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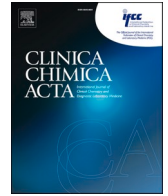
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Human cystatin C in fibrotic diseases

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ABSTRACT

Human cystatin C (hCC), which has a pervasive distribution within body fluids and is ubiquitously expressed by numerous cells and tissues, is a highly potent extracellular inhibitor of cysteine proteases. Besides measurement of serum creatinine, which is the most widely used technique for appraising glomerular filtration rate (GFR), hCC has emerged as a relevant GFR biomarker, because its quantification in serum is less sensitive to interferences with factors such as age, muscle mass or diet. Moreover, there are growing body of evidence that hCC over-expression and/or oversecretion, which is primarily driven by TGF- β 1, occur during fibrogenesis (cardiac, liver, oral, and lung fibrosis). Even though molecular mechanisms and signaling pathways governing the regulation of hCC remain to be deciphered more acutely, current data sustain that hCC expression relates to myofibrogenesis and that hCC could be a specific and valuable biomarker of fibrotic disease.

1. Introduction

The term "cystatin" was initially introduced in the early 1980s by Dr. Alan J. Barrett [1]. Cystatins, that belong to the MEROPS family I25 (clan IH), encompass numerous structural and functional similarities and are classified into three subfamilies: stefins (type 1), cystatins (type 2) and kininogens (type 3) [2,3]. Cystatins are powerful inhibitors of papain-related cysteine cathepsins, operating both intracellularly and in the extracellular environment [4,5]. Moreover, they share additional functions, participating in oncogenic pathways [6,7] and acting as potent immunomodulators [8,9,10]. Within the family, human type 2 cystatins (i.e., cystatins C, D, E/M, F, G, S, SA, and SN; subfamily I25B) are unglycosylated proteins (except cystatins E/M and F), which consist of about 120 amino acids (circa 13 kDa). Type 2 cystatins, which are synthesized with a signal peptide, are primarily secreted proteins and are found in all biological body fluids [11]. Cystatin C is the best-characterized inhibitor of this subfamily, whose regulation, physiological and pathophysiological roles have been the focus of intense investigation for over decades. It displays an ubiquitous and broad distribution with particularly high concentrations in cerebrospinal fluid

and seminal plasma [12]. Glomerular filtration rate (GFR) corresponds to the volume of fluid filtered per unit time from the renal glomerular capillaries into the Bowman capsule, which performs the first step in the filtration of blood to form urine. GFR is the pivotal parameter, in conjunction with albuminuria, in the assessment of kidney function and the measurement of extent of chronic kidney disease in patients. Of note, most of reports (several thousand clinical quotes in PubMed) have focused on the use of serum human cystatin C (hCC) as a relevant GFR biomarker, at least as suitable as serum creatinine and possibly more appropriate [13].

2. Cystatin C: General features

The name "cystatin C" was proposed for human γ -trace, also called post- γ -globulin, which was first identified in 1961 as a constituent of cerebrospinal fluid [1]. Both immunochemical and Northern blot studies have shown that hCC is distinctive among other type 2 cystatins since hCC and its mRNA are found in all nucleated cell types and human tissues. The molecular organization of its *CST3* gene (location: chromosome 20 at p.11.2) was deciphered in the late 1980 s [14,15]. Nucleotide

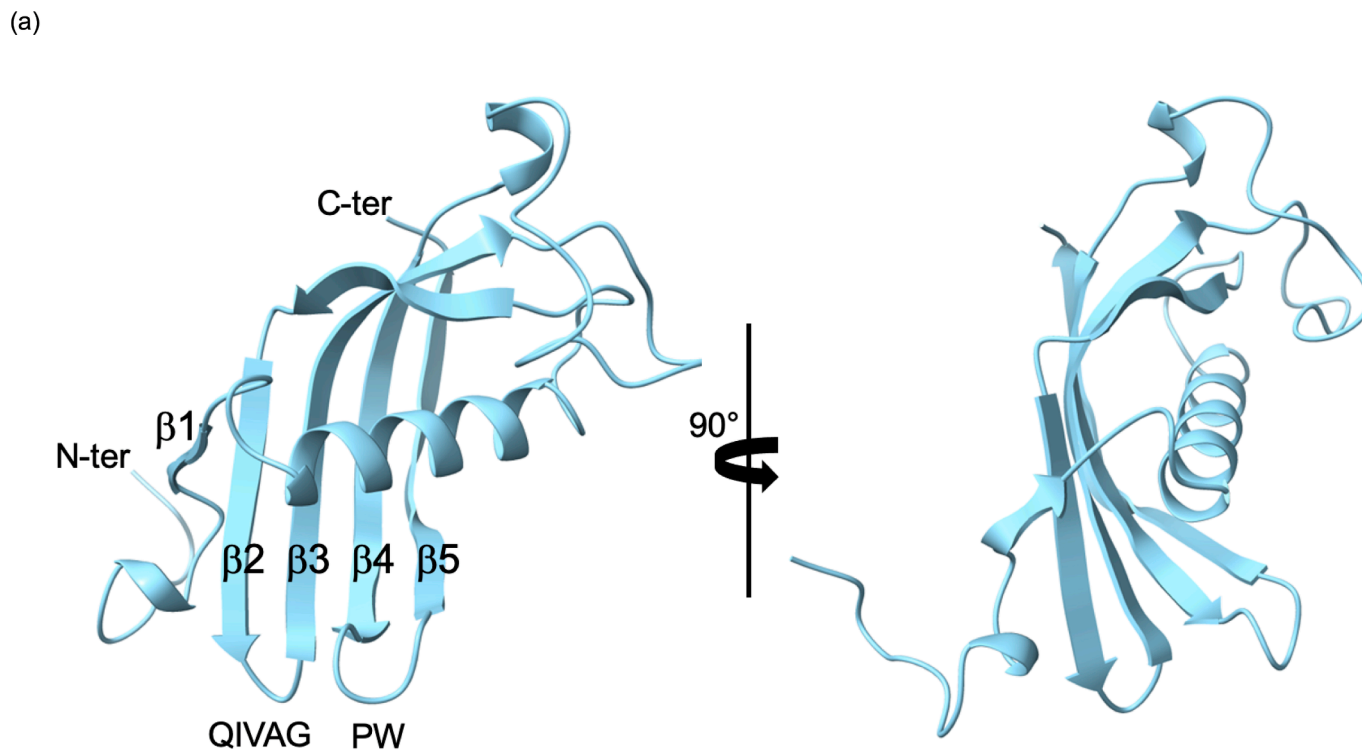
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sequence data are available – from the EMBL, GenBank, and DDBJ Nucleotide Sequence Databases – under the accession number X52255. The *CST3* promoter region has several of the characteristics of a housekeeping gene (i.e., no CAAT box, multiple GGGCGG regions, presence of an AT-rich sequence in the transcription regulation region). Nevertheless, there is a GATAAA site and there are also binding sites for transcription factors AP-2 and MEP-1. The *CST3* gene encodes the complete 120 residues mature hCC and a hydrophobic leader sequence of 26 amino acids, supporting its extracellular location following secretion (Fig. 1). The unglycosylated protein exhibits a molecular mass of ~ 13.3 kDa and possesses two disulfide bonds between residues 73 and 83 and between residues 97 and 117 [16]. Additionally, hCC may carry a hydroxylated proline residue at position three. Its basic isoelectric point (9.3) sustains that extracellular hCC is positively charged in body fluids. Following an initial three-dimensional study by 2D NMR [17], its crystal structure was finally solved in 2010 [18]. It exhibits the canonical features of chicken cystatin [19], including a long α -helix running across a large, five-stranded anti-parallel β -sheet, and contains the conserved QXVXG region in the central part of the molecule and the PW pair in the C-terminal part of cystatins.

