Cysteine Protease Cathepsins in Atherosclerotic Cardiovascular Diseases

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Atherosclerotic cardiovascular disease (ASCVD) is an inflammatory disease characterized by extensive arterial wall matrix protein degradation. Cysteine protease cathepsins play a pivotal role in extracellular matrix (ECM) remodeling and have been implicated in the development and progression of atherosclerosis-based cardiovascular diseases. An imbalance in expression between cathepsins (such as cathepsins S, K, L, C) and their inhibitor cystatin C may favor proteolysis of ECM in the pathogenesis of cardiovascular disease such as atherosclerosis, aneurysm formation, restenosis, and neovascularization. New insights into cathepsin functions have been made possible by the generation of knockout mice and by the application of specific inhibitors. Inflammatory cytokines regulate the expression and activities of cathepsins in cultured vascular cells and macrophages. In addition, evaluations of the possibility of cathepsins as a diagnostic tool revealed that the circulating levels of cathepsin S, K, and L, and their endogenous inhibitor cystatin C could be promising biomarkers in the diagnosis of coronary artery disease, aneurysm, adiposity, peripheral arterial disease, and coronary artery calcification. In this review, we summarize the available information regarding the mechanistic contributions of cathepsins to ASCVD.

Key words: Cathepsins, Atherosclerosis, Aneurysm, Restenosis, Neovascularization

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extracellular space in the development and progression of cardiovascular disease such as atherosclerosis, aneurysm formation, neointima formation, and neovascularization. This review focuses on recent findings in this field, highlighting the cathepsin biology and the significance of lysosomal cysteine proteases in ECM remodeling, pharmacological intervention, and the prediction of the development and progression of ASCVD.

1. The Properties, Structure and Molecular Regulation of Cysteiny1 Cathepsins

To date, 11 human cathepsins have been identified: B, C, H, F, K, L, O, S, V, W, and X. Human cathepsins have been shown to share a conserved active three-dimensional pocket, which formed with histidine, asparagine, and cysteine residues. Cathepsins are synthesized as preproenzymes, containing a signal peptide, a proregion, a heavy chain, and a light chain. Procathepsin is formed by removing the signaling peptide during the passage to the endoplasmic reticulum. Next, the active cathepsins can be produced in the acidic compartments of the late endosomes or lysosomes after proteolytic removal of the proregion. The process of the maturation of cathepsin from synthesis to activation has been reviewed.

Cathepsins are typically located in the lysosomes and endosomes to degrade endocytosed and intracellular "unwanted" proteins. However, exogenous oxidants (reactive oxygen species) may induce lysosomal leakage and permeabilization, causing the release of cathepsins into the cytoplasm. Most strikingly, cathepsin proteins have been observed in cell-cultured medium and in the circulation of both experimental animals and human beings under physiological conditions. Moreover, the levels of cathepsins increased after cells were cultured with inflammatory cytokines or in patients with inflammatory diseases. Reiser et al. proposed that active cathepsins might be recruited from late endosomes or lysosomes for secretion into the extracellular space through a Ca^{2+}-dependent fusion of these organelles with the cell membrane. The activities of most cathepsins, such as cathepsin B, F, H, K, L and V, are optimal in acidic environments, and weakly active at neutral pH. In contrast, cathepsin S is optimally active at neutral pH.

The activities of cathepsins are found to be regulated intracellularly by stefins (stefin A and stefin B) and extracellularly by cystatins (cystatin C) and kininogens. Cystatin C shows the highest inhibiting properties toward cathepsin L and cathepsin S, followed by cathepsin B and cathepsin H. In primary cultured human endothelial cells (ECs), vascular smooth muscle cells (SMCs), and monocyte-derived macrophages, inflammatory cytokines or growth factors induced the expression of cathepsin L and its activity against extracellular elastin and collagen.

2. Cathepsins in Atherosclerosis

Some cathepsins such as cathepsin S, K, L, B, and V are potent collagenases and/or elastases. Atherosclerosis is an inflammatory disease, the complex development of which occurs in three main stages: initiation, progression, and complication. Throughout the three stages, arterial wall remodeling involves collagenolysis and elastinolysis, including the initial transmigration of inflammatory cells from the vascular lumen or the vasa vasorum through the basement membrane into the subendothelium; the progressive migration of SMCs from the media into the intima through the elastic lamina; and finally, disruption of the arterial wall—outward in aneurysmal disease and luminally in athero-occlusive disease.

