human atherosclerotic lesions.[107-109] The affinity of these complexes is relatively high (association constants values for versican and biglycan of 23 and 170 nM, respectively)[110] and is mediated by an ionic interaction between the negatively-charged GAGs on the proteoglycans and positively-charged residues in apolipoprotein B.[111] The result of this interaction is an increased internalisation of LDLs by vascular smooth muscle cells (VSMCs) and macrophages

compared with GAG-free LDL.[112,113] This causes intracellular accumulation of cholesterol, chosteryl-esters, triglycerides and phospholipids.[114] By regulating proteoglycan levels, proteoglycanases may reduce LDL uptake by VSMCs and macrophages. Recombinant ADAMTS-5 has been shown to reduce the LDL-binding ability of biglycan and releases LDLs from human aortic lesions.[96] The aortas of *Adamts5* knockout mice show increased levels of both biglycan and versican and no ADAMTS-generated versican fragments.[96] Importantly, *ApolipoproteinE (ApoE)* knockout mice, which spontaneously develop atherosclerotic lesions, also show accumulation of biglycan and versican which was associated to a marked reduction in ADAMTS-5 expression at the protein level.[96] In addition to ADAMTS-5, human VSMCs and macrophages express ADAMTS-1 and -4.[86,115,116] Why do these proteoglycanases not compensate for the absence of ADAMTS-5? A possible answer may come from our *in vitro* kinetic analysis of versican cleavage (Glu441↓Ala442 bond), using purified enzymes and proteoglycans.[41] This revealed that ADAMTS-5 is a 20-fold more potent versicanase than ADAMTS-4, whereas ADAMTS-1 versicanase activity is negligible, at least in the absence of cofactors.[41] Importantly, adult *Adamts5* knockout mice have been recently reported to show reduced versican cleavage in the cardiac ECM, but this was not associated with any significant impairment of cardiac function.[117] Only when kept on a high fat diet (HFD), *Adamts5* knockout mice show subtle cardiac anomalies such as an increased diastolic posterior wall thickness and left ventricle volume, and increased diastolic and systolic blood pressure,[117] suggesting that the pathological consequences of *Adamts5* knockout may be exacerbated by a pro-atherosclerotic diet. In contrast, ADAMTS-4 seems to exert a detrimental role in atherosclerosis, since *Adamts4/ApoE* double knockout mice exhibit enhanced plaque stability.[118]

Taken together, these data point to an important role exerted by ADAMTS-5 in the cardiovasculature where it is necessary to maintain adequate levels of proteoglycans. Due to its much higher versicanase[41] and aggrecanase activity[46], a reduction in ADAMTS-5 levels/activity is only partially compensated by both ADAMTS-1 and -4. Any therapy aiming at targeting ADAMTS-5 must take into account this cardiovascular function.

4. OTHER ROLES OF ADAMTS-5

Analysis of *Adamts5* knockout mice have uncovered a range of unexpected functions and phenotypes (**Table 1 and Figure 2**). However, caution must be taken in extrapolating mouse models to human diseases. Not only do pathophysiologies differ between human and mice, but also the specific mouse strain used to investigate ADAMTS-5 ablation may affect the observed phenotypes. To date, two different *Adamts5* knockout strains have been developed. To investigate the effect of ADAMTS-5 ablation on aggrecan cleavage, Pfizer, in collaboration with Lexicon Genetics, generated so called *Adamts*5 P mice (*Adamts5*$^{tm1.1Lex}$),[59-61,117-120] whereas for investigating versican cleavage, *Adamts*5 J mice (*Adamts5*^{tm1Dgen}) were generated by Deltagen Inc. (now commercially available through the Jackson Laboratory, Stock No:005771).[50,94,95,102,103] Whereas the *Adamts5* J mice show an out-of-frame deletion of exon 2 (coding for the catalytic site),[94] the *Adamts5* P mice show an in-frame deletion of exon 2.[119] The *Adamts5* P mice are similar to the *Adamts5* knockout mice originally reported in 2005.[53,54] Both *Adamts*5 P and *Adamts*5 J mice do not show expression of the enzyme at the protein level.[119] Although phenotypes shown by these mice are similar, qualitative and quantitative differences were observed in several models.[119-123] Importantly, no conditional knockout has been reported so far. In Sections 2 and 3 I discussed the involvement of ADAMTS-5 in OA and cardiovascular diseases, respectively. In the following sections, the involvement of ADAMTS-5 in other biological/pathological processes will be discussed. Whenever discrepancies emerged between the two aforementioned *Adamts5* knockout models, these are explicitly stated, otherwise I refer to the references for the description of the particular knockout model investigated.