Cystatin C is a highly potent, reversible, and competitive tight-binding inhibitor of cysteine cathepsins, including cathepsins B, K, L, and S, the first hairpin loop (with the conserved QXVXG pentapeptide) contributing circa 50 % of the total free energy of binding to its protease targets [5,20]. Cleavage of the Val10-Gly11 bond in the N-terminal substrate-like region of hCC by leucocyte elastase decreases inhibition of human cathepsins B and L by three orders of magnitude, supporting that elastase could play a regulatory function with respect to hCC at sites of inflammation [21]. Moreover, hCC is a powerful inhibitor of asparaginyl endopeptidase (AEP, a.k.a. legumain; clan CD, family C13) [22]. Recently, we have pinpointed a new way of regulating the biological activity of cystatins. A glycosaminoglycan, heparan sulfate (HS), was identified as a compelling modulator of the inhibitory activity of hCC. The tight binding of HS and HS-derived oligosaccharides to hCC is associated to a deep structural rearrangement, and weakening of its inhibitory potential may reflect dysregulation of the proteolytic activity of lung cathepsin L in patients with mucopolysaccharidosis [23]. NMR and site-directed mutagenesis studies carried out at the same period by another group reported convergent data, advocating that HS-hCC interactions are pH-dependent [24].



(b)

Leader sequence (residues: Met²⁶ to Gly¹):

Met Ala Gly Pro Leu Arg Ala Pro Leu Leu Leu Leu Ala Ile Leu Ala Val Ala Leu Ala
Val Ser Pro Ala Ala Gly

Mature hCC (residues: Ser¹ to Ala¹²⁰):

Ser Ser Pro Gly Lys Pro Pro Arg Leu Val Gly Gly Pro Met Asp Ala Ser Val Glu Glu
Glu Gly Val Arg Arg Ala Leu Asp Phe Ala Val Gly Glu Tyr Asn Lys Ala Ser Asn Asp
Met Tyr His Ser Arg Ala Leu Gln Val Val Arg Ala Arg Lys **Gln Ile Val Ala Gly** Val
Asn Tyr Phe Leu Asp Val Glu Leu Gly Arg Thr Thr **Cys** Thr Lys Thr Gln Pro Asn Leu
Asp Asn **Cys** Pro Phe His Asp Gln Pro His Leu Lys Arg Lys Ala Phe **Cys** Ser Phe Gln
Ile Tyr Ala Val **Pro Trp** Gln Gly Thr Met Thr Leu Ser Lys Ser Thr **Cys** Gln Asp Ala

Fig. 1. Structure of human cystatin C. (a) 3-D structure of human cystatin C (PDB: 3GAX). The conserved “QXVXG” region and the PW pair of human cystatin C are depicted. Molecular graphics were created with UCSF Chimera, developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco. (b) Primary structure. The sequence consists of the hydrophobic propeptide (26 amino acids) and the mature hCC (120 residues). The “QXVXG” region and the PW pair are depicted in blue (bold). Disulfide bonds (Cys73-Cys83 and Cys 97-Cys117) are highlighted in red (bold).

Cystatin C has a widespread distribution and is expressed by a broad variety of human cells and tissues [25]. For example, hCC was early identified as one of the most abundant secretion products of human alveolar macrophages [26]. It is also the most abundant and ubiquitous extracellular cysteine protease inhibitor found in biological fluids, especially in seminal plasma. Its half-life was estimated circa 20 min in blood plasma. The average value (mg/L) of hCC concentration for healthy individuals is, respectively: ~3.5 for milk (2.2–3.9), ~2 for saliva (0.36–4.8), ~1 for blood plasma (0.6–1.8), ~1 for amniotic fluid (0.8–1.4), ~0.1 for urine (0.03–0.29), ~6 for cerebrospinal fluid (3.2–12.5), ~2.5 for tears (1.3–7.4), and ~ 50 for seminal plasma (41–62) (range in parentheses, mg/L) [13]. Of note, it has been proposed that hCC disappearance is associated to a decrease of sperm viability. An abnormal expression level was also reported in the semen of patients with type 2 diabetes mellitus (T2DM), which could relate to a lower sperm motility [27]. Despite hCC is primarily a circulating secreted molecule, hCC could be internalized in significant amounts via a specific pathway by cells *in vitro*, especially by cancer cells [28,29]. Variations in expression and/or localization of cystatins are observed following diverse stimuli on immune cells, such as dendritic cells (DCs), which are present in most tissues and organs and act as potent antigen-presenting cells (APCs). Cystatins, including hCC, interfere with antigen processing and presentation, phagocytosis, expression of cytokines (e.g., interleukin 10 and tumor necrosis factor) and induction of nitric oxide, modifying the immune response and supporting that they are key molecules involved in immunity. For an outline of the roles of cystatins in the immune system, readers may refer to previous reviews [8,9,10].

3. Cystatin C in pathophysiological conditions

3.1. Kidney

Serum hCC, which was clinically thought as a better GFR biomarker than complement factor D, retinol binding protein, and β_2 -microglobulin, is considered as relevant as serum creatinine (Fig. 2). Benefits and limitations of validated and emerging GFR biomarkers as reliable tools for early diagnosis and prognosis were summarized and discussed in a recent and comprehensive review [30]. Moreover, readers could consult profuse reports dedicated to the use of hCC as GFR marker (sometimes called “The Lund Model”), see for example [31]. Interestingly, hCC production does not depend on muscle mass, age, or sex compared to creatinine [32]. Nevertheless, quantification of serum creatinine is a simple, inexpensive, and robust assay which is widely available; conversely, hCC remains less used, mainly due to the higher cost of laboratory analysis in the clinical routine. Since pioneering methods aiming to determine hCC concentration and besides conventional procedures (e.g., enzyme linked immunosorbent assay, chemiluminescence immunoassay, or particle-enhanced turbidimetric immunoassay), innovative methodological approaches are continuously developed, including time-resolved fluorometry-based immunoassays [33], electrochemical nano sensing based on surface-enhanced Raman spectroscopy [34], label-free electrochemical immunosensor based on a ferrocene functionalized-graphene platform [35], aptamer biosensor [36], magnetic solid phase chemiluminescent immunoassay [37], or LC-MS/MS procedure [38].