These inflammatory cells, including monocytes/macrophages, lymphocytes, neutrophils, and mast cells, can express high levels of cathepsins, and the pro-inflammatory cytokines can also induce vascular cell cathepsin expression. Inflammatory cells and activated vascular cells then produce cathepsins or other proteases such as MMPs to degrade extracellular elastic and collagen in the arterial wall, thus causing athrogenesis.

2.1. Cathepsin S

Cathepsin S (CatS) was one of the first cathepsins observed in human atherosclerotic lesions, whereas normal arteries contain little or no CatS. In early human atherosclerotic plaques (or fatty streaks), vivid CatS immunoreactivity was observed in intimal and medial SMCs. In advanced human atherosclerotic lesions, CatS localized in SMCs of the fibrous cap and macrophages and in areas of elastin fragmentation. CatS deficiency (CatS<sup>−/−</sup>) lead to marked reductions in the atherosclerotic plaque area (by 60%) after 12 weeks of a western-style diet in athrogenic low-density lipoprotein receptor-deficient (Ldlr<sup>−/−</sup>) mice. CatS<sup>−/−</sup> also reduced elastinolytic activities and the number of elastin breaks, and the mice also had significantly reduced contents of plaque macrophages, smooth muscle cells, CD4<sup>+</sup> T lymphocytes, lipids, collagen, and levels of interferon-gamma (IFN-γ). Macrophages from CatS<sup>−/−</sup> mice had attenuated transmigration ability through an artificial aortic wall made with an endothelial cell monolayer and collagen types I and IV.

In another study using apolipoprotein E-deficient
(ApoE−/−) mice30), CatS−/− reduced the atherosclerotic plaque area by 46% after 12 weeks of a western-style diet and lead to a reduction in the number of plaque ruptures by 73%. Bone marrow transplantation technology was used to test the importance of leukocyte-derived CatS in atherogenesis. Leukocyte CatS deficiency showed significantly altered plaque morphology with decreased apoptosis, smaller necrotic cores, and reduced SMC content and collagen deposition36). These data point to a pivotal role of CatS in atherogenesis through the degradation of ECM proteins.

2.2. Cathepsin K

Like CatS, it is difficult to detect CatK expression in normal arteries. In early human atherosclerotic plaques, CatK expression was found in the intimal and medial SMCs. In advanced atherosclerotic lesions, CatK localized in SMCs of the fibrous cap and macrophages17, 37). Lutgens and colleagues38) showed that CatK deficiency (CatK−/−) resulted in a 42% reduction in the atherosclerotic plaque area in ApoE−/− mice, with increased lesion collagen and decreased elastin breaks. Samokhin et al. found that CatK−/− increased lesion stability in brachiocephalic arteries by retaining the integrity of the tunica media and by reducing plaque vulnerability to rupture in ApoE−/− mice39). These findings highlight a protective role of CatK deficiency in atherogenesis through a reduced degradation of ECM components.

2.3. Cathepsin L

CatL, one of the most potent mammalian collagenases and elastases, is found to be widely expressed at basal levels in most tested tissues and cell types, and regulated by pro-inflammatory cytokines14, 22). Liu and colleagues demonstrated increased expression of CatL in human atheromata and abdominal aortic aneurysms (AAAs), and they identified its expression in lesional SMCs, ECs, and macrophages20). A quantitative immunohistochemical analysis of human carotid atherosclerotic lesions also showed an increased expression of CatL in atherosclerotic lesions with formation of the necrotic core and rupture of the fibrous cap40).

In human carotid atherosclerotic lesions, plaques from symptomatic patients showed higher levels of CatL compared to those from asymptomatic patients40). In Ldlr−/− mice, Kitamoto et al. showed that the deficiency of one or both alleles of the CatL gene decreased atherosclerotic lesions at both 12- and 26-week time points30). The plaque lipid core areas, the contents of plaque inflammatory cells and collagen, medial SMC content, and elastin fragmentation were all decreased in a gene dose-dependent fashion14, 30). Kitamoto et al. demonstrated that CatL promotes atherosclerosis by degrading elastin and collagen and regulates blood-borne leukocyte transmigration and lesion progression30).

2.4. Cathepsin C

CatC gene and protein expression were significantly increased in ruptured and advanced human carotid artery lesions41), and CatC was abundantly expressed by plaque macrophages and foam cells. Leukocyte CatC deficiency caused by bone marrow transplantation (in murine CatC−/− Ldlr−/− chimeras) presented reduced plaque burdens in carotids, the descending aorta and the aortic arch and root at both the early and advanced plaque stage, indicating that leukocyte CatC deficiency attenuates atherosclerotic lesion progression.