4.1. Wound healing

*Adamts*5 *P* knockout mice show delayed contraction in an excisional wound healing model, a phenotype associated with accumulation in the dermal layer of cell aggregates and fibroblasts surrounded by a pericellular ECM enriched in full-length aggrecan.[122] These changes in the ECM composition result in an altered signalling of TGF-β1, a key regulator of multiple processes in wound healing. In contrast, *Adamts4* knockout mice exhibit a normal wound healing response.[122] On the other hand, using *Adamts5* J mice knockout mice, Hattori et al.[123] observed normal proliferation and migration by isolated dermal fibroblasts, but increased contractility, associated with accumulation of uncleaved pericellular versican and upregulation of α-smooth muscle actin as well as increased

canonical TGF-β signalling. Although contradictory to some extent in their conclusions, these studies showed that loss of ADAMTS-5 in dermal fibroblasts results in an abnormal ECM enriched in aggrecan and versican, suggesting a crucial role of ADAMTS-5 in regulating proteoglycan levels in the skin.

4.2. Liver disease, obesity and metabolic disorders

As discussed in Section 3, lipoprotein retention increases in *Adamts5* knockout mice due to accumulation of uncleaved proteoglycans such as versican and biglycan.[96] Both versican and heparan sulfate proteoglycans such syndecan-1, an ADAMTS-5 substrate (**Table 2**),[124] also regulate lipid uptake in the liver.[117,124-126] Surprisingly, when *Adamts5* knockout mice are subjected to HFD to induce obesity, their plasma lipid levels are higher than wild-type. [117,124] Their total body weight does not differ from wild-type mice, but their liver weight and hepatic lipid accumulation are significantly reduced.[117,124] Additionally, *Adamts5* knockout mice are protected from steatohepatitis in a methionine/choline deficient diet (MCD) model.[121,124] Although cleavage of versican remains unaffected probably due to compensation from other ADAMTS proteoglycanases, *Adamts5* knockout mice show a reduced expression of versican at the mRNA level when subjected to HFD.[117,124] On the other hand, *Adamts5* knockout mice show both reduced cleavage and increased expression of its substrate syndecan-1.[124] More studies are necessary to ascertain the mechanism behind the hepatic phenotype of *Adamts5* knockout mice.

To support a role of ADAMTS-5 in obesity, *Adamts5* expression is upregulated in mice on HFD.[127, 128] Moreover, mice knockout for *Timp3* (Tissue Inhibitor of Metalloproteinase-3), the most potent endogenous inhibitor of ADAMTS-4 and -5,[129] are more susceptible to develop hepatic steatosis when kept on HFD.[130]

ADAMTS-5 may be also involved in glucose metabolism. *Adamts5* knockout mice have more interscapular brown adipose tissue and cold exposure induce in these mice a more pronounced browning of white adipose tissue compared to wild-type as a result of an enhanced glucose metabolism.[121,131] Adipose-derived stromal cells from *Adamts5* knockout mice show an increased uptake of glucose, a precursor for the synthesis of CS chains, compared with wild-type mice, resulting in an increased expression of aggrecan.[119] Therefore, one function of ADAMTS-5 may be to depress

glucose metabolism. Intriguingly, injection of leptin, a hormone that downregulates lipid accumulation by adipocytes, increases ADAMTS-5 expression in rats.[132]

These data suggest that ADAMTS-5 may be involved in metabolic disorders, although the specific mechanisms have not been fully elucidated.

4.3. Inflammation and cancer

The N-terminal versican fragment generated by ADAMTS-5 cleavage, called versikine, has been shown to exert a pro-apoptotic function.[50] Versikine has been shown to accumulate in regions of blood vessels undergoing atrophy and cell death as well as in tumour vasculature.[99,133, 134] Versikine acts as a damage associated molecular pattern by triggering Toll-like Receptor-2 signalling and activation of the NF-kB pathway and thus supporting T cell activation though the release of pro-inflammatory cytokines such as IL-6 and IL-12 and interferon.[135- 138] Versikine has been associated with a higher frequency of activated $CD8^+$ T cells in myeloma and colorectal cancer.[135,139] To corroborate these findings, decreased versikine levels were associated with decreased numbers of total $CD4^+$ and $CD8^+$ T cells in the spleen and lung of *Adamts5* knockout mice following infection with influenza virus, resulting in delayed virus clearance and higher weight loss compared with wild-type mice.[140] These results suggest that ADAMTS-5 versicanase activity contributes to migration of T cells which may have implications in other pathological conditions such as cancer.