Measurements of increased concentrations of serum hCC appears to be suitable for evaluating the differentiation and early diagnosis of acute kidney injury (AKI), while hCC monitoring is less pertinent for

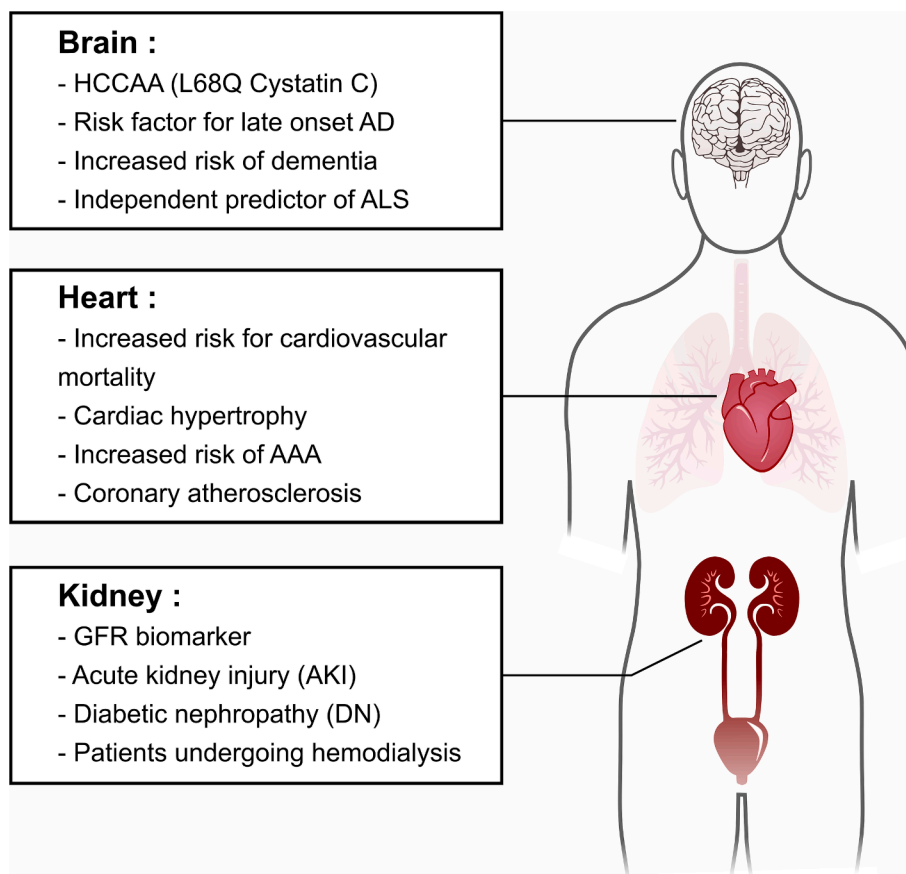


Fig. 2. Human cystatin C in pathological conditions (brain, heart, kidney). AAA, abdominal aortic aneurysm; AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; GFR, glomerular filtration rate; HCCAA, hereditary cystatin C amyloid angiopathy. Variations of hCC expression levels are associated to heart and kidney diseases. A punctual mutation (Leu68Gln cystatin C) is related to HCCAA.

assessment of AKI severity, as well for predicting progress of the disease and mortality in AKI [39]. Recently, the potential of serum hCC for the risk prediction of hospital-acquired acute kidney injury (HA-AKI) in patients with acute exacerbation of chronic obstructive pulmonary disease was also reported [40]. Moreover, both hCC and urinary neutrophil gelatinase-associated lipocalin identify AKI or progression of AKI earlier than serum creatinine in patients with cirrhosis [41]. Of note, a machine learning (ML) model to predict post-transplant AKI stage 3, a common and severe complication in patients with heart transplantation, was developed. Besides creatinine or estimated glomerular filtration rate (eGFR), serum hCC was one of the top six features selected for the ML model. The effective predictive performance of the ML model was significantly higher compared with that of the Cleveland-clinical model, supporting that the ML model may be an helpful support for timely improve prognosis [42]. Nephropathy is a noteworthy cause of morbidity and mortality in patients with type 2 diabetes mellitus (T2DM), which is characterized by lasting albuminuria and progressive renal structural changes associated with GFR decline. Both serum hCC and creatinine were significantly higher in patients with diabetic nephropathy (DN) than in normoalbuminuric patients. The usefulness of hCC appraisal as both an early indicator, as already reported in young patients with type 1 diabetes mellitus, and predictor of the development of diabetic nephropathy was proposed [43].

Serum hCC measurement, which is a practical alternative to measure residual renal function for dialysis patients [44], could also represent a suitable marker for predicting clinical outcomes of patients undergoing both hemodialysis (HD) and peritoneal dialysis (PD) [32,44]. Urinary matrix metalloproteinase 7 (MMP-7), combined with hCC, was reported to provide valuable information on tubular injury in focal segmental glomerulosclerosis [45]. As recently summarized by Lees and colleagues, hCC improves sensitivity and specificity of chronic kidney disease diagnosis, improves detection of harmful acute and chronic changes in kidney function, enriches precision of treatment eligibility and safety, and subsequently may reduce healthcare dissimilarities [46].

3.2. Heart

Analysis of serum samples collected from a sizeable cohort (>4500 participants) demonstrated that the highest quintile of hCC (≥ 1.29 mg/L) was associated with an elevated risk of death from cardiovascular causes, myocardial infarction, or stroke, supporting that hCC is a strong predictor of the risk of cardiovascular events in elderly persons [46,47] (Table 1). Another study confirms that patients with hCC levels above 1 mg/L (fifth quintile) had a significantly increased risk for cardiovascular mortality [48]. Higher hCC levels were also associated with increased left ventricular (LV) mass and a hypertrophy phenotype, sustaining hCC could be useful to identify individuals with preclinical structural heart abnormalities [49]. Serum hCC, but not serum creatinine nor eGFR, was related to LV mass index in hypertensive patients and could be a marker for cardiac hypertrophy [50]. Analysis of the mechanisms underlying hCC-induced myocardial injury of acute myocardial infarction (AMI) supports that the Wnt/ β -catenin pathway serves as an upstream

Table 1
Cystatin C and cardiovascular diseases.

Study findings	References
– High serum hCC levels are associated with an amplified risk for cardiovascular mortality	[48]
– High serum hCC levels are associated with an elevated risk of myocardial infarction or stroke in elderly persons	[46,47]
– High serum hCC levels are associated with an increased left ventricular mass (possible marker for cardiac hypertrophy)	[49]
– Serum hCC level is correlated with a risk of abdominal aortic aneurysm	[52]
– An hCC shortage could favor atherosclerosis	[54,55]
– Coronary artery plaque burden and hCC level are associated	[56]

protective system, which may reduce the expression of cytoplasmic hCC. Accordingly, the impairment of hCC expression attenuates mitochondrial oxidative stress injury and mitigates myocardial hypoxia injury, supporting that hCC exerts deleterious effects on the hypoxic aging cardiomyocytes through the reactive oxygen species (ROS) mitochondrial signaling pathway [51]. A randomized population-based study showed that plasma hCC levels correlate negatively with aortic diameter and is associated to a risk of abdominal aortic aneurysm (AAA) [52]. A similar hCC reduction was observed in both atherosclerotic and aneurysmal aortic lesions, while elastolytic cysteine cathepsins (e.g., cathepsin S) are overexpressed at sites of arterial elastin damage [53]. Accordingly, it was proposed that hCC deficit promotes atherosclerosis by favoring lamina degradation [54,55]. A Mendelian randomization analysis has demonstrated the association between hCC level and coronary artery plaque burden and indicated that genetic predicted hCC amount is associated with an increased risk of coronary atherosclerosis [56]. Also, associations between hCC level and retinal blood flow in patients with primary hypertension [57] as well hCC and early cardiac impairment in T2DM patients without renal dysfunction were identified [58].