2.5. Cystatin C

Little or no expression of CatS and CatK was demonstrated in normal arteries, but these cathepsins were overexpressed in atherogenesis. In contrast, cystatin C (CystC), the most important endogenous inhibitor of the collagen- and elastin-degrading cysteine proteases of the cathepsin family, is found to express normally in vascular SMCs, and this cysteine protease inhibitor is severely reduced in atherosclerotic aortic lesions33). These findings point to an imbalance in expression between cathepsins and their inhibitor CystC in cardiovascular disease.

By characterizing the polymorphisms in the promoter region of the CystC gene that influence CystC production, Eriksson et al.42) showed that the plasma CystC concentration was significantly lower in carriers of the mutant haplotype. They also reported that the mutant haplotype was associated with a greater average number of stenosis per coronary artery segment in unselected post-infarction patients undergoing routine coronary angiography. These results provide clinical evidence of an important role of CystC gene in the focal progression of coronary artery disease. To further determine the functional role of CystC in atherosclerosis, CystC and ApoE double-deficient mice were crossed (CystC−/− ApoE−/− mice)43), and after 25 weeks of an atherogenic diet the mice lacking cystatin C had larger subvalvular plaques compared to the control mice (CystC+/− ApoE−/− mice)43). These results suggest a protective role of cystatin C in atherogenesis.

2.6. Cathepsins and Lipid Metabolism

2.6.1. Lipid Uptake and Modification

It is widely known that lipoprotein modification and uptake by atherosclerotic lesion cells (mainly macrophages and SMCs), are important pathological steps in the process of atherosclerotic plaque formation27, 44-47).
The treatment of low-density lipoprotein (LDL) particles with human recombinant CatF, CatK and CatS led to the extensive degradation of apoB-100, triggered the aggregation of extracellular LDL particles, lipid droplet formation, and LDL retention to arterial proteoglycans. This proteolysis has been demonstrated to enhance the LDL particle fusion to macrophages.

After taking up the lipoproteins and modified lipoproteins, macrophages and SMCs become foam cells filled with lipid droplets. In addition, lipids or modified lipoproteins impact cathepsins’ cellular expression and translocation. Li et al. reported that when macrophages were exposed to oxidized LDL or 7β-hydroxycholesterol, the cells expressed high levels of CatB and CatL, in addition to forming foam cells. Another study demonstrated that free cholesterol accumulation stimulated CatK expression via activation of toll-like receptors and p38 mitogen-activated protein kinase. These cathepsins, which translocated from lysosomes to the cytosol or nuclei, cause foam cell apoptosis and may play important roles in plaque development and rupture.

2.6.2. Cholesterol Efflux

Cathepsins are also involved in the modulation of cholesterol efflux. CatF and CatS were reported to reduce the ability of cholesterol efflux from macrophages by 50% in vitro due to the proteolysis of preβ-HDL. CatF and CatK were reported to partially degrade lipid-free apolipoprotein A-1 (apoA-1) and reduced its ability to induce cholesterol efflux (by 30% and 15%, respectively), whereas CatS totally degraded apoA-1, leading to a complete loss of the ability of apoA-1 to stimulate cholesterol efflux. Therefore, different cathepsins show different functions in lipid metabolism and contribute to atherosclerosis via different mechanisms.

3. Cathepsins in Restenosis

The process of vascular neointima formation is common to various forms of vascular diseases, such as atherosclerosis, in-stent restenosis, and transplant vasculopathy. Restenosis currently limits the long-term success of percutaneous coronary intervention (PCI) and related procedures. Despite widespread improvements in PCI technology, restenosis remains the major challenge of percutaneous revascularization techniques after successful dilation. The migration of vascular SMCs from the media to the intima and the proliferation of intimal SMCs are key events in restenotic lesion development in response to vascular injury. One essential factor required for vascular SMC migration is degradation of the basement membrane and surrounding ECM. Like the role of cathepsins in the process of atherogenesis, cathepsins have also been suggested to play a possible role in neointima formation and restenosis, contributing to ECM degradation.

We previously demonstrated that the levels of CatS and CatK mRNAs and proteins increased in the neointima of balloon-injured rat carotid arteries, whereas CystC mRNA and protein showed no significant change. Immunohistochemistry showed that increased expression of both cathepsins localized in SMCs and infiltrated macrophages of the developing neointima. Similarly, a rabbit balloon-injury model, Burns-Kurtis et al. reported that the neointima had higher levels of CatS mRNA and protein compared to the neointima in uninjured control iliac arteries, whereas CystC expression was only minimally up-regulated. These findings highlight the importance of maintaining a fine balance between cathepsins and CystC; and the disruption of this balance leads to a pathological state of maladaptive vascular remodeling due to a deficiency or excessive degradation of collagen and other components of the cardiovascular wall ECM protein.