High *ADAMTS5* expression in colorectal cancer patients indeed correlated with higher levels of lymphatic invasion and lymph node metastasis but no differences in survival rate were observed.[141] The *ADAMTS5* promoter has also been found to be hyper-methylated in colorectal cancer[142] and T-cell acute lymphoblastic leukaemia.[143] Moreover, *ADAMTS5* expression has been found to be down-regulated in breast cancer,[144] prostate cancer,[145] in head and neck squamous cell carcinoma,[146,147] and hepatocellular carcinoma.[148] ADAMTS-5 has also been shown to display an anti-tumorigenic activity which is independent of its catalytic activity. The central thrombospondin-type I motif of ADAMTS-5 has been suggested to act as an anti-angiogenic and proapoptotic peptide.[149-151] Suppression of angiogenesis is mediated by down-regulation of pro-angiogenic factors,[150] although the mechanism by which the central thrombospondin-type I modulates transcriptions of these genes is not known. On the other hand, a pro-tumorigenic, catalytic-dependent role has been suggested for ADAMTS-5 in

glioblastoma, where its expression is greatly increased.[152] By cleaving brevican, a proteoglycan specific to the central nervous system, ADAMTS-5 may contribute to the invasiveness of glioblastoma cells.[153,154] ADAMTS-5 is also highly expressed in laryngeal squamous cell carcinoma,[155] where it may contribute to the severe decrease in aggrecan content observed in late stage cancers.[156]

4.4. Neural plasticity

Proteoglycans inhibit neural plasticity under physiological and pathological conditions and this is mainly due to their GAG groups which restrict the formation of new neuronal nets.[157] Among the neuronal proteoglycans, versican and brevican are strongly upregulated immediately after spinal cord injury (SCI),[158-160] whereas aggrecan is down-regulated.[161] Expression of *Adamts5* is also upregulated in a SCI model.[162] Recombinant ADAMTS-5 has been shown to promote neurite outgrowth *in vitro* by cleaving proteoglycans:[163] Following SCI, *Adamts5* knockout mice show severely reduced versican cleavage but significant cleavage of other proteoglycans such as aggrecan and brevican suggests compensation from other ADAMTS family members, such as ADAMTS-1 and -4.[164]

ADAMTS-5 also cleaves the N-terminal region of reelin, a secreted glycoprotein that is mainly expressed in the brain, where it is essential for proper neurodevelopment and synaptic plasticity.[165] This proteolytic processing results in the degradation of the N-terminal reelin fragment, a region that is prone to aggregate and, therefore, plays a role in Alzheimer's disease.[165] AD mice show decreased expression of *Adamts5* in the hippocampus, therefore decreased processing of the reelin N-terminal fragment by ADAMTS-5 may be responsible for accelerated reelin aggregation in the hippocampus of AD mice.[165]

4.5. Muscle maturation and intervertebral disc disease

Although *Adamts5* knockout mice do not show apparent myopathy or deficit in gait and mobility,[53,54] they show delayed muscle maturation during postnatal growth as a result of decreased versican cleavage.[166]

Genome wide association studies have identified *ADAMTS5* as a risk locus for degenerative intervertebral disc disease.[167] To support a role for ADAMTS-5 in this pathology, injection of anADAMTS-5 siRNA suppressed invertebral disc degeneration in the rabbit anular needle-puncture

model.[168] So far, *Adamts5* knockout mice have not been investigated in relation to intervertebral disc disease.

5. CONCLUSIONS AND PERSPECTIVE

5.1. Anti-ADAMTS-5 therapy: the jury is still out

Inhibition of metalloproteinases has a long and troubled history. In the '90s, the failure of broad spectrum MMP inhibitors in cancer clinical trials came as a shock.[169] The major causes of this failure were attributed to a lack of specificity of these inhibitors and incomplete knowledge of the role which the targeted enzymes exert in the disease, especially a naivety in assuming that endogenous proteases are destructive agents only active in cancer.[170] The first problem was addressed by the development of inhibitory mAbs which can be used to "post-translationally knock down" the activity of a certain protease.[171] However, a lot still needs to be done to uncover the multiple biological processes orchestrated by metalloproteinases. The history of ADAMTS-5 research show this in a dramatic way. In few years, ADAMTS-5 turned from an OA target to a cardiovascular anti-target while yet other unpredicted biological roles were uncovered by analysing the phenotypes of *Adamts5* knockout mice (**Table 1 and Figure 2**).

