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**Molecular and tissue alterations of collagens in fibrosis****Sylvie Ricard-Blum<sup>1\*</sup>, Georges Baffet<sup>2</sup> and Nathalie Th  ret<sup>2</sup>**

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**ABBREVIATIONS**

ADAM: A disintegrin and metalloproteinase; ADAMTS: A disintegrin and metalloproteinase with thrombospondin motifs; Akt: RAC-alpha serine/threonine-protein kinase; AP1: transcription factor AP-1; BMP: Bone morphogenetic protein; DNMT: DNA methyl-transferase; ECM: Extracellular matrix; ERK: Extracellular signal-regulated protein kinase; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HDAC: histone deacetylase activity; HIV: Human immunodeficiency virus; HSC: hepatic stellate cells; IFN: interferon; IL: interleukin; LARP6: La ribonucleoprotein domain family member 6 or La-related protein 6; LOX: lysyl oxidase; LTBP: Latent TGF- $\beta$  binding protein; MMP: Matrix metalloproteinase; NF $\kappa$ B: Nuclear factor NF-kappa-B; PDGF: Platelet-derived growth factor; PIK3: phosphatidylinositol-3-kinase; SHG: Second Harmonic Generation; SMA: Smooth Muscle Actin; SP1: transcription factor Sp1; STRAP: serine-threonine kinase receptor-associated protein; TGF: Transforming growth factor; TIMP: tissue inhibitor of metalloprotease; TNF: Tumor necrosis factor; TPEF: two-photon excitation microscopy; TAZ also known as WWTR1: WW domain-containing transcription regulator protein 1; YAP (also known as YAP1): transcriptional coactivator YAP1

## ABSTRACT

The collagen network is altered in fibrotic diseases associated with extracellular matrix (ECM) biosynthesis and remodeling. This mini-review focuses on the quantitative and qualitative modifications of collagens occurring at the molecular and tissue levels in fibrosis. They result from changes in collagen expression, biosynthesis, enzymatic cross-linking and degradation by several protease families. These molecular modifications, which are mostly regulated by TGF- $\beta$ , are associated with altered collagen organization at the tissue level, leading to a fibrotic signature that can be analyzed by Second Harmonic Generation (SHG) microscopy.

## INTRODUCTION

Collagens comprise 30% of the total proteins of the organism, and are the major structural components of the extracellular matrix (ECM). They form ECM building blocks in association with other components [1], namely proteoglycans [2], elastic fibers [3–6], fibronectin [7], laminins [8,9] and matricellular proteins [10,11]. Collagen molecules are homo- or hetero-trimers made of one, two or three different polypeptide chains, called  $\alpha$  chains, and contain at least one triple-helical domain. The collagen family comprises twenty-eight members, numbered with Roman numerals according to the chronological order of their discoveries. It is divided into subfamilies based on common molecular and supramolecular features such as sequence motifs, domain organization, supramolecular assemblies and/or locations. Four collagens (XIII, XVII, XXIII, and XXV) are type II transmembrane proteins. The diversity of the collagen family also arises from the existence of several molecular isoforms for several collagen types (e.g collagens IV, V, VI, VIII and IX), of several networks for a single collagen type (e.g. collagen IV [12], and of splicing variants (e.g. collagens XII, XIII, XIV, and XVIII) [13,14]. In addition to their well-known structural roles in ECM assembly and organization and in providing tissues with mechanical properties, collagens regulate cell proliferation and migration, cell-matrix interactions, and cell signaling.

The collagen network is altered in many diseases involving ECM remodeling such as fibrosis [15,16]. Fibrosis is associated with an increase in collagen expression and deposition in the ECM, which can reach a several hundred-fold increase in the biosynthesis of collagen I [17]. The expression of fibril-forming collagen V, regulated by transforming growth factor- $\beta$  (TGF- $\beta$ ), is increased in fibrotic adipose tissue, lung, skin, kidney, and liver [18], and collagen VI, forming beaded-filaments, is overexpressed in lung and liver fibrosis [19,20]. Collagens found in basement membrane such as collagens IV [21], VIII and XVIII also contribute to fibrosis. Lack of collagen VIII reduces fibrosis and promotes cardiac dilatation in response to pressure overload in mice [22]. Collagen XVIII is up-regulated in idiopathic pulmonary fibrosis as are collagens III, VII, XIV,

XV, XVII, and XXVII [23]. Collagen I homotrimer was found in cirrhotic livers [24], in hypertrophic scar and inflamed gingiva where it represents 6% [25] and 1.5% [26] of total collagen respectively. Although it is present in low amount in fibrotic tissues, homotrimeric collagen I may play a crucial role in fibrotic disorders by interfering with ECM remodeling because it is resistant to all mammalian collagenases [27]. Some collagens play an indirect role in fibrosis as shown for collagen XIII, induced in vascular endothelium, which mediates  $\alpha 1\beta 1$  integrin-dependent transmigration of monocytes in renal fibrosis [28]. The dynamics of collagen synthesis in a fibrotic context has been investigated in bleomycin-induced lung fibrosis in which newly synthesized collagen was labeled with deuterated water to discriminate it from “old” collagen deposited in tissue before the onset of fibrosis. Neosynthesis of collagen correlates with expression of collagen V, elastin, tenascin C, the collagen cross-linking enzyme lysyl oxidase (LOX) and Wnt-1 inducible signaling pathway protein 1, which are upregulated by TGF- $\beta 1$  *in vitro*, and with cell proliferation [29].