This notion is further supported by the direct evidence that extracts of balloon-injured carotid arteries show an increase in collagenolytic and elastolytic activity. The results of a recent study using a murine vein graft model suggested that CatS also contributes to vascular SMC proliferation and macrophage migration through degradation of elastic lamina to facilitate vein graft neointimal hyperplasia. The role of cathepsins in neointima formation is further supported by the direct evidence that genetic deletions of CatS and CatK reduced neointimal lesion formation in response to injury, indicating a novel therapeutic strategy for the treatment of endovascular therapy-related restenosis by regulating CatS or CatK activity. Moreover, it has also been demonstrated that CatS and CatK degrade fibronectin, collagen type I, and laminin, and that the ability of SMCs transmigration through a basement membrane matrix gel can be inhibited by a selective Cat inhibitor.

4. Cathepsins in AAAs

The formation of an abdominal aortic aneurysm (AAA) is characterized by extensive medial and adventitial inflammatory cell infiltration, medial SMC depletion, and the degradation of collagen and elastin in the media. The inflammatory cell invasion mainly includes macrophages, neutrophils, CD4+ T cells, natural killer T cells, and mast cells. These cells are recruited from the lumen or the vasa vasorum to the
media and adventitia of animal or human AAAs, followed by releasing cytokines, chemokines, and proteases. Increased expression of these inflammatory molecules lead to further inflammatory cell recruitment, thereby inducing vascular cell infiltration and apoptosis, degrading the vasculature ECM proteins (e.g., collagen and elastin), and promoting cell migration, angiogenesis, and apoptosis.

In human AAA, the protein levels of CatS, CatK, and CatL are increased, and the expression of their endogenous inhibitor CystC is reduced. Similarly, CatB, CatK, and CatS have shown to be highly expressed in cerebral aneurysms, whereas CystC is sparse. The expression and activities of CatB, CatC, and CatL were also increased in the aneurysm wall and parietal thrombus of human aortic aneurysms compared to normal arteries, indicating that these cathepsins may participate in process of aneurysm formation.

Significantly increased CatS expression was observed in mouse AAA lesions. Lohoefer et al. performed immunohistochemistry for the expression of CatB, CatD, CatK, CatL and CatS, and CystC in all cells localized within AAAs, and they reported that the luminal ECs of the AAAs were positive for CatD and partially positive for CatB, CatK and CatS; in addition, endothelial cells of the neovessels and SMCs in the media were positive for CatD, CatK, CatL and CatS.

4.1 CatS

The role of CatS in AAA has been tested in angiotensin II (Ang-II) minipump perfusion-induced mice. CatS−/− significantly reduced the AAA incidence in these mice compared to ApoE−/− mice (10% vs. 80%). CatS deletion greatly reduced the luminal and external abdominal aortic diameters, medial elastin fragmentation, and adventitia collagen content. Absence of CatS significantly reduced the aortic lesion expression, the activity of MMP-2, MMP-9, and CatK, AAA lesion media SMC apoptosis, inflammatory cell accumulation and proliferation, and lesion adventitia microvessel content. Moreover, in vitro studies demonstrated that CatS promoted SMC apoptosis, angiogenesis, monocyte and T-cell transmigration, and T-cell proliferation, all of which are essential for the pathogenesis of AAA. These findings provide direct evidence that CatS plays a pivotal role in the formation of AAA and suggest that targeting CatS is a new therapeutic strategy for human AAA.

4.2. CatK

CatK function has also been tested in Ang-II perfusion-induced experimental AAA, but the findings are contradictory. In Ang-II perfusion-induced AAA, CatK−/− did not affect AAA development. Bai et al. reported that the aneurysm lesion area, rupture numbers, and aortic wall elastin breaks were not affected by CatK deletion. Contrary to their expectations, AAA lesions in CatK−/− mice contained markedly more CD45+ leukocytes and macrophages, and increased CatS and CystC expression. However, Sun et al. demonstrated that CatK absence prevented AAA formation in elastase perfusion-induced experimental AAA in mice. They further demonstrated that CatK contributed to AAA lesion T-cell proliferation, medial SMC apoptosis, and elastin fragmentation, all of which are essential to AAA pathogenesis.

The contradictory observations between the Bai and Sun groups might be attributable to the differences in experimental models—Ang-II perfusion versus elastase perfusion-induced AAA. Based on the observations from both laboratory investigations, Sun et al. raised the possibility that Ang-II infusion enhances both peripheral active CD4+/CD25+ T cells and Lg6G neutrophils and AAA lesional CD45+ leukocytes and macro-3+ macrophages, and thereby both hyperinflammatory responses may have obscured the CatK deficiency-mediated vascular protective actions.