3.3. Brain

Amyloidosis comprises a heterogeneous group of disorders of distinctive etiology characterized by the relentless (mainly extracellular) deposition of fibrillar proteins. In a seminal article, a protein, which is comparable to human urinary gamma-trace basic protein (i.e., cystatin C) with a punctual Leu68Gln substitution, was identified as the major constituent of amyloid fibrils in patients suffering from hereditary cerebral hemorrhage with amyloidosis, a rare inherited disorder [59]. This disease, so called hereditary cystatin C amyloid angiopathy (HCCAA), occurs naturally in Iceland and has been traced to a northwest region, around Breidafjörður bay. It manifests in the form of intracerebral hemorrhages and strokes followed by dementia caused by amyloid deposition, leading to premature death (30–40 years of age) [60]. Mutated Leu68Gln hCC forms amyloid, predominantly in the vessel walls of brain arteries and arterioles, inducing thickening then rupture of the walls, resulting in brain hemorrhage [61]. Leu68Gln hCC variant was also found to a lesser extent in skin, lymph nodes, testis, spleen, salivary glands, and adrenal cortex. Furthermore, the highly amyloidogenic hCC variant displays a high potential for dimerization and self-association. The molecular mechanism leading to dimerization has been elucidated [62]. It occurs through three-dimensional propagated domain swapping, a mechanism for forming oligomeric proteins [63,64]. Using chicken cystatin as a structural model of the type 2 cystatin family, the domain-swapped dimer further oligomerizes to a tetramer, prior to fibrillization leading to the formation of amyloid deposits [65].

Human cystatin C is commonly considered as a risk factor for late onset Alzheimer's disease (AD), although seemingly contradictory results have been reported in several studies. Overexpression of murine cystatin C leads to an extensive drop in cerebral β -amyloidosis in an experimental AD model. It was proposed that murine cystatin C binds to amyloid- β peptide and inhibits fibril formation, sustaining that cystatin C has a direct effect on β -amyloidosis rather than a neuroprotective effect [66]. An independent study published simultaneously reached a similar conclusion: cystatin C may bind to soluble amyloid- β peptide and inhibits cerebral amyloid deposition in a transgenic model, reinforcing the hypothesis that murine cystatin C can have a protective role in AD pathogenesis [67]. Interestingly, an increased expression of hCC, which co-localized with amyloid- β , was detected within the core of plaques. On the other hand, Tau hyperphosphorylation, which causes neurofibrillary tangle formation, microtubule instability, and neurodegeneration, is probably a crucial process in the development of AD pathology. Under these circumstances, it was reported that murine cystatin C assists phosphorylation of Tau protein, thus promoting AD pathogenesis [68].

Despite mechanisms by which hCC may promote AD pathogenesis or have beneficial effects remain blurred, high hCC levels appear deleterious, while lower hCC concentrations may protect neurons against amyloid deposition and degeneration [69]. Cystatin C would also have a neuroprotective role by inducing neuronal autophagy via inhibition of mTOR (Mammalian Target of Rapamycin) pathway [70]. Besides A β 1–42 and A β 1–40, biochemical analyses of AD brain deposits and fluids have unraveled an A β peptide consisting of numerous additional N- and C-terminally truncated A β peptides of diverse toxicity [71]. In a recent study, the intermolecular interactions between hCC and different fibrils formed by such amyloid- β peptides (e.g., A β 1–42, A β 1–40, as well as less abundant A β -related species), which are co-deposited in the brain of patients with AD, were scrutinized to better understand their pathological aggregation into neurotoxic oligomers. Results demonstrated a dependency of the sequence length of A β variants on the ability of hCC to unpack β -stranded fibril aggregates. Of interest, hCC secreted by astrocytes displays a higher proficiency to vanish A β 3–28 fibrils than longer amyloid- β peptides, which may partly explain contradictory findings about the role of hCC towards amyloid deposit [72]. Another reason of such apparent conflicting findings is that its target protease, cathepsin B, might be anti-amyloidogenic, helping in amyloid- β clearance or, instead, might have a detrimental effect by promoting amyloid- β production [73]. Furthermore, as recently summarized by Hook and colleagues, genetic deletion of the murine *CTSB* gene results in noteworthy improvements in behavioral deficits, neuropathology, and/or biomarkers in traumatic brain injury, ischemia, inflammatory pain, opiate tolerance, AD, epilepsy, or aging [73].

Vascular cognitive impairment (VCI) is, along with AD, the most conventional cause of cognitive decline in elderly people, and commonly reflect the effects of cerebral microvascular disease. Performance on measures of global cognition, executive function, and visuospatial ability declined at higher tertiles (corresponding to lower kidney function) of hCC (cohort: mean age was 93.9 years; 61 % were women). Elevated hCC level was significantly associated with a lower gray matter volume and an increased risk of dementia, probably associated to the presence of cerebral microbleeds. Taken as a whole, cystatin C is associated with cognitive performance and decline [74].

An extensive cohort of sporadic amyotrophic lateral sclerosis (ALS) patients was enrolled in a study to evaluate the prognosis interest of hCC. While the median survival time of all ALS patients was 42 months, patients with high hCC levels had significantly shorter median survival time than those with lower hCC levels (38 months vs. 48 months). Elevated hCC levels were associated with greater disease severity, faster progression rate and shorter ALS survival, supporting that serum hCC could be an independent predictor of ALS survival [75,76]. Also, hCC, which is strongly up-regulated in sporadic Creutzfeldt–Jakob disease (sCJD), has been proposed as a putative sCJD marker, according to a foremost differential proteomic analysis of cerebrospinal fluids (CSF) [77].

3.4. Cancer

Change in the expression of cystatins (e.g., hCC or cystatin M/E) may shape all stages of cancer progression including tumor growth, tumor invasion, metastasis, as well as angiogenesis and apoptosis [78]. Readers are invited to refer to an outstanding review discussing on both tumor-suppressing properties vs tumor-promoting effects of cystatins [6]. Indeed, cystatins are distinctly involved in regulatory mechanisms in cancer. They primarily unveil anti-tumor properties by inhibiting their target proteases. Instead, cystatins may modulate critical oncogenic pathways via alternative non-protease inhibitory functions. In a classic way, high tumor tissue levels of hCC are related to low tumor grade and favorable clinical outcome. More specifically, hCC encompasses an antitumor function on a few cancer types (i.e., pancreatic cancer, leukemia), which is generally related to its potent inhibition of cathepsin B, acting as a suppressor of cell migration, thereby inhibiting tumor

invasion and metastasis [7]. Accordingly, its proteolytic inactivation by secreted aspartyl cathepsin D enhances extracellular proteolytic activity of cathepsins of breast cancer cells and favor tumor progression [79]. Conversely, cystatin C shortage may suppress tumor growth in a mouse model of breast cancer induced by expression of the polyoma middle T oncoprotein (PyMT) in the mammary epithelium [80]. An *in vitro* study proposed that hCC reduces cell proliferation of leukemic cells and enhances apoptosis induced by oxidative stress [81]. An additional mechanism may correspond to the decreased growth of melanoma cells by the increase of cell cycle time [82]. Several findings revealed that elevated hCC levels correlate with a poor prognosis (i.e., colon, ovary, and esophageal cancer). However, the mechanism by which hCC could favor tumor progression remains unclear and may involve signaling cascades leading to cancer progression [6]. It has been proposed to use hCC as a marker for cancer prognosis. But renal function being often altered in cancer, this could negatively impact its usefulness as a valuable biomarker for cancer prognosis. Of note, a salient article indicates that hCC directs recruitment of TREM (Triggering receptor expressed on myeloid cells) 2⁺ macrophages and may predict failure of cancer immunotherapy [83].