Notwithstanding the adverse side effects observed in *Cynomolgus* upon administration of GSK2394002,[67,83] pharmaceutical companies are still actively pursuing ADAMTS-5 as a therapeutic target for OA. After all, it may well have been that those effects were drug-related rather than target-related. In this regard, it will be interesting to compare the effects of this anti-ADAMTS-5 mAb with those exhibited by other mAbs or small molecule inhibitors.

Agg-523, Wyeth's ADAMTS-4/ADAMTS-5 small molecule inhibitor, was investigated in Phase I clinical trials in patients with mild to moderate and severe knee OA (NCT00454298 and NCT00427687), but no results are currently available. Another orally-available small molecule inhibitor of ADAMTS-5 developed by Galapagos N.V., GLPG1972, with a modest (5-fold) selectivity over ADAMTS-4, is protective against surgically-induced OA in mice and rats.[172,173] GLPG1972 has been tested in a phase 1 trial (ID: NCT02612246) and shown to be well tolerated in healthy male subjects with doses up to 1050 mg/day.[174] The absence of adverse effects is remarkable

and suggests differences in target engagement compared with GSK2394002. A phase 2 clinical trial (ID: NCT03595618) comparing its effects with placebo is currently underway (results expected December 2020).

EMDSerono has developed an-anti-ADAMTS-5 nanobody (M6495, Ablynx) able to block OA progression in mice following DMM[175] and reduce circulating levels of aggrecanase-generated aggrecan fragments in *Cynomolgus* monkeys.[176] Nanobodies are single domain mAbs whose antigen binding site is composed only of one heavy chain, rather than one light and one heavy chain; by being much smaller than conventional mAbs, nanobodies have higher tissue penetration *in vivo*.[177] M6945 is a bifunctional nanobody binding to ADAMTS-5 metalloproteinase/disintegrin domains and serum albumin to extend its *in vivo* half-life.[178] Phase 1 clinical trials (ID: NCT03583346 and NCT03224702) after subcutaneous administration were completed but results have yet to be posted on https://clinicaltrials.gov.

Whatever will be the outcome of these clinical trials, there are a few problems that ADAMTS-5 targeting therapies should address in a clinically-relevant context. For example, administration of ADAMTS-5 inhibitors may require a careful follow-up in OA patients, as they may make them more susceptible to adverse cardiovascular effects such as aortic aneurysms, myxomatous valves, atherosclerosis, and influenza infection.[96,140] Given the administration of ADAMTS-5 inhibitors to an aged patient population with multiple co-morbidities, it is important to fully understand their effects beyond cartilage degradation alone. Moreover, an important application of ADAMTS-5 therapy may be on young athletes with post-traumatic OA, who may better respond to pharmacological treatment. Intra-articular injections may circumvent potential systemic side effects, although this is not a preferable route of administration due to the discomfort for the patients and the necessity to be administered by expert physicians.

Substrate-specific inhibitors have been hypothesised as a way to limit the side-effects of protease inhibitors by restricting their actions to a specific substrate or set of substrates,[171] but in the case of ADAMTS-5 this approach may be unsuccessful for two reasons. First, in the ADAMTS-5 spacer domain, the substrate binding-sites (exosites) are shared between aggrecan and versican.[41] Second,

aggrecan has been recently shown to be expressed not only in cartilage but also in the blood vessels,[84-89,105] where it exerts similar functions to versican.

In the end, only the outcome of clinical trials and, eventually, their follow up will tell if ADAMTS-5 represents a viable target for OA therapy.

5.2. The next twenty years

Scientifically, many questions about ADAMTS-5 biology still need an answer. Other potential biological roles of ADAMTS-5 discussed in Section 4 should be thoroughly investigated and assessed in relevant mouse models.