4.3. CatL

Gacko et al. were the first to report increased activities of CatD and CatL in the human aortic aneurysm wall and parietal thrombus. Liu and colleagues also demonstrated increased expression of CatL in human AAA and atheromata, and they localized its expression to lesional SMC, macrophages, and ECs, suggesting the involvement of CatL in AAA formation. The role of CatL in AAA has been tested in aortic elastase perfusion-induced experimental AAA. Sun et al. showed that CatL−/− mice were fully protected from elastase perfusion-induced AAA at 14 days post-perfusion, none of the CatL−/− mice had developed AAA.

Mechanistic studies have shown that CatL absence reduced the lesion monocyte chemotactic protein-1 (MCP-1) content, macrophage and T cell in vitro trans-
migration, and angiogenesis, and altered the expression and activities of MMPs and other cysteine proteases in inflammatory cells, vascular cells, and AAA lesions. These findings suggest that CatL contributes to the formation of AAA by increasing lesion inflammatory cell infiltration, angiogenesis, and protease expression.80)

4.4. CatC.
One report noted that elastase-induced AAAs in the mouse was accompanied by increased aortic wall expression of CatC/dipeptidyl peptidase I73). Pagano et al.81) showed that mice with a loss-of-function mutation in CatC−/− are resistant to the development of elastase-induced experimental AAAs. They revealed that the protective effect against AAA development seen in CatC−/− mice might be due to impaired early influx of neutrophils into the aortic wall.

4.5. CystC
The decreased expression of CystC points to the possibility that CystC may play a role in the pathogenesis of AAAs. To investigate the direct role of CystC in AAA, Sukhova et al. generated CystC−/− ApoE−/− mice77). The CystC−/− ApoE−/− mice showed significantly dilated thoracic and abdominal aortas compared to the ApoE−/− control mice. Mechanistic studies demonstrated that CystC deletion yielded greatly increased tunica media elastic lamina fragmentation, reduced aortic lesions medial size, and increased lesions smooth muscle cell and collagen content in ApoE−/− mice after 12 weeks on an atherogenic diet77).

Schulte and colleagues further directly examined the roles of the imbalance of cysteine proteases and their inhibitor in AAA formation in Ang II-induced AAA76). CystC−/− led to increased inflammatory cell accumulation (macrophage content, CD4⁺ T cells, leukocytes), more severe elastic elastin fragmentations, and fewer SMCs in aortic lesions of ApoE−/− mice76). CystC deletion enhanced the cathepsin activity by 5.5-fold in AAA, yielding greater elastic lamina fragmentations and production of the proangiogenic peptide laminin-5 gamma 2, which may contributing to the increased lesions microvascularization in CystC−/− ApoE−/− mice compared to ApoE−/− mice70). Schulte et al. concluded that the effect of CystC deficiency in promoting experimental AAA formation can be attributable to enhanced cysteine protease activity, which favors inflammation in AAA lesions by promoting microvascularization, SMC apoptosis, and leukocyte adhesion and proliferation70). The expression of cathepsins in atherosclerosis, restenosis, and AAA or other aneurysms is summarized in Table 1.

5. Cathepsins in Neovascularization
The growth of atherosclerotic plaques is accompanied by neovascularization from vasa vasorum microvessels extending through the tunica media into the base of the plaque and by lumen-derived microvessels extending through the fibrous cap82). Moreno and colleagues showed that plaque neovascularization is increased in ruptured atherosclerotic lesions of human aorta, indicating a contributory role for neovascularization in the process of plaque rupture82). Our recent observation showed that neovessel formation and CatS protein expression were increased in advanced plaque in ApoE−/− mice83), whereas CatS deficiency reduced the plaque neovessel formation, indicating that CatS plays an important role in plaque neovascularization. Our previous study demonstrated that deficiency of CatS impaired wound repair related-microvessel growth despite normal expression of the angiogenic factors basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF)84).