3.5. Cystatin C and pathogens (bacteria, virus)

Following a preliminary report claiming that chicken cystatin may partly prevent poliovirus replication [84], it was demonstrated that recombinant hCC displays stronger inhibitory effects than acyclovir on herpes simplex virus type 1 (HSV) replication, but not on poliovirus replication [85]. Cystatin C exhibits also a potent inhibitory effect on human coronaviruses and rotavirus [86,87]. Interestingly, N-benzyloxycarbonyl-leucyl-valyl-glycine diazomethyl ketone (Z-LVG-CHN₂), an activity-based probe mimicking the N-terminal substrate-like of cystatin C, demonstrated a potent activity against many bacterial strains. Z-LVG-CHN₂ blocks the growth of all strains of group A streptococci, comparing with antibiotics such as tetracycline and bacitracin [88]. Diverse peptidyl or pseudopeptidyl derivatives of the pivotal structure (e.g., N-benzyloxycarbonyl-leucyl-valyl-glycine diazomethyl ketone) were further designed. Among these molecules, Cystapep1 (e.g., (2S)-2-(N α -benzyloxycarbonyl-arginyl-leucylamido-1-[(E)-cinnamoylamido]-3-methylbutane) displays a potent antibacterial activity against clinically relevant gram-positive bacteria (*Staphylococcus aureus* and *Streptococcus pyogenes*). Cystapep1 also showed prominent potency against methicillin-resistant *S. aureus* (MRSA) and multiantibiotic-resistant coagulase-negative staphylococci (CNS) but has no inhibitory potential towards gram-negative bacteria [89]. Besides its antipathogenic properties, hCC acts as an unexpected facultative cofactor for IdeS of the human pathogen *S. pyogenes*. IdeS activity does not depend on hCC, but is extensively enhanced in the presence of hCC, supporting that the host protease inhibitor is hijacked by the bacterial IdeS to increase its proteolytic activity [90]. In a recent study, it was demonstrated that cystatin C-deficient mice were significantly more sensitive to a lethal LPS-induced sepsis than wild-type mice. This exacerbated inflammatory response to sepsis is not related to a dysregulation of the proteolytic balance associated with cysteine cathepsins but depends on an increased caspase-11 expression and impaired autophagy [91].

3.6. Miscellaneous

While treatment of monocytic cells by lipopolysaccharide (LPS) induces a drop in the expression level of hCC, its production is raised by corticoids (e.g., dexamethasone) [92]. Also, thyroid dysfunction may modulate hCC production and serum hCC concentration [13]. The clinical use of hCC levels remains controversial and a careful clinical interpretation must be done for patients suffering from thyroid pathologies, especially in hypothyroid patients under hormone therapy by 3,5,3',5'-tetraiodo-L-thyronine (a.k.a., levothyroxine) [93]. Graves' disease (GD), which is an organ-specific autoimmune disease primarily

associated to an overstimulation of the thyroid gland, is a common cause for hyperthyroidism. Besides hyperthyroidism and diffuse goiter, Graves' ophthalmopathy (GO) is diagnosed in approximately 25 % to 50 % of patients with GD caused by dysfunctions of lymphocytes, autoantibodies, and cytokines. Hyperthyroid patients with GO exhibit upper hCC concentration than euthyroid patients with GO [94].

Both expression and secretion of hCC as well its intracellular distribution in the submandibular glands could be modulated by a treatment with isoproterenol, a β -adrenergic agonist. Conversely, these effects are efficiently abolished by propranolol, a β -adrenergic antagonist [95].

4. Fibrotic disorders

4.1. Some etiologic characteristics of fibrosis

Fibrosis, which predominantly results of chronic inflammation and repeated injuries, is defined as an uncontrolled wound healing in which connective tissues replace normal parenchyma, piloting an extensive tissue remodeling and a prevalent accumulation of constituents of the extracellular matrix (ECM) and basement membrane (BM) (e.g., collagens, elastin, fibronectin). This buildup which is primarily due to fibroblastic cells conducts to the establishment of a permanent fibrotic scar. The damaged epithelium supplies growth factors and cytokines that promote chemotaxis, stimulate the differentiation of fibroblasts into myofibroblasts and further deposit of ECM components. Major cytokines are transforming growth factor-beta1 (TGF- β 1), a powerful and the best described profibrotic effector, platelet-derived growth factor (PDGF) and tumor necrosis factor-alpha (TNF- α), which induce the proliferation, differentiation, and secretion of ECM proteins [96,97]. TGF- β 1 that is secreted by macrophages, epithelial cells, and fibroblasts, participates in cellular processes such as motility, differentiation, proliferation, or apoptosis, and disturbance of TGF- β 1 signaling pathways has been linked to diverse disorders including cancer, autoimmune diseases, and cardiovascular diseases. Also, TGF- β 1, which is overexpressed in patients with fibrosis and is a momentous mediator of differentiation, acts via both autocrine and paracrine mechanisms [98]. TGF- β 1 binds to TGF- β 1 receptor II, a membrane serine/threonine kinase receptor, which in turn forms a heterodimer with TGF- β 1 receptor I and elicits its phosphorylation. In addition to the canonical Smad pathway, non-canonical TGF- β 1-related signaling pathways may participate in the fibrotic process: mitogen-activated protein kinase (p38/MAPK), extracellular signal-regulated kinase (ERK), and stress-associated protein kinase/c-jun amino terminal kinase (SAPK/JNK) [98,99]. Likewise, overexpression of TNF- α triggers fibrosis in a murine experimental model, while TNF- α knock-out mice demonstrate an increased resistance to bleomycin-induced fibrosis [100]. Similarly, inhibition of PDGF by 5-methyl-1-phenylpyridin-2-one (i.e. pirfenidone) mitigates the development of fibrosis in an experimental rodent model [101]. A vasoactive peptide, endothelin-1 was also reported as a potent profibrotic molecule [102]. During fibrogenesis, injured and inflammatory cells release additional cytokines and growth factors: insulin growth factor-1 (IGF-1), chemokines (CCL18, CCL2 and CCL12) as well various interleukins (IL-4, IL-13, IL-1 β , IL-17A) [103].