In recent years, the number of ADAMTS-5 substrates has increased (**Table 2**). However, this number is still relatively small, compared to other enzymes and, with the exception of proteoglycans, most of these substrates are cleaved at a slow rate, suggesting that ADAMTS-5 has a quite strict substrate specificity. Future degradomic studies can help to define its substrate repertoire and therefore clarify if some elusive aspects of its biology are due to unknown substrates or unknown non-proteolytic functions. Moreover, researchers should assess the biological activity of ADAMTS-5-generated cleavage products. In Section 4.3, I discussed the pro-apoptotic activity of versikine, the N-terminal versican cleavage fragment generated by ADAMTS cleavage at Glu441↓F361.[50] This is not the only example, as a 32-amino acid aggrecan fragment (32-mer) generated by the combined proteolytic activity of aggrecanases (clevage at Glu392↓Ala393) and MMPs (cleavage at N360↓F361) is endowed with cytokine-like activities.[179] The 32-mer excites nociceptive neurons in chondrocytes through Toll-Like Receptor 2[179] and may be at least partially responsible for pain-related OA. Mice bearing a mutated MMP cleavage site in aggrecan which, for this reason, cannot produce the 32-mer aggrecan fragments, are protected from increased pain sensitivity (hyperalgesia) although they still show severe cartilage degradation due to ADAMTS-5 activity at Glu392↓Ala392.[38] The generation of such matrix-derived cryptic cytokines (matrikines) may be a more general strategy which ADAMTS-5 employs to fulfil its biological functions.

Finally, much work still needs to be done on the biochemical characterisation of ADAMTS-5. Although it is known that the spacer and cysteine-rich domains contain exosites important for proteoglycanase activity,[41,46,48] the three-dimensional structure of these domains have not been

reported. The only spacer/cysteine-rich domains structures reported so far for any ADAMTS family member are those of ADAMTS-13, whose proteolytic activity, domain composition and regulation are completely different.[180,181] Solving these structures may help to clarify the way that ADAMTS-5 interacts with proteoglycans and at the same time inform the rational design/optimisation of more selective small molecule inhibitors.

In conclusion, after twenty years the field of ADAMTS-5 research has developed considerably and reached a complexity that was unimaginable when it was first discovered. The complicated teenager is getting more complicated, but, hopefully, more manageable.

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Conflict of interest

The author declares no conflicts of interest.

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List of Tables

Table 1. Phenotypes of *Adamts5* knockout mice

Organ/tissue	Model	Phenotype compared to wild-type (penetrance)	Reference
Adipose tissue	HFD	Increased brown adipose tissue	[121,131]
Adipose tissue	Cold exposure	Reduce WAT mass; increased BAT mass; enhanced insulin sensitivity	[121, 131]
Aorta/Heart	-	Myxomatous cardiac valve (100%)	[95]
		Bicuspid pulmonary and aortic valves (10-20%)	[102]
		Bicuspid and tricuspid aortic valves (100%)	[103]
	HFD	Increased LV wall thickness and volume	[117]
Cartilage	DMM	Reduced cartilage degradation	[53]
Cartilage	DMM	Reduced cartilage degradation and mechanical allodynia	[59]
Cartilage	AIA	Reduced cartilage degradation	[54]
	AIA	Reduced cartilage degradation	[120]
Cartilage	TTR	Reduced cartilage degradation and fibrosis	[60]
CNS	SCI	Reduced versican cleavage	[164]
Immune system	Influenza virus infection	Delayed virus clearance, reduced T cell infiltration, increased weight loss	[140]
Limb	-	Syndactyly (44%)	[50]
Liver	HFD	Reduced liver weight and hepatic triglyceride accumulation	[121, 124]
Liver	MCD	Reduced steatosis and fibrosis	[121]
Skeletal muscle	-	Delayed myotube formation	[166]
Skin	Wound healing	Delayed contraction	[122]
Tendon	-	Reduced maximum tensile strength	[62]
Tendon	TTR	Reduced maximum tensile strength	[61]

Abbreviations: AIA, antigen-induced arthritis; BAT, brown adipose tissue; CNS, central nervous system; DMM, destabilisation of medial meniscus; HFD, high-fat diet; LV, left ventricle; MCD, methionine/choline-deficient diet; SCI, spinal cord injury; TTR, TGF-β injection and enforced uphill treadmill running; WAT, white adipose tissue;. Data for combinatorial knockout models are not shown.

Table 2. List of ADAMTS-5 substrates. Note the predominance of glutamic acid in P1 position. Abbreviations: α2M, α2-macroglobulin; CILP, cartilage intermediate layer protein; Cm-Tf, carboxymethylated transferrin. References in black identified the cleavage site by mass-spectrometry/Edman sequencing. Note that for aggrecan, the cleavage sites reported by the original references were for the bovine homologue (Uniprot ID: P13608).