In an experimental model of ischemia-induced neovascularization in mice, we demonstrated that either CatS−/−85) or CatK−/−86) impaired functional recovery following hindlimb ischemia. In established animal models of retinal and choroidal neovascularization, Shimada et al. showed that both genetic and pharmacological interventions of CatL led to a significant decrease of intraocular neovascularization87). Despite the implication of CatK and CatL involvement in atherosclerotic plaque vessel formation, the direct evidence of in vivo intervention studies using inhibitors or genetically modified mice to define the role of these cathepsins in atherosclerosis-related vasa vasorum have been lacking. The effect of cathepsins or their inhibitor cystatin C deficiency on the progression of athero-

Table 1. Cathepsin expression in atherosclerosis, restenosis, and AAA or other aneurysms

| Disease               | CatS        | CatK        | CatL        | CatC        | Cystatin C |
|-----------------------|-------------|-------------|-------------|-------------|------------|
| Atherosclerosis       | Increase²⁷, ³³, ³⁴ | Increase²⁷, ³³, ³⁷ | Increase²², ⁴⁰ | Increase⁴¹  | Decrease³³ |
| Restenosis            | Increase⁵⁷, ⁶⁰ | Increase⁵⁸, ⁵⁹, ⁶² | Increase⁵⁹   | Increase⁵⁹  | Increase or No Change⁵⁷ |
| AAA or other aneurysms| Increase⁷¹, ⁷⁵ | Increase⁷¹, ⁷⁵ | Increase²², ⁷², ⁷⁵ | Increase⁷³  | Decrease³³, ⁷¹, ⁷² |

CatS indicates cathepsin S; CatK, cathepsin K; CatL, cathepsin L; CatC, cathepsin C; AAA, abdominal aortic aneurysms.
sclerosis, restenosis, AAA, and angiogenesis or vasa vasorum is summarized in Table 2.

### 6. Circulating Cathepsins as Biomarkers for ASCVD

#### 6.1. Circulating Cathepsins in Atherosclerosis and Restenosis

Liu et al.\(^88\) reported significantly higher serum levels of CatS in patients with either atherosclerotic stenosis or diabetes, indicating that increased serum CatS may serve as a biomarker for both diseases. Liu et al. also showed that patients with acute or previous myocardial infarction or unstable angina pectoris had elevated levels of CatS.\(^88\) Similarly, Gu et al.\(^89\) reported that plasma CatS and CystC levels were significantly higher in patients with unstable angina (UA) or stable angina (SA) compared to controls. Plasma CatS and CystC were higher in the UA group than in the SA group. Plasma CystC was positively correlated with plaque area and plaque burdening in the UA group. Gu and colleagues concluded that in angina patients, higher plasma CatS may suggest the presence of vulnerable plaque, and higher plasma CystC may be a clue regarding larger atherosclerotic coronary plaque.\(^89\)

We recently reported that patients with coronary artery disease (CAD) had higher CatK levels compared to controls.\(^90\) Patients with acute coronary syndrome had higher CatK levels than those with stable angina pectoris. CatK levels were also correlated positively with percent plaque volumes by intravascular ultrasound. Liu et al.\(^22\) reported that patients with coronary artery stenosis had higher levels of serum CatK than those without lesions detectable by coronary angiography. In patients with rheumatoid arthritis, high CystC serum levels indicated a high risk of subclinical atherosclerotic disease.\(^91\) These findings indicate that serum cathepsins or CystC could be a biomarker for CAD, and that the measurement of circulating cathepsins or CystC levels may be useful in the diagnosis of atherosclerosis-based cardiovascular disease.

#### 6.2. Circulating Cathepsins in AAA

Lv et al.\(^92\) collected plasma samples from 476 male AAA patients and 200 age-matched male controls to measure the circulating levels of CatS and CystC by enzyme-linked immunosorbent assay (ELISA). The AAA patients had higher plasma levels of total, active, and pro-CatS than the controls. They further showed that the plasma levels of total, active, and pro-CatS were positively correlated with aortic diameter, whereas the plasma CystC levels were negatively correlated with aortic diameter, suggesting these serological parameters as biomarkers for human AAA.

Qin et al.\(^93\) reported that patients with AAA had higher serum levels of CatS and high-sensitivity C-reactive protein (hs-CRP) than controls. Since the human serum CatS and hs-CRP levels were positively correlated with AAA diameter size, Qin et al. suggested that combined serum CatS and hs-CRP levels could be used to predict the inflammatory activity of AAA lesions in clinical settings. Shi et al.\(^33\) reported that the serum CystC concentration was inversely corre-