4.2. Cysteine cathepsins and fibrotic disorders

As mentioned earlier, hCC is a potent endogenous inhibitor of cysteine cathepsins. These proteases are primarily involved in the endocytic pathway by cleaving endocytosed proteins within lysosomal compartments but participate also in dedicated biological processes such as histone proteolysis during embryonic stem cell differentiation, apoptosis, processing and maturation of some neurotransmitters and hormones, antigen presentation. Moreover, cysteine cathepsins are implied in various pathophysiological events (e.g., angiogenesis, adiposity, atherosclerosis, tumor invasion, pulmonary hypertension, inflammation, musculoskeletal and bone diseases) [104,105,106,107].

Cysteine cathepsins have been identified as key players in the recycling and remodeling of BM and ECM components [108]. Accordingly, dysregulation of the proteolytic balance between cystatins (i.e., including hCC) and cysteine cathepsins has emerged as a central issue in fibrotic mechanisms. It may be noticed that cathepsin B, which is overexpressed during activation of hepatic stellate cells (HSCs) drives HSCs *trans*-differentiation and participates in fibrogenesis, supporting that targeting cathepsin B is relevant in the treatment of liver fibrosis [109,110]. Accordingly, genetic impairment of cathepsin B in a bile duct ligation model reduced collagen deposition and fibrogenesis [111]. Other members of the family (cathepsins K, F, L, S, and V) were also identified as putative players of liver cirrhosis as well liver, cardiac, and skin fibrotic disorders and their respective roles have been recently discussed [112,113,114,115,116].

4.3. Cysteine cathepsins and lung fibrosis

Interstitial lung diseases (ILDs) form a heterogeneous group of 150 rare diseases characterized by inflammation and/or fibrosis of the pulmonary interstitium with various causes, including exposure to organic dusts, occupational exposures, pollutants, ionizing radiations, smoking, or autoimmune diseases (e.g., scleroderma, rheumatoid arthritis) [117]. ILDs may evolve into a progressive pulmonary fibrosis phenotype (PPF), characterized by the irreversible worsening of scar tissue and fibrotic areas, and associated with high mortality and morbidity. PPFs are estimated to account for 30 % of ILDs, but this proportion increases to 95 % for idiopathic pulmonary fibrosis (IPF). Thus, IPF is the most severe and common form of ILD, with an average survival of 3–5 years after diagnosis. The widespread proliferation, and differentiation of fibroblasts into α -smooth muscle actin (α -SMA)-expressing myofibroblasts and accumulation of ECM components induce an aberrant repair, the destruction of alveolar architecture and an irreversible decline in respiratory function [103].

Numerous studies were conducted on a murine model of bleomycin-induced fibrosis, leading to the activation of type II pneumocytes and apoptosis of type I pneumocytes, besides the rise of cytokines and growth factors and deposit of ECM components. The dysregulated ECM remodeling/turnover and its subsequent contribution to pathogenesis is closely associated with a compromised proteases/antiproteases balance [118,119,120]. Non-invasive imaging by a near-infrared fluorescent optical probe revealed a specific labeling of cysteine cathepsins at sites of fibrotic lesions, which correlated with the extent of disease burden [121]. Bleomycin-treated cathepsin K-deficient mice are more sensitive to fibrosis than wild-type mice, and display a thicker epithelium and an increase in the expression of α -SMA, while primary cathepsin K-/- fibroblasts exhibit a lower collagenolytic activity in agreement with the pivotal role of cathepsin K in degradation of collagen and its protective role in pulmonary homeostasis [122,123]. Moreover, cathepsin K could proteolytically inactivate TGF- β 1, thus restraining excessive ECM deposition [123]. Conversely, cathepsin B acts as a profibrotic signaling scissor by triggering TGF- β 1-driven canonical Smad pathway, and consequently contributes to lung myofibrogenesis [124]. Moreover, impairment of cathepsin B expression by genetic silencing or cathepsin B activity by a pharmacological inhibitor reduced α -SMA expression, delayed fibroblast differentiation, and led to an accumulation of intracellularly inactive pro-TGF- β 1. Subsidiarily, cathepsin B could also participate in the release of mature and active TGF- β 1, via the proteolytic activation of urokinase plasminogen activator [125].

5. Cystatin C and fibrotic disorders

5.1. Cardiac fibrosis

Interstitial fibrosis is a current part of the pathological cardiac remodeling process in cardiomyopathy. A rise of plasma hCC was observed. Moreover, hCC increase correlates with the inhibition of CatB

activity and accumulation of fibronectin and type I and III collagens [114,126] (Table 2). Interestingly, enlarged abdominal aortic diameters among patients relate inversely to serum hCC levels in both atherosclerotic and aneurysmal aortic lesions, highlighting a potentially important role for hCC in arterial wall remodeling [53]. Noteworthy, plasma hCC level is elevated in people living with HIV and is associated with myocardial stress and cardiac fibrosis and was also proposed as a possible biomarker of cardiac dysfunction and fibrosis. Nevertheless, the predictive value of hCC and its use as a risk marker especially for women should be further explored [127].

Aortic valve stenosis (AVS) is a sexually dimorphic cardiovascular disease that is directed by fibrosis and calcification of the aortic valve leaflets. Annexin A2 and hCC were identified as new inflammatory factors, which drive the activation of valvular interstitial cells (VICs) to myofibroblasts and were proposed as putative AVS biomarkers. Moreover, hCC correlates with both AVS severity and myofibroblast activation via p38 MAPK signaling [128].

5.2. Liver fibrosis

As extensively discussed in previous articles, progression of fibrosis is markedly linked to the hazard of evolution to advanced liver fibrosis and cirrhosis, sustaining that the monitoring of serum hCC level could be a useful device for the follow-up of patients to evaluate fibrosis progression [129]. Of interest, the measurement of creatinine level to estimate GFR is strongly challenged in cirrhotic patients. While some patients with liver cirrhosis display stable serum creatinine levels, serum concentration of hCC is closely related to the progression of the disease. Accordingly, hCC appears as the most accurate candidate for monitoring the renal failure in cirrhotic patients [130]. Also, hCC level was proposed as an independent predictor of poor survival in patients with liver cirrhosis [131], while creatinine/hCC ratio was proposed as an independent risk factor of sarcopenia in patients with cirrhosis complicated with primary hepatocellular carcinoma [132]. Despite hCC-based equations tend to underestimate GFR in patients with cirrhosis, incorporating hCC reduces differences between ethnic groups and improved accuracy of GFR in patients with cirrhosis [133]. Besides hCC and creatinine, the relevance of using neutrophil gelatinase-associated lipocalin to improve both the diagnosis and prognosis of decompensated cirrhosis was also considered [134]. On the other hand, elevated hCC concentrations are found in sera of patients and are closely correlated to the progression of hepatic fibrosis [129,135,136]. A similar increase of

cystatin C level was reported for profibrogenic rat hepatic stellate cells (HSCs). Of note, similarly to that observed for smooth muscle cells [53], TGF- β 1 triggers both production and secretion of cystatin C during HSC differentiation [137]. Consequently, extracellular hCC participates in the inhibition of collagenolytic activity of cysteine cathepsins. Likewise, decrease of collagen deposits following cystatin deletion sustains that hCC could act as a potent profibrogenic regulator by inhibiting cathepsins produced by activated HSCs during liver fibrogenesis [138].