This mini-review focuses on the quantitative and qualitative modifications of collagens, which occur at the molecular and tissue levels during fibrosis, and are mediated by changes in collagen expression, biosynthesis and degradation, and on the ECM reorganization characterized at the tissue level by a fibrotic collagen signature visualized by Second Harmonic Generation.

## 1. REGULATION OF COLLAGEN EXPRESSION IN FIBROSIS

Following the cloning of the first collagen genes in 1980's, numerous studies analyzed their promoter regions to identify regulatory elements driving collagen expression [30–32]. A main feature is the modular arrangement of regulatory domains that explains cell-specific expression. In addition, epigenetic events including methylation, histone modification and miRNA regulation appear as critical processes for controlling collagen expression during fibrosis leading to complex regulatory networks [17,33,34].

### 1.1 Transcriptional regulation

Among the cytokines implicated in the up-regulation of interstitial collagens TGF- $\beta$  is considered to be the major pro-fibrogenic inducer [35]. TGF- $\beta$ -mediated signaling depends on heteromeric complexes of two types of transmembrane serine/threonine kinases, type I (TGF $\beta$ RI) and type II (TGF- $\beta$ RII) receptors. TGF- $\beta$  binding to TGF- $\beta$ RII induces the recruitment and phosphorylation of TGF- $\beta$ RI, which transduces signals to downstream specific intracellular substrates, namely R-Smad proteins, Smad2 and Smad3. R-Smad proteins are phosphorylated and heterodimerize with a common partner, CoSmad Smad4. The heterodimeric complexes move into the nucleus where they regulate the transcription of TGF- $\beta$ -target genes either by binding directly to DNA or in association with other factors. Despite the fact that Smad2 and Smad3 have been generally described as equivalent downstream mediators, Smad3-dependent pathways mainly regulate the fibrotic process. The Smad-binding element (SBE), or CAGA box, has been identified in proximal promoters of most of collagens involved in fibrosis, and Smad3 binds to COL1A2, COL3A1, COL5A1, COL6A1 and COL6A3 promoters [36–38]. Of note, the lack of the classical Smad recognition element has been reported in the COL1A1 promoter where the ubiquitous zinc-finger family transcription factor SP1 and the complex Smad2-Smad4 bind to a CC(GG)-rich element to mediate the expression of the human  $\alpha$ 1(I) collagen chain induced by TGF- $\beta$  [39]. However, the deletion of Smad2 enhances collagen expression, and its overexpression decreases TGF- $\beta$ -induced collagen expression suggesting that Smad2 may prevent TGF- $\beta$ 1/Smad3-mediated collagen synthesis in renal and liver fibrosis [40,41]. Consistent with the differential role of Smad2 and Smad3, Smad2-deficient mice die early in development while Smad3-deficient mice have defects in immunity and are protected against fibrosis [42–44]). Other transcriptional factors synergize with Smads to activate TGF- $\beta$ -dependent gene expression. SP1, AP1 and the complex p300/CBP participate in positive regulation of COL1A2 expression [45–48]. SP1 is also involved

in the down-regulation of type I collagen through interaction with NF $\kappa$ B or Fli-1 [49–51]. NF $\kappa$ B signaling pathway contributes to fibrogenesis by promoting TGF- $\beta$ 1-induced activation of fibroblasts [52].

Alternatively, non-Smad pathways are activated by TGF- $\beta$  to modulate downstream cellular responses [53,54]. These non-Smad pathways include mitogen-activated protein kinase (MAPK) such as p38 and Jun N-terminal kinase (JNK), Rho-like GTPase, and phosphatidylinositol-3-kinase PI3K/Akt pathways. Hence, combinations of Smad and non-Smad pathways contribute to the high heterogeneity of cell responses to TGF- $\beta$ . Additionally, the kinases from these pathways are part of other cell signaling pathways activated by other microenvironment inputs such as cytokines and ECM components leading to complex cross-talks. The contribution of non-Smad signaling pathways to TGF- $\beta$ -induced collagen I expression has been widely documented and involves the PI3K-Akt [55,56], JNK and p38 MAPK pathways [57–60]. Similarly, MAPK signaling pathway regulates TGF- $\beta$ -induced collagen IV expression in mesangial cells [61].

Other pro-fibrogenic cytokines modulate collagen expression. Interleukin-4 (IL-4) and interleukin-13 (IL-13) play a critical role in chronic type 2 immune responses associated with fibrotic disease [62]. IL-4 or IL-13 induces collagen I expression in human fibroblasts [63–67]. In addition, IL-13 induces TGF- $\beta$  expression in macrophages, amplifying the fibrotic response [68]. While IL-4 and IL-13 share receptor complexes that induce a Stat6-dependent signaling pathway [69], IL-13 acts as a dominant effector of fibrosis as demonstrated in liver fibrosis due to *Schistosoma mansoni* infection [70–72]. The complexity of signaling cascades explains qualitative differential effects of IL-4 and IL-13 according to the biological context [73]. Furthermore IL-1 $\beta$  downregulates TGF- $\beta$ 1-induced myofibroblast formation and collagen synthesis in dermal and lung fibroblasts [74].