### Table 2. The effect of cathepsins or their inhibitor Cystatin C deficiency on the progression of atherosclerosis, restenosis, AAA, and angiogenesis or vasa vasorum

| Disease | CatS | CatK | CatL | CatC | Cystatin C |
|---------|------|------|------|------|-----------|
| Atherosclerosis | CatS\(^{-/-}\)/Ldlr\(^{-/-}\)\(^\text{35}\) | CatK\(^{-/-}\)/ApoE\(^{-/-}\)\(^\text{36, 39}\) | CatL\(^{-/-}\)/Ldlr\(^{-/-}\)\(^\text{30}\) | CatC\(^{-/-}\)/Ldlr\(^{-/-}\)\(^\text{41}\) | CystC\(^{-/-}\)/ApoE\(^{-/-}\)\(^\text{43}\) |
| Restenosis | CatS\(^{-/-}\)/Ldlr\(^{-/-}\) \(^\text{40}\) | CatK\(^{-/-}\)/ApoE\(^{-/-}\)\(^\text{62}\) | – | – | – |
| AAA | CatS\(^{-/-}\)/ApoE\(^{-/-}\) \(^\text{74}\) | CatK\(^{-/-}\)/ApoE\(^{-/-}\)\(^\text{78}\) | CatL\(^{-/-}\)\(^\text{40}\) | CatC\(^{-/-}\)\(^\text{81}\) | CystC\(^{-/-}\)/ApoE\(^{-/-}\)\(^\text{76, 77}\) |
| Angiogenesis or vasa vasorum | CatS\(^{-/-}\)/ApoE\(^{-/-}\) \(^\text{84, 85}\) | CatK\(^{-/-}\)\(^\text{86}\) | CatL\(^{-/-}\)\(^\text{87}\) | – | – |

CatS indicates cathepsin S; CatK, cathepsin K; CatL, cathepsin L; CatC, cathepsin C; CystC, cystatin C; AAA, abdominal aortic aneurysms; Ldlr, low-density lipoprotein receptor; ApoE, apolipoprotein E; \(^{\text{35}}\), deficiency.
lated with the abdominal aortic diameter in 122 patients screened by ultrasonography. Similarly, Lindholt et al.\(^{98}\) showed that the serum CystC level was negatively correlated with AAA size and the annual expansion rate in 142 patients with small AAAs. Their data showed that the levels of serum CystC were a significant predictor of operation in patients with an abdominal aortic diameter expanded to \(>50\) mm, with 61% sensitivity and 57% specificity. These findings indicate that serum CatS or CystC could be a biomarker for AAA, and that increased serum levels of CatS or lower CystC may be helpful in the diagnosis of AAA.

### 6.3. Circulating Cathepsins in Adiposity

Taleb et al.\(^{95}\) demonstrated that obese subjects had increased levels of CatS mRNA and protein in subcutaneous white adipose tissue (scWAT) and serum CatS compared to lean subjects. They showed that both CatS mRNA in scWAT and circulating CatS levels were positively correlated with body mass index (BMI), body fat, and plasma triglyceride levels, identifying CatS as a novel marker of adiposity. Taleb et al.\(^{96}\) further demonstrated that weight loss by surgery decreased the circulating CatS levels and CatS enzymatic activity and adipose tissue CatS content. They proposed that the decrease in circulating CatS may contribute to vascular improvement in obese individuals after weight loss. Since CatS has been implicated in the development of atherosclerotic lesions, Taleb et al. proposed that CatS represents a molecular link between obesity and atherosclerosis\(^{95}\).

### 6.4. Circulating CystC in Peripheral Arterial Disease (PAD)

Arpegard et al.\(^{97}\) collected blood samples from 103 patients with peripheral arterial disease (PAD) and 96 controls matched for age and sex to measure serum CystC. They reported that the circulating CystC concentration was significantly higher in the PAD patients than that in controls, suggesting that CystC may be an independent marker of PAD\(^{97}\). Urbonaviciene et al.\(^{98}\) investigated the relationships of CystC, CatL and CatS to lethal outcome in patients with PAD. They reported that the patients with serum CystC levels \(>1\) µg/l had a significantly enhanced adjusted risk for all-cause and cardiovascular mortality compared to the patients with CystC levels \(\leq 1\) µg/l. They concluded that higher CystC levels independently predicted 5-year all-cause mortality and cardiovascular death in PAD patients\(^{98}\). However, no significant relationships between CatS and CatL with all-cause and cardiovascular mortality were observed.

### 6.5. Circulating Cathepsins in Coronary Calcification

Our group recently investigated the relationships among CatK, coronary artery calcification and major adverse cardiac and cerebrovascular events (MACCEs) in patients with chronic kidney disease (CKD)\(^{89}\); 113 consecutive CKD patients were divided into two groups by plasma CatK levels and followed up for up to 3 years. Our analysis demonstrated that the high-CatK group had a significantly higher incidence of MACCEs. We also observed that the circulating CatK level was greatly higher in the patients with MACCEs than that in the patients without MACCEs. Moreover, we showed that in non-diabetic patients with CKD, there was a significant correlation between CatK levels and the coronary artery calcification score. These data suggest that higher circulating CatK may be an indicator of more severe coronary calcification and worse clinical outcome in CKD patients. Circulating cathepsins concentrations in patients with coronary artery disease, AAA, adiposity, PAD, or coronary artery calcification are summarized in Table 3.