Cystatins, including hCC, hold immunomodulatory properties by governing the activity of cysteine proteases, but also via mechanisms unrelated to its inhibitory role. It should be stated that in some specific circumstances, murine fibroblast cystatin C could be involved in a feedback loop that reduces TGF- β 1 signaling pathway by antagonizing the binding of TGF- β 1 to its receptor, thus lessening the phosphorylation of Smad2, as well p38 mitogen-activated protein kinase and extracellular signal-regulated kinase 1/2 [139]. Remarkably, significant variations of hCC expression are reported in autoimmune diseases [140]. Among them, autoimmune hepatitis (AIH) is a chronic liver disorder inducing lasting inflammatory destruction of liver parenchyma, cirrhosis and ultimately liver failure [141]. Notably, AIH is associated with the presence of autoantibodies directed against α -SMA, the archetypal marker of fibroblast differentiation. This brings up the question of assessing serum hCC levels for the clinical survey of patients with AIH that are potential candidates for a liver transplantation [142].

5.3. Oral fibrosis

Oral submucous fibrosis (OSF) is a sickness associated with betel (areca) nut chewing as well chronic exposure of buccal mucosa to pepper and chili. After a timely phase of stomatitis, an epithelial atrophy occurs, followed by an ongoing enlargement of collagen deposits in the submucosal tissues (lamina propria and deeper connective tissues). Protein expression level as well hCC transcription are significantly increased in OSF patients and correlated to the disease status [143].

5.4. Lung fibrosis

While hCC is not transcriptionally regulated during TGF- β 1-induced differentiation of CCD-19Lu fibroblasts, its secretion is significantly enhanced (~5-fold increase; $p < 0.001$), as observed in profibrogenic HSCs and human vascular smooth muscle cells. Consequently, secreted hCC impairs proteolytic activities of collagenolytic cysteine cathepsins, supporting that TGF- β 1 (via the canonical Smad pathway) drives hCC-dependent inhibition of extracellular cysteine cathepsins, and thus may promote lung fibrosis [124] (Fig. 3). Despite molecular mechanisms need to be better decoded both *in vivo* and *in vitro*, the proteolytic balance was similarly shifted in favor of cystatins in the alveolar lumen and bronchoalveolar lavage fluids (BALFs) in a rodent model of bleomycin-induced lung fibrosis [144]. Of note, curcumin, a potent antifibrotic drug used in traditional Asian medicine, impairs the expression of both α -SMA and mature TGF- β 1 and inhibits the differentiation of human lung fibroblasts. Consistently, the amount of both insoluble and soluble type I collagen decreased, reaching levels like those observed for undifferentiated fibroblasts. We untangled molecular mechanisms and signaling pathways involved in its antifibrotic properties. Curcumin inhibits hCC production at the transcriptional level. Also, curcumin triggers the expression of nuclear peroxisome proliferator-activated receptor γ (PPAR γ) and PPAR γ response element (PPRE)-like sequences were identified in the promoter regions of cathepsins B and L. We established that the transcriptional induction of both cathepsin B and cathepsin L depends on the binding of PPAR γ to PPRE sequences as a PPAR γ /Retinoid X Receptor- α heterodimer. Taken together upregulation of ECM-degrading cathepsins B and L and hCC downregulation, curcumin may participate in the recovery of the collagenolytic activity of secreted cathepsins [145]. A recent article confirmed the protective antifibrotic role of curcumin with respect to the progression of

Table 2
Cystatin C and fibrotic diseases.

	Study findings	References
Heart	– Increased expression in cardiomyocytes and plasma. Role in arterial wall and ECM remodeling.	[114]
	– Candidate marker of cardiac dysfunction and fibrosis.	[127]
	– Correlation with AVS (aortic valve stenosis) severity.	[128]
Liver	– Proposed as a more pertinent marker of liver cirrhosis than serum creatinine for estimating GFR.	[130]
	– Independent predictor of poor survival (cirrhosis).	[131]
	– Elevated concentrations in sera of fibrotic patients. Potential use as a biomarker.	[129,135,136]
	– TGF- β 1 induces cystatin C secretion during <i>trans</i> -differentiation of hepatic stellate cells.	[137]
Oral mucosa	– Strong immunostaining in fibroblasts, endothelial and inflammatory cells	[143]
Lung	– Increase of immunoreactive hCC in BALFs of IPF patients. Potential use as a biomarker.	[148]
	– Protective effect of curcumin, a potent antifibrotic molecule, is associated to hCC downregulation	[145,146,147]
	– Serum hCC, but not creatinine, as a possible CF marker.	[155]