The pro-inflammatory cytokines interferon gamma (IFN- $\gamma$ ) and tumor necrosis alpha (TNF- $\alpha$ ) are the major anti-fibrotic agents that down-regulate collagen expression. IFN- $\gamma$  was first shown

to inhibit COL1A2 and COL1A1 expression through the binding of Y box-binding protein YB-1 to an IFN- $\gamma$  response element [75,76]. Binding of IFN- $\gamma$  to its receptors induces phosphorylation of JAK tyrosine kinase, leading to STAT1 phosphorylation. The IFN-STAT1-dependent signaling pathway antagonizes the TGF- $\beta$ -Smad-dependent signaling pathway for COL1A2 regulation by interfering at the level of p300/Smad3 transcriptional coactivators [77,78]. The antagonist cross-talks between IFN- $\gamma$  and TGF- $\beta$  signaling pathways is further supported by the up-regulation of Smad7 induced by IFN- $\gamma$  and YB-1 [79,80]. Similarly, TNF- $\alpha$  inhibits TGF- $\beta$ -induced collagen expression by interfering with co-activator complexes regulating TGF- $\beta$  target genes. TNF- $\alpha$  binding to its receptors leads to the repression of COL1A2 expression through two pathways including either Jun N-terminal kinase and AP1 or NF $\kappa$ B pathways [81]. Similarly, TNF- $\alpha$  and TGF- $\beta$ -responsive elements are co-localized in the promoter of COL1A1, and the down-regulation of COL1A1 expression by TNF- $\alpha$  involves transcriptional complexes containing p20C/EBP $\beta$ , p35C/EBP $\beta$ , and C/EBP $\delta$  [82]. p38 MAPK signaling pathway has been proposed to mediate the inhibitory effect of TNF- $\alpha$  on COL1A1 [59]. Other factors modulate collagen I expression such as lipid peroxidation products that act as positive regulators [83], and the transcription factor peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) that acts as a negative regulator [84]

The examples discussed above highlight the complexity of collagen regulation in fibrosis. Although TGF- $\beta$ -dependent signaling pathways orchestrate collagen expression and fibrogenesis, they cross-talk with other signaling pathways, which either synergize or antagonize their functions. Indeed signal integration occurs between the TGF- $\beta$ , Wnt, and YAP (also known as YAP1)/TAZ (also known as WWTR1) pathways in the development of fibrosis [85]. The understanding of the molecular mechanisms underlying the role of TGF- $\beta$  in the regulation of collagen expression thus requires a systemic approach integrating all the signaling pathways it is connected to. We have recently developed for this purpose a dynamic model of the TGF- $\beta$  network that encompasses more than 9000 biomolecules [86].



## 1.2. Epigenetic regulation

Epigenetic mechanisms play a key role in mammalian gene regulation [87]. The involvement of epigenetic regulation in the pathogenesis of fibrosis is supported by numerous studies and has been recently reviewed [34,88]. The major mechanisms include DNA methylation, histone modification and non-coding RNA expression that regulate the transcription of pro-fibrotic genes such as those encoding collagens.

### 1.2.1 DNA methylation

DNA methylation involves the transfer of a methyl group from S-adenosyl methionine to a cytosine in CpG island promoters found in more than 70% of eukaryote genes [89]. The DNA methyl-transferase (DNMT) family is responsible for DNA methylation of promoters leading to down-regulation of gene expression. Deregulation of DNA methylation is associated with fibrosis [90–92]. Genome-wide analysis of DNA methylation in liver shows hypomethylation of fibrogenic genes in a CCl<sub>4</sub> murine model of fibrosis [93], and during the activation process of hepatic stellate cells (HSC) [94]. DNA methylation of promoters also down-regulates COL1A1 and COL1A2 expression [95,96]. In contrast, the increase in collagen expression during fibrosis is associated with the epigenetic repression of either collagen suppressor genes such as the Proto-Oncogene Fli-1 [97,98], or negative regulators of fibroblast activation such as fra-2 through the methylation of H3 histone on the residue lysine 27 [99], or negative regulators of TGF- $\beta$  signaling such as Smad7 through inhibition of DNMT 1 [100] and p300 histone acetyltransferase [101]. The inhibitory effect of TGF- $\beta$  on global DNMT activity leads to DNA demethylation of COL1A1 promoter, thereby inducing its expression [102].

### 1.2.2 Histone post-translational modifications

Histones play a critical role in the regulation of chromatin structure and gene transcription [103]. By catalyzing the acetylation of histones and of the transcription factor Smad3, the co-activator p300 is required for TGF- $\beta$ -induced stimulation of collagen expression [104]. The histone methyltransferases ASH1 binds to fibrogenic gene promoters including collagen I and TGF- $\beta$  in activated liver hepatic stellate cells, and silencing ASH1 abolishes gene expression [105]. Conversely, many reports describe the beneficial effect of inhibitors of histone deacetylase activity (HDAC) in fibrotic diseases. The HDAC inhibitors, suberoylanilide hydroxamic acid and the pan-HDAC inhibitor, prevent TGF- $\beta$ -induced collagen I expression and deposition in human lung fibroblasts [106,107]. Suberoylanilidehydroxamic acid also decreases collagen III expression in bleomycin-induced pulmonary fibrosis [108]. Sodium valproate, an inhibitor of class I histone deacetylase, reduces expression of collagen I in diabetic renal injury and fibrosis [109], and diminishes collagen deposition in *Schistosoma mansoni*-induced liver fibrosis [110]. Tubastatin, a HDAC6 selective inhibitor, decreases TGF- $\beta$ -induced genes including collagen I in angiotensin II-induced renal fibrosis [111]. Physiological regulators such as the transcription factor KLF11 (Kruppel Like Factor 11) also repress COL1A1 expression through the recruitment of SIN3A/HDAC on its promoter [112].