### 7. Pharmacological Therapeutics for Cathepsins in ASCVD

Studies using genetic interventions targeting cathepsins and CystC in mice have provided invaluable insights regarding the roles of cathepsins in cardiovascular diseases such as atherosclerosis and AAA. The roles of cathepsins, mainly CatS, in these diseases have been tested by using selective cathepsin inhibitors. Figueiredo et al.\(^{100}\) reported that selective CatS inhibitor by RO5444101 attenuated the progression of atherosclerosis in ApoE\(^{-/-}\) mice with chronic renal disease. Their histological assessment of atherosclerotic plaques demonstrated that RO5444101 reduced plaque size, immunoreactive CatS, elastin degradation, macrophage accumulation, growth differentiation factor-15, and calcification.

Another study showed that a cysteine cathepsin inhibitor (NC-2300) significantly reduced the activities of CatK and CatS in rat aneurysm walls and led to the reduced incidence of advanced cerebral aneurysm\(^{71}\). The expression of collagenase 1 and IV in aneurysm walls was also decreased and the elastin content was increased in the NC-2300-treated group\(^{71}\). The CatS inhibitor LY3000328 recently showed potent efficacy in a CaCl\(_2\)-induced mice experimental AAA. LY3000328 treatment resulted in a dose-dependent aortic diameter reduction at 1, 3, 10, and 30 mg/kg\(^{100}\).

The CatS inhibitor LY3000328 was selected for development as a clinical candidate, and it has com-
Conflicts of Interest

All authors declare that they have no conflict of interest.

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Author Contributions

All authors contributed equally to the preparation of the manuscript and approved the final manuscript.

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Table 3. Circulating cathepsins in patients with coronary artery disease, AAA, adiposity, PAD, or coronary artery calcification

| Disease                         | CatS  | CatK  | CatL  | Cystatin C |
|---------------------------------|-------|-------|-------|------------|
| Coronary artery disease         | Increase⁷⁸, ⁸⁹ | Increase ⁹⁰ | Increase ²² | Increase ⁸⁹, Decrease ⁴² |
| AAA                             | Increase ²², ³³ | –     | –     | Decrease ³³, ⁹², ⁹⁴ |
| Adiposity                       | Increase ⁹⁵, ⁹⁶ | –     | –     | –          |
| PAD                             | –     | –     | –     | Increase ⁷⁷ |
| Coronary artery calcification   | –     | Increase (in non-diabetic patients with CKD)⁹⁹ | –     | –          |

CatS indicates cathepsin S; CatK, cathepsin K; CatL, cathepsin L; AAA, abdominal aortic aneurysms; PAD, peripheral arterial disease; CKD, chronic kidney disease.

Completed Phase 1 single ascending dose studies⁵⁰, ⁵¹. Pharmacological interventions against cathepsins are under evaluation for other human diseases and may be used as clinical therapies for cardiovascular diseases in the near future⁵².

Conclusions and Perspective

Many data from basic and clinical studies of ASCVD highlight a role for cathepsins in these diseases, including atherosclerosis, aneurysm, and vasa vasorum and its complications. The pathogenesis of atherosclerosis and aneurysm formation involves substantial proteolysis of the arterial extracellular matrix⁷⁷. It has become clear that lysosomal cysteine proteases serve as regulatory enzymes beyond the traditional role as simple housekeeping proteases, and they possess important functions outside the lysosome, exerting potent collagenolytic and elastolytic activity⁷⁷. The increased cysteine protease content and decreased levels of the endogenous inhibitor CystC in atherosclerotic cardiovascular diseases such as atherosclerosis and abdominal aortic aneurysms suggest an imbalance that would favor matrix degradation in the arterial wall³³, ⁷⁷, ¹⁰⁴. Increased serum levels of CatL, CatK and CatS and decreased levels of their endogenous inhibitor CystC could be useful predictive biomarkers in patients with coronary artery disease and aneurysm.

Cathepsins have been targeted by pharmacological drugs and inhibitors in several animal experimental models of cardiovascular diseases such as atherosclerosis and AAA. However, no data are currently available on the effect of these inhibitors in human atherosclerotic cardiovascular disease. More research is required to investigate whether selective and reversible cathepsin inhibitors will be pharmacologically effective and physiologically safe in treating human atherosclerotic cardiovascular diseases¹⁰⁵.
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