Substrate	Uniprot ID	Cleavage site (P1↓P1')	Reference
ADAMTS-5	Q9UNA0	Glu753↓Gly754	[**182**]
Aggrecan	P16112-1	Glu392↓Ala393	[**1,2**]
		Glu1679↓Gly1680	[**48**]
		Glu1848↓Gly1849	[**183**]
		Glu1953↓Ala1954	[**47**]
		Glu2053↓Leu2054	[**183**]
α2M	P01023	Met713↓Gly714	[**184**]
Biglycan	P21810	Asn186↓Cys187	[48,**185]**
Brevican	Q96GW7-1	Glu400↓Ser401	[**152**]
CILP-1	O75339	Glu591↓Val592	[**186**]
		Val592↓Val593	[**186**]
		Ser607↓Phe608	[**186**]
		Trp699↓Ser700	[**186**]
		Glu717↓Asn718	[**186**]
		Asn718↓Gln719	[**186**]
		Gln719↓Arg720	[**186**]
		Asn722↓Lys723	[**186**]
CILP-2	Q8IUL8	Thr810↓Ala811	[**186**]
		Ala811↓Thr812	[**186**]
		Leu813↓Gly814	[**186**]
		Ala830↓Thr831	[**186**]
		Val832↓Gly833	[**186**]
Clusterin	P10909	Asn43↓Lys44	[**186**]
Cm-tf	P02787-1	Cys58↓Val59	[**48**]
		Cys213↓Leu214	[**48**]
		Ser409↓Leu410	[**48**]
		Cys542↓Leu543	[**48**]
Collagen type II α1	P02458	Ala902↓Gly903	[**186**]
		Pro922↓Ser923	[**186**]
		Ser923↓Gly924	[**186**]
		Gly1053↓Val1054	[**186**]
		Pro1078↓Ala1079	[**186**]
Collagen type III α1	P02461	Gln374↓Gly375	[**186**]
		Asn389↓Gly390	**[186]**
Decorin	P07585	Lys74↓Val75	[48,**186**, 187]
		Leu85↓Leu86	[48,**186**, 187]
Fibromodulin	P13605	Tyr63↓Ala64	[**187**]
Fibronectin	P02751	Phe2113↓Val2114	[48, **186**]
		Pro2135↓Ser2136	[48, **186**]
Inter-α-inhibitor HC2	P19823	Arg359↓Asn360	[**188**]
		Thr366↓Lys367	[**188**]
		Tyr490↓Asn491	[**188**]
		Asn491↓Gln492	[**188**]
		Leu681↓Ala682	[**188**]
Prolargin	P51888	His340↓Asp341	[**186**]
		His351↓Leu352	[**186**]
Reelin		-	[165]
Syndecan-1		-	[124]
Tenascin	P24821	Cys64↓Ser65	[**186**]
		Ser70↓Ala71	[**186**]
		Glu89↓His90	[**186**]
Versican V1	P13611-2	Glu441↓Ala442	[41, 97, 98]