### 1.2.3 MicroRNAs (miRNAs) and fibrosis

They are highly conserved non-coding RNA molecules that regulate gene expression post-transcriptionally [113] and play a role in fibrosis. A meta-analysis of literature reported thirty three miRNAs linked to fibrosis in lung, heart, liver, and kidney [114]. Among these miRNAs eighteen have anti-fibrotic effects, and sixteen have pro-fibrotic effects. The expression of collagen I is regulated by the miR-29 family, human COL1A1, COL1A2 and COL1A3 genes being direct miR-29 targets (data from mirTarBase <http://mirtarbase.mbc.nctu.edu.tw>, see [115]). TGF- $\beta$  decreases the expression of the miR-29 family members leading to up-regulation of collagen

production in renal tubular epithelial cells [116], SP1 being involved in this process [117]. Conversely, overexpression of miR-29b down-regulates collagens I and III expression in intestinal fibroblasts [118]. Other microRNAs regulate collagen biosynthesis. Indeed the down-regulation of microRNA let-7a, miR-196a and miR-150 is associated with collagen overexpression in dermal and keloid fibroblasts [119–121], while knockdown of miR-135a reduces collagen I synthesis in diabetic kidneys [122]. The role of miR-143 and miR-241 in promoting fibrosis through an increase in collagen synthesis has been reported in stromal fibroblasts of scirrhous gastric cancer [123] and in cardiac fibroblasts [124]. In the same way, increased miR-21 levels have been associated with fibrosis in numerous tissues including lung [125], liver [126,127] and kidney [128]. miR-21 targets Smad7, a negative regulator of TGF- $\beta$  signaling [129–131]. MMP-9 and TIMP-1 have been proposed as miR-21 targets [132].

### 1.3 Stabilization of collagen mRNAs

The stability of mRNAs plays a critical role in protein levels, and numerous molecular mechanisms including interactions with noncoding RNAs are involved in the control of mRNA decay [133]. The studies from Stefanovic's group contributed to highlight the importance of increased stability of collagen I mRNA in fibrosis [134] (for review). Up-regulation of collagen genes transcription is not sufficient to explain the increase in protein accumulation during fibrogenesis. Indeed, collagen mRNA half-life increases with activation of fibroblasts, TGF- $\beta$  treatment and modification of the microenvironment stiffness.

The stability of collagen mRNA is governed through the binding of proteins to specific sequences in the 3' or 5'-untranslated region of collagen I mRNA such as  $\alpha$ CP,  $\alpha$ 1-RBF67 (type I collagen  $\alpha$ 1 chain RNA-binding factor) and La ribonucleoprotein domain family member 6 (or La-related protein 6, LARP6).  $\alpha$ CP binds to the C-rich sequences located 3' to the stop codon and protects them from degradation [135]. Turnover of COL1A1 mRNA mediated by dexamethasone

in fibroblasts decreases binding of alpha 1-RBF67 to 3' untranslated region [136]. LARP6 is the only RNA-binding protein specifically involved in collagen I regulation [17]. It directly interacts with the stem-loop structure encompassing the start codon, and stabilizes mRNA through the recruitment of other proteins [137]. Indeed LARP6 recruits other proteins such as vimentin, serine-threonine kinase receptor-associated protein (STRAP), peptidyl-prolyl cis-trans isomerase FKBP3 (FKBP25) and RNA helicase A, which participate in collagen regulation [17]. Vimentin filaments associate with collagen mRNAs in a 5' stem-loop- and LARP6-dependent manner, and stabilize collagen mRNAs. They may thus play a role in fibrogenesis [138]. STRAP interacts with the C-terminal domain of LARP6 and prevents unrestricted translation of COL1A2 mRNA, thereby playing a key role in the coordinated translation of collagen mRNAs [139]. The interaction between LARP6 and STRAP is regulated by the mammalian target of rapamycin mTORC1, which phosphorylates serine residues 348 and 409 of LARP6, facilitating its release from endoplasmic reticulum [140]. The role of another RNA binding protein, HuR (Hu-antigen R or ELAV-like protein 1), has been reported in liver fibrosis [141,142]. HuR is a member of the embryonic lethal abnormal vision (ELAV)-like/Hu-protein family of RNA binding proteins, which regulate stability, splicing and translation of RNAs [143]. Its expression is increased during the activation of HSCs, and silencing of HuR reduces liver fibrosis after bile duct ligation in mice [142]. HuR-mediated HSC activation requires binding to sphingosine kinase 1 (SphK1) mRNA, a known regulator of TGF- $\beta$ 1-dependent expression of COL1A1 or  $\alpha$ -SMA [141]. More recently, the long non-coding RNA TSIX has been identified as a new regulator of collagen mRNA stability in scleroderma fibroblasts [144].

#### **1.4 Mechanical stress and collagen expression**

Mechanical properties of the extracellular matrix affect cell behavior through regulation of gene expression [145,146]. The switch from a soft towards a stiff extracellular matrix is at the heart of fibrosis progression, and mechanical stress acts as the main motor of the pathology [147].

Fibroblasts are able to sense matrix elasticity through cell-ECM and cell-cell adhesion sites. Such mechanosensory systems include integrins and adherens junctions, adaptor proteins and kinases that transduce signal to intracellular effectors [148]. Among the latter, YAP and TAZ are now considered as the major nuclear transducers of cell mechanics [149]. The growing importance of Hippo and YAP/TAZ pathways in fibrosis is supported by the role of YAP and TAZ in controlling hepatic stellate cell activation [150,151]. In accordance with a critical role of these regulators, YAP deficiency in Dupuytren myofibroblasts results in decreased expression of COL1A1 [152]. The crosstalk among the different cell mechanosensing systems has been recently enlightened by the finding that YAP plays a role in regulating focal adhesion assembly and cell mechanisms [153]. Furthermore, mechanosensing pathways involving the P21-activated kinase PAK-1 and YAP have been recently identified as core mediators of pro-fibrotic  $\beta$ 1-integrin signaling and collagen deposition [154]. Collagen expression is indirectly regulated by ECM stiffness through the extracellular activation of TGF- $\beta$ 1. TGF- $\beta$  is synthesized as an inactive, homodimeric, large precursor molecule that is intracellularly cleaved but remains non-covalently associated with its latency-associated protein (LAP). LAP-TGF- $\beta$  binds to latent TGF- $\beta$ -binding protein (LTBPs) to form the large latent complexes (LLC) that are sequestered within ECM and prevent TGF- $\beta$  to bind to its receptors. The activation process of TGF- $\beta$  requires the dissociation of TGF- $\beta$  from the ECM-bound LLC, and implicates integrin- and protease-dependent mechanisms [155,156]. Matrix elasticity controls TGF- $\beta$  activation, and the activation of latent TGF- $\beta$  by traction forces exerted by myofibroblasts depends on the stiffness of the matrix [157]. Once activated, TGF- $\beta$ 1 activates in turn different signaling pathways depending of the context [158].

## **2. CHANGES IN COLLAGEN POST-TRANSLATIONAL MODIFICATIONS**

Collagens undergo a series of post-translational modifications including hydroxylation and glycosylation of certain lysyl residues, which are involved in the formation of covalent

intermolecular cross-links stabilizing collagen supramolecular assemblies [159,160], and preventing its proteolytic degradation [161]. Lysine and hydroxylysine residues located in the N- and C-telopeptides of collagen molecules undergo oxidative deamination catalyzed by lysyl oxidases leading to the formation of reactive aldehydes that spontaneously condense with either aldehyde or amino groups to form covalent intra- and inter-molecular cross-links such as pyridinoline, an intermolecular cross-link [160,162].

### 2.1 Lysyl oxidase-mediated cross-linking in fibrosis

We have demonstrated for the first time the presence of increased formation of pyridinoline in fibrotic tissues by measuring its level in fibrotic livers from patients with alcoholic cirrhosis and with alveolar echinococcosis, a severe and irreversible fibrosis due to *Echinococcus multilocularis* infection [163]. Cross-linked collagen I is predominantly found in collagen bundles deposited in the periparasitic granuloma, and pyridinoline is the major cross-link involved in the stabilization of fibrotic collagen in irreversible fibrosis. Indeed the level of the cross-link pentosidine, formed during the glycation process, decreases in fibrotic livers from patients with echinococcosis [164]. We have also shown that transglutaminase-mediated cross-linking, leading to the formation of N<sup>ε</sup>( $\gamma$ -glutamyl)lysine cross-linking residue, occurs in fibrotic liver but the major substrate of the enzyme is the matricellular protein SPARC [165]. Furthermore transglutaminase-induced cross-linking forms during the early, inflammatory, stage of liver fibrosis, whereas cross-linking by pyridinoline occurs later in the fibrotic process [165]. Increased pyridinoline levels in fibrotic liver have also been found in a CCl<sub>4</sub> model of liver fibrosis [166], and in murine models of alveolar echinococcosis and schistosomiasis due to infection by *Schistosoma mansoni*, which leads to a milder fibrosis largely reversible following chemotherapy [167,168]. Comparative analysis of both models of parasitic liver fibrosis suggests that liver pyridinoline level is related to the severity of liver fibrosis. This relationship has also been observed in skin fibrosis. Indeed the level of

pyridinoline in the skin of patients with chromoblastomycosis, a fungal infection leading to an extensive and chronic dermal fibrosis, is higher than in the skin of patients with localized cutaneous leishmaniasis, an acute inflammatory process leading to an extensive and reversible remodeling of the ECM [169]. The type of collagen cross-link influences collagen degradation and determines the reversibility of experimental skin fibrosis [161].

Pyridinoline is excreted in urine upon degradation of mature collagen. In *Schistosoma mansoni*-infected patients pyridinoline is excreted in lower amount in the urine of patients with fibrosis than in infected patients without fibrosis [170]. The urinary level of pyridinoline is positively correlated to the collagen content of granulomas in *Schistosoma mansoni*-infected mice and negatively correlated to the length of the treatment follow-up in infected mice treated by an antiparasitic drug [171]. Furthermore urinary pyridinoline decreases upon treatment in a murine model of echinococcosis associated with severe liver fibrosis [172]. Urinary and skin levels of pyridinoline have been measured in one patient with skin alveolar echinococcosis [173], and in patients with chromoblastomycosis treated by a fungicidal drug [174]. Urinary pyridinoline may reflect changes occurring in fibrotic collagen during fibrogenesis and drug administration.

Pyridinoline is derived from hydroxylated lysine residues located within the collagen telopeptides *via* the hydroxyallysine pathway [162]. The hydroxylation of these residues is catalyzed by the telopeptide lysyl hydroxylase [175]. The mRNA level of this enzyme is increased in fibroblasts derived from fibrotic skin of patients with systemic sclerosis. The ECM deposited by these fibroblasts contains a higher amount of pyridinoline, suggesting a role of this enzyme in increased pyridinoline formation in fibrotic tissues [175]. The telopeptide lysyl hydroxylase plays an important role in fibrosis (reviewed in [159]). Increased formation of pyridinoline due to a specific increase in telopeptide lysyl hydroxylase or lysyl hydroxylase 2b (LH2b) level is indeed a general fibrotic phenomenon [176]. The association between increased pyridinoline level and

increased lysyl hydroxylase 2b expression has also been found in osteoarthritis-related fibrosis [177]. In contrast, a connective tissue disorder characterized by a loss of telopeptide lysine hydroxylation, results in reduced collagen pyridinoline cross-linking. This disease, the Bruck syndrome, is due to mutations in genes coding for LH2 or peptidyl-prolyl cis-trans isomerase FKBP65, which mediates the dimerization of LH2 [178]. The three isoforms of the pro-fibrogenic growth factor TGF- $\beta$ , interleukin-4, activin A, and TNF- $\alpha$  increase LH2b mRNA levels, and pyridinoline formation [179]. TGF- $\beta$ , but not Connective Tissue Growth Factor, induces LH2 expression in osteoarthritic human synovial fibroblasts *via* ALK5 (TGF- $\beta$  receptor type-1) Smad2/3P [180].

LOX and the isoforms of the LOX family [181] catalyze the oxidative deamination of specific lysyl and hydroxylysyl residues of collagens and elastin, which is the first step of cross-linking, and hence of pyridinoline formation [160]. LOX is involved in fibrosis [182], and some examples of LOX contribution to fibrotic processes are given below.

LOX activity increases in liver fibrosis induced by CCl<sub>4</sub> [183] in a higher proportion than prolyl and lysyl hydrolases [184] and in non-parenchymal cells from human fibrotic livers [185]. LOX contributes to collagen stabilization during liver fibrosis progression, and limits spontaneous fibrosis reversal in a CCl<sub>4</sub> murine model of liver fibrosis [186]. Hepatic stellate cells and portal fibroblasts are the major cellular sources of collagens, and lysyl oxidases in normal liver and early after injury in a model of liver fibrosis. LOX promotes fibrogenic activation of hepatic stellate cells [186], and LOX enzymatic activity increases during differentiation into myofibroblast [166]. In a bile-duct ligation model, LOX is expressed very early in portal connective tissue surrounding proliferating ductules, and precedes myofibroblastic differentiation [187]. Lysyl oxidase plays a role in myocardial fibrosis as shown in experimental models and in patients (reviewed in [188]). Inhibition of LOX by  $\beta$ -aminopropionitrile decreases myocardial fibrosis, and modulates the



TGF- $\beta$  pathway and collagen synthesis [189]. LOX also contributes to bone marrow fibrosis by regulating megakaryocyte expansion by platelet-derived growth factor-BB [190–192]. Furthermore LOX is critical in providing fibrotic tissue with an environment favoring the growth of metastatic tumor cells [193].

LOX expression is also markedly increased in bleomycin-induced lung fibrosis [193–195]. LOX and LOXL2, but not LOXL1, LOXL3 and LOXL4, are overexpressed in this model, where LOX expression correlates with fibroblast activation and secretion of collagen I and fibronectin [196]. LOXL1 and LOXL2 are increased in fibrotic lung of patients with idiopathic pulmonary fibrosis [197]. The inhibition of LOX by heparin, which selectively modulates the oxidative deamination of collagen lysyl residues by lysyl oxidase [198], may have anti-fibrotic potential in idiopathic pulmonary fibrosis [199]. LOX promotes lung fibrosis through modulating inflammation in bleomycin-induced fibrosis [194]. In this model, LOX inhibition at the inflammatory stage, but not at the fibrogenic stage, decreases collagen deposition and impairs inflammatory cell infiltration, TGF- $\beta$  signaling, and myofibroblast accumulation [194]. Inhibition of LOX activity reverses fibrosis and improves cardiac function in a rat model of volume overload with a possible role of oxidative stress in the beneficial effects of LOX inhibition [200]. Targeting lysyl oxidase with  $\beta$ -aminopropionitrile or LOX miRNA reduces peritoneal fibrosis and collagen deposition in mice treated intraperitoneally with multiwalled carbon nanotubes [201]. Targeting another member of the lysyl oxidase family, namely LOXL2, which cross-links collagen IV [202], suppresses liver fibrosis progression induced by thioacetamide, and speeds up its reversal [203]. LOXL2 is overexpressed in cardiac interstitium, and its levels correlate with collagen cross-linking and cardiac dysfunction [204]. It promotes cardiac interstitial fibrosis by stimulating cardiac fibroblasts through PI3K/AKT to produce TGF- $\beta$ 2, by inducing differentiation of fibroblast into myofibroblast, and stimulating myofibroblast migration [204].

Serum LOX level is increased in a bleomycin-model of lung fibrosis, where it induces fibrosis by upregulating interleukin-6 [195], and in patients with systemic sclerosis where it correlates with skin fibrosis [205]. LOX is thus a potential diagnostic marker of systemic sclerosis and may help to discriminate this disease from primary Raynaud's phenomenon [205,206]. The concentration of LOXL2 is increased in the serum of patients with heart failure, where it is correlated with that of TIMP1, a tissue fibrosis marker [204].

## **2.2 Peroxidasin, collagen IV cross-linking and fibrosis**

The network of collagen IV is covalently cross-linked by peroxidasin, a heme peroxidase found in basement membranes [207], which catalyzes the formation of sulfilimine bond between a methionine sulfur and a hydroxylysine nitrogen [208,209]. Peroxidasin is secreted by myofibroblasts in the ECM, where it co-localizes with fibronectin. Its expression is increased in a murine model of kidney fibrosis and localizes to the peritubular space in fibrotic kidneys [207]. Peroxidasin may thus play a role in fibrogenesis.

## **3. SECOND-HARMONIC GENERATION (SHG) MICROSCOPY TO CHARACTERIZE COLLAGEN DEPOSITS IN FIBROTIC TISSUES**

The role of stromal tissue microenvironment and extracellular matrix on tumor cells behaviour has been widely documented. Numerous studies have demonstrated that cell homeostasis depends on physical constraints of the microenvironment and on mechanochemical control of cell fate switching by ECM [210]. These forces generate mechanical strength that directly affects intracellular signaling pathways and the cell response to many exogenous factors. Indeed, the increase in ECM stiffness during wound healing fibrosis can influence physical interactions between cells and their environment. It is becoming clear that the tumor physical environment influences cancer development and cell motility linked to metastatic properties

[211,212]. Tumor stiffness tends to be associated with poor prognosis and local forces can amplify the proliferation/motility of cancer cells [213,214]. These mechanical forces influence the organization of tissues, where fibrosis could in turn greatly influences carcinogenesis. Qualitative remodeling of collagen fibrils could thus impact stiffness, cells behavior and intra-tissue metastasis. Several techniques such as confocal reflection microscopy, differential interference contrast (DIC), optical coherence tomography (OCT), magnetic resonance imaging (MRI), and second-harmonic generation have been developed in the last decades to investigate the ECM at high resolution in a non-destructive manner [215,216]. In this review, we will focus on SHG microscopy, a method of choice for collagen quantitative and qualitative analysis, which can be used in association with two-photon excitation microscopy (TPEF, also referred to as non-linear, multiphoton, or two-photon laser scanning microscopy) to image the fibrillar structure within the ECM and around cells *in vivo* and *in vitro* [217–220]. In breast cancer, invasion is facilitated by specific organization of collagen fibers microenvironment, and tumor-associated collagen signatures detected by SHG microscopy could provide collagen markers to locate and characterize breast tumors. Well-aligned fibers, analyzed by SHG microscopy, near groups of epithelial cells, enhance the efficiency of migration of breast cancer cells by increasing directional persistence and restricting protrusions along aligned fibers [221]. This collagen alignment could be an independent prognostic signature that correlates strongly with poor patient survival [222].

### **3.1 SHG-based collagen signatures, ECM reorganization and heterogeneity**

Two-photon excitation microscopy is an alternative to confocal and deconvolution microscopy that provides advantages for high deep three-dimensional imaging. TPEF and SHG enable observation of endogenous auto-fluorescent unstained samples and hyperpolarizable fibrillar proteins like collagens respectively. They can be used in tandem to provide spatially resolved 3-dimensional structure of cells and collagen matrix [223]. SHG microscopy is a label-

free, 3D-resolved laser scanning technique. One of the advantages of SHG microscopy is its high reliability and sensitivity that lead to evaluation of fibrosis without tissue staining (and fixation if necessary). Collagens have a highly crystalline triple-helical structure, are not centro-symmetric, and have first hyperpolarizability large enough for SHG microscopy, which is an invaluable tool for imaging tissue structure with submicron resolution [224]. The main advantage of SHG microscopy is its unique capability to provide 3D images of the organization of collagen fibers with micrometer resolution, its ability to distinguish immature fibrils from mature fibrils and its absence of bleaching [225]. The combination of SHG microscopy with TPEF allows the localization of collagen via SHG signals within a tissue/organ, the morphology of which is visualized by endogenous TPEF signals. Fibrosis assessment by SHG microscopy was first mentioned in liver [226] but it has been used for fibrosis imaging and scoring in lung, kidney and liver [225,227–230]. SHG provides sensitive resolution information on collagen distribution, discriminates between collagens I and III when compared to Sirius red staining, and a sensitive test for cirrhosis of the liver [226]. Imaging of SHG microscopy, fluorescence intensity, fluorescence spectroscopy and fluorescence lifetime imaging (FLIM) have been combined in a single instrument (5-dimensional multiphoton laser scanning microscopy) and used to discriminate collagen I, which generates a higher SHG intensity and a longer fluorescence lifetime, from collagen III in human skin [231]. SHG microscopy can also discriminate collagen I from collagen V in collagen gels [232], and collagen I and III from collagen IV in human fibrotic livers [227]. The lack of SHG signal from collagen IV results from the centrosymmetric arrangement of this non-fibrillar collagen [225]. A polarization-resolved SHG microscope has been used to distinct peaks corresponding to collagens I and III in normal human skin [233]. SHG and a phasor approach to fluorescence lifetime imaging allow to separate pixels having different fluorescence lifetimes. They have been used to analyze FLIM images, and to separate collagens I and III in mouse femur [234]. Recently, Campbell and Campagnola implemented wavelength-dependent SHG circular

dichroism to discriminate collagens I and III in collagen gels [235]. Elastic fibers can be clearly distinguished from collagenous structures by using SHG microscopy [236]. It is also possible to measure changes in the elastin/collagen ratio in tissues by simultaneously measuring the elastin autofluorescence and SHG signals as shown in tissues from patients with idiopathic pulmonary fibrosis, which are poorer in elastin relative to collagen. This is useful to improve the discrimination of patients with rapid and slow diseases progression [237]. SHG-based morphometry in unstained kidney tissues has been shown to be comparable to polarized super-resolution microscopy (SRM) for the quantification of fibrillar collagens, but has a higher sensitivity to detect treatment-induced changes in renal interstitial fibrosis [238]. The combined use of TPEF/SHG microscopy allows to visualize 3D remodeling of ECM induced by fibroblast contraction [228], and 3D structural remodeling of a collagen matrix during wound repair [239].

The imaging techniques and signature identified in breast tumor tissue may provide useful diagnostic tools to rapidly assess tissue biopsies. Associated with genomic methods, SHG microscopy may improve the prediction of metastases, and hence may help to reduce patient over-treatment [240]. SHG image segmentation algorithms show that elongated collagen fibers significantly correlate with poor clinical outcome [241]. Furthermore, texture analysis, based on first and second order statistics, helps to extract images features for collagen remodeling in skin disorders, fibrosis and muscular-skeletal diseases affecting ligaments and cartilage [242]. A gradient-based BoF (Bag-of-Features) strategy has been used to exploit TPEF and SHG images associated with fibrosis progression for diagnostic purpose [243]. The use of Bessel beam excitation for SHG imaging extends depth SHG-TPEF imaging of fibrotic mouse kidney in histological slides [244]. SHG-based signatures associated with texture parameters and TPEF microscopy might greatly improve the characterization of ECM architecture in tissues and the localization of areas providing cellular contact guidance cell and of metastatic potential.

### 3.2 Quantification of liver fibrosis by SHG microscopy

The scoring and qualification of the main component of the ECM, the fibrillar collagens, by SHG microscopy provide an accurate SHG score, and permit a better follow-up of liver fibrosis [227,230,245]. SHG thus emerges as an original and powerful tool in the assessment of liver fibrosis [227,246–249]. The SHG index, calculated from the surface density of binary distribution of the SHG signal above an optimal threshold, allows for the discrimination of fibrosis level assessed by the F-Metavir scores. It has been validated by us and others in human fibrotic livers against the classical Metavir and Elastography scores. SHG technique allows discriminating patients with moderate to severe fibrosis and cirrhosis, whereas TPEF microscopy images the auto-fluorescence of the parenchymal cells. Furthermore, a capsule index can be calculated, based on significant parameters from the SHG microscopy images of capsule collagen from the anterior surface of rat livers [250]. TPEF and SHG of unstained lung tissue allow the discrimination of inflammatory from fibrotic stages [251]. Multicolor two-photon fluorescence lifetime imaging (FLIM) permits simultaneous co-registration of endogenous fluorophores (NADH and FAD) a major source of endogenous auto-fluorescence, along with SHG imaging of collagen in living tissues [252]. Based on the strategy of combining pathology-relevant collagen architectural features with automated computer-aided image analysis tools, a method for liver biopsy assessment in animal models and in patients with chronic hepatitis B has been developed [249]. Automated biphasic morphological assessment of hepatitis B-related liver fibrosis can be applied using SHG microscopy [253]. More recently, Pirhonen *et al.* have reported that SHG imaging detects fibrillar collagen deposition more sensitively than routine histological techniques and enables quantification of early human fibrosis in nonalcoholic fatty liver disease [254]. SHG and TPEF microscopy quantitatively characterize the hepatic capsule and parenchymal morphologies respectively, associated with liver disease progression at the early stage of a fibrotic non-alcoholic steatohepatitis murine model [255].

In order to illustrate the high potential of the SHG microscopy, we imaged liver tissues from healthy mice. The excitation wavelength was set at 810 nm and ad'hoc filters were used to separately collect specific endogenous signals at 405 nm (SHG transmission mode) and the FAD and NAD(P)H autofluorescence (TPEF mode). At low magnification, TPEF signals show the typical autofluorescence of liver tissues. The SHG response visualized fibrillar components corresponding to collagens I and III, mainly located around and between the portal tracts (Figure 1A). Using SHG microscopy, the change in collagen deposition during the CCl<sub>4</sub> treatment can be visualized with a great sensitivity (Figure 1B). The accumulation of fibrillar collagen in portal triads and within the parenchymal tissue of CCl<sub>4</sub>-treated mice, quantified by the SHG index, was clearly observed 2 weeks after the beginning of CCl<sub>4</sub> administration and thereafter compared to control liver (oil-treated mice). As previously described fibrosis SHG index was defined as the pixels area (in percentage) corresponding to collagen-based SHG signal after image thresholding and excluding normal collagen of portals triads [227]. This SHG-scoring method enabled the follow-up of liver fibrosis gradation during CCl<sub>4</sub> treatment, peaking at week 6, (Figure 1C). This model of liver fibrosis is characterized by a mild and constant fibrosis with a fibrosis area that does not exceed 6-8 % of the total liver area. The precise quantification of moderate changes in liver collagen deposition occurring in this model is thus a major challenge using conventional histological methods but it can be achieved by SHG microscopy. SHG images at higher magnification enable high spatial resolution and reconstitution of the 3D fibrillar networks (Figure 1D).

### **3.3 Polarization-dependence of SHG microscopy**

One of the major challenges of SHG imaging in the study of fibrillar deposits is to correlate changes in image contrast with changes in the organization of the fibrillar network at a submicron scale. Computer-assisted interpretation and automated tracking algorithm of fiber-level collagen

patterns have the potential to generate more reliable and reproducible results. This method has been successfully applied to track collagen fiber shape changes over time in an *in vivo* murine model of breast cancer [256]. Change in polarization of the incident laser beam allows measurement of signal orientation in collagen at a sub-micron scale well below the optical resolution [248,257]. Pixel-by-pixel analyzes of polarized SHG images combined with *a priori* modeling of the 3D fibrils architecture could provide structural insight and relationship between SHG signature and collagen fibril 3D organization. This model takes into account the hierarchical structural organization of collagen molecules into fibrils and bundles of fibrils [258]. Qualitative analyses of the collagen network using polarized light permit to determine the fiber orientations at sub-micrometric level. SHG polarimetric data evidence the presence of birefringence from molecular and biological samples, demonstrating the importance of accounting for such effect to retrieve sample structural information [259]. Modulating the polarization angle of the laser during scanning allows visualization of the 3D structure of collagen tissues [260–263]. Measurements