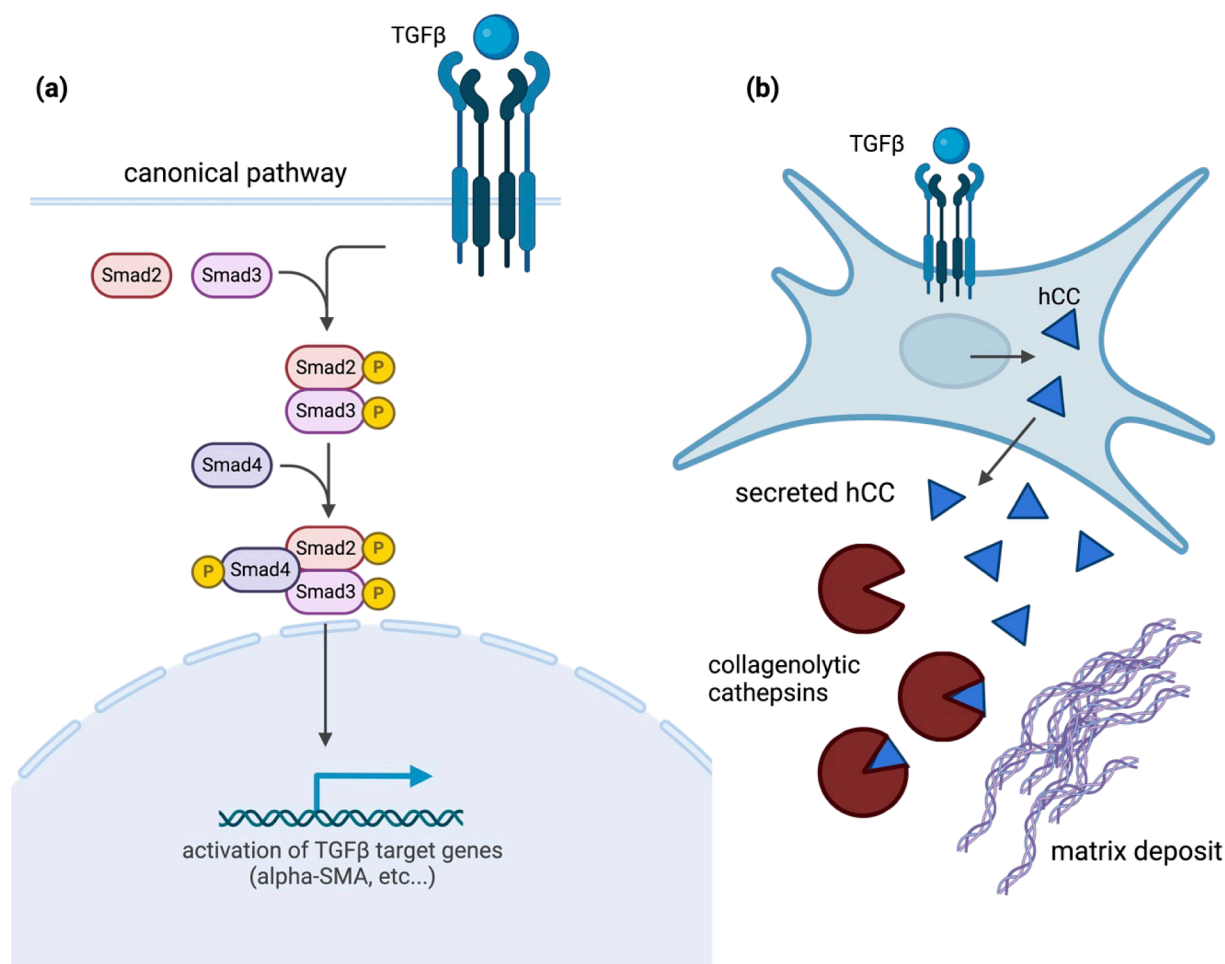


Fig. 3. TGF- β 1-driven secretion of human cystatin C. (a) TGF- β 1 binds to its specific receptors and triggers Smad 2/3 phosphorylation (TGF- β 1 canonical pathway) and myodifferentiation of fibroblasts. (2) In addition, TGF- β 1 upregulates secretion of cystatin C. Subsequently, hCC may inhibit collagenolytic cysteine cathepsins and thus promotes fibrogenesis (Figure prepared with BioRender).

pulmonary fibrosis [146]. Also, curcumin further reduces expression levels of inflammatory cytokines IL-1 β , IL-6 and TNF- α , as well hCC [147]. Additionally, a critically enhanced protein level of alveolar hCC was reported in BALFs of IPF patients with diverse severity grades (stages I, II and III) compared to non-IPF BALFs ($p < 0.001$) [148]. On the other hand, serum creatinine levels do not show notable variations, sustaining that the increased concentration of hCC in IPF BALFs do not rely on renal clearance and subsequent kidney damage. Taken together, data support that increase of BALF hCC relates directly to IPF pathogenesis and that hCC could be a specific and valuable biomarker of human lung fibrosis. Conversely, we demonstrated that murine cystatin C is not a reliable marker during experimental lung fibrosis induced by bleomycin challenge, pointing out that transposition of murine experimental results to human IPF should be done with caution [149]. Of note, despite supplementary studies are required for validation, the serum creatinine/hCC ratio was proposed as a substitute marker to detect sarcopenia in IPF patients [150]. Furthermore, association between aging and IPF being established, correlations between aging-related biomarkers and interstitial lung abnormalities have been recently evaluated. No aging-related biomarkers as well serum biomarkers, including hCC, were significantly associated with increased mortality, except GDF15 (growth differentiation factor 15), which could be a likely candidate to clarify alleged associations among age, interstitial lung abnormalities, and mortality [151]. However, the number of patients in the cohorts would need to be increased [152].

Cystic fibrosis (CF) is a rare genetic autosomal recessive disease,

which is associated with mutations of the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel gene. The disturbance of ion transport generates an accumulation of mucus in the airways and an increase of its viscosity. Periodic exacerbations of inflammation and infection by opportunistic pathogens (e.g., *Pseudomonas aeruginosa* and *Staphylococcus aureus*) lead to a decline in lung function, respiratory failure, and ultimately death [153]. Among the symptoms, collagen deposits significantly increase in alveolar tissues of CF patients as compared to age-matched, healthy individuals. Dysregulation of the proteolytic balance affects airway clearance and airway surface liquid homeostasis in CF. Using a proteomics approach, it was shown that hCC, participates in the pH-dependent coupling of the CFTR chloride channel and epithelial sodium channel (ENaC), amending airway hydration and mucociliary clearance of submucosal glands [154]. While eGFR level is decreased in CF patients compared to healthy patients, hCC concentration is significantly increased ($p < 0.01$ and $p < 0.001$, respectively). Conversely, no substantial difference in serum creatinine, serum urea and urinary neutrophil gelatinase-associated lipocalin (uNGAL) concentrations between CF patients with CF and controls were detected [155], sustaining that hCC could be a possible CF marker. On the other hand, concentrations of active cathepsins B and S, its proteolytic targets in the lung, are similar in *Pseudomonas aeruginosa*-positive and *P. aeruginosa*-negative samples of CF patients, indicating that cathepsins B and S are not appropriate markers of bacterial infection in cystic fibrosis [156].

In addition to IPF, current analyses are underway to determine

whether hCC could also be a broad-spectrum biomarker of interstitial lung diseases (ILDs), which display a progressive fibrosing phenotype characterized by an excessive and persistent differentiation of lung myofibroblasts. In the absence of effective ILD treatments, a better understanding of the dysregulation of the proteolytic homeostasis in favor of protease inhibitors is indeed an urgency to offer the possibility of exploring potential innovative therapeutic avenues.

6. Conclusion

Human cystatin C is now a well-established and relevant GFR marker, even if some complementary studies need to be conducted and refined, according to upper levels of serum hCC have been associated with some chronic diseases such as adiposity or hyperthyroidism, or during treatments with glucocorticoids [152]. In addition, the increased expression level of hCC, which is predominantly triggered by TGF- β 1 during myodifferentiation of fibroblasts, appears to be a general feature occurring during fibrogenesis (cardiac, liver, oral and lung fibrosis). Rising experimental evidence support that hCC could be an effective biomarker of cardiac, liver, or lung fibrosis, even if more systematic studies on cohorts with a significant number of patients still need to be achieved to validate hCC as a specific and unambiguous biomarker of fibrosis. Also, regulatory mechanisms driven hCC upregulation during fibrotic diseases as well its pathophysiological consequences on matrix deposits remain to be meticulously scrutinized.

Different interventional strategies are currently being considered. A promising research direction is the identification and characterization of new natural small molecules with antifibrotic properties (e.g., bufalin or lycorine) based on high-throughput natural compound library screenings to develop novel therapeutics [157]. Flavonoids, polyphenol compounds, and terpenoids, which target TGF- β 1 pathway are also under investigations to restore proteolytic balance during fibrogenesis [158], as previously suggested for curcumin [145]. Alternatively, drug repositioning is an increasingly attractive option. According to PPAR- γ triggers both transcription and translation of collagenolytic cysteine cathepsins, PPAR activators are currently under investigation for evaluation of their antifibrotic properties [159].

7. Authorship contribution statement

G.L. wrote the article; G.L., B.R. and F.L. prepared the tables and the figures; All authors participated in bibliographic research; B.R., A.D., M. T-J., F.L., S.M-A. and A.S. edited the article. All authors have read and approved the final manuscript.

CRedit authorship contribution statement

Gilles Lalmanach: Writing – original draft, Supervision, Conceptualization. **Baptiste Rigoux:** Writing – review & editing. **Alexis David:** Writing – review & editing. **Mounia Tahri-Joutey:** Writing – review & editing. **Fabien Lecaille:** Writing – review & editing. **Sylvain Marchand-Adam:** Writing – review & editing. **Ahlame Saidi:** Writing – review & editing.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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Data availability

No data was used for the research described in the article.

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