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Figure 1

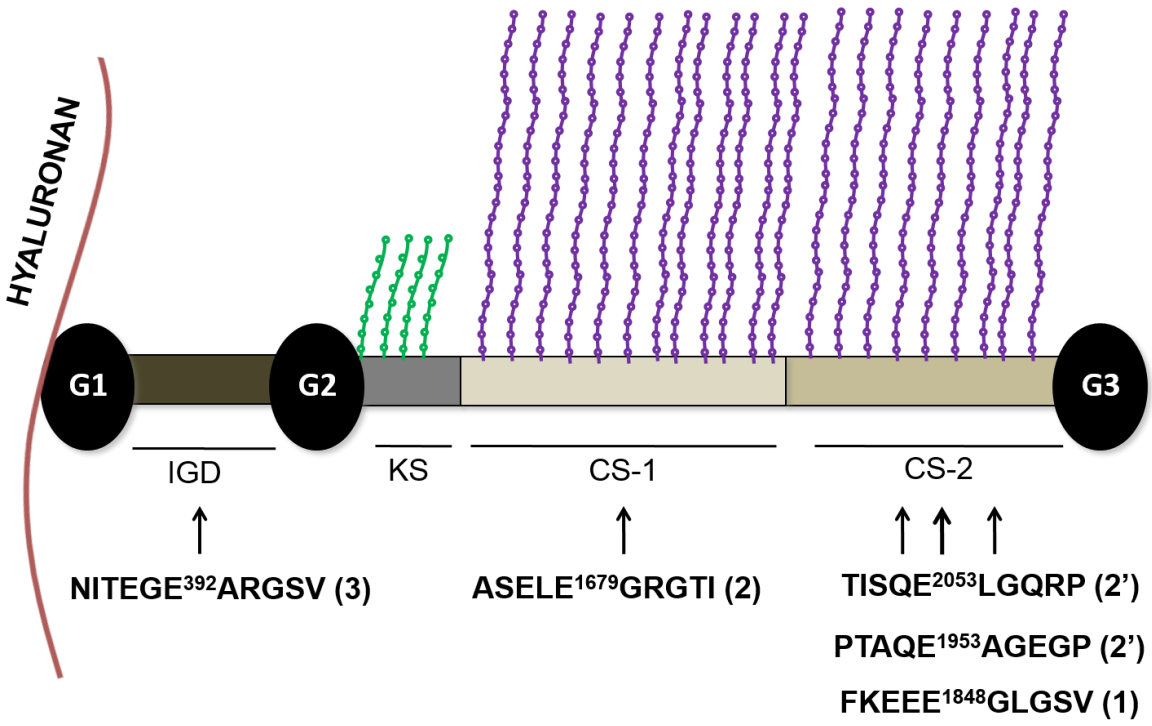
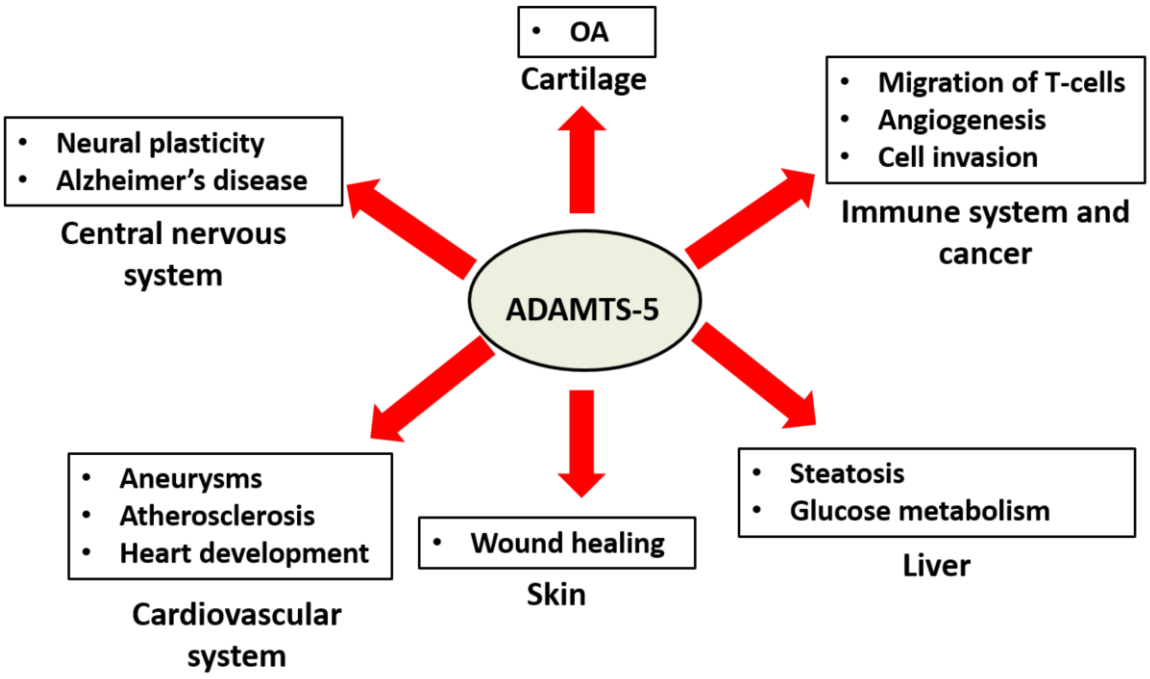

HYALURONAN
G1
G2
G3
IGD
KS
CS-1
CS-2
NITEGE392ARGSV (3)
ASELE1679GRGTI (2)
TISQE2053LGQRP (2')
PTAQE1953AGEGP (2')
FKEEE1848GLGSV (1)

Figure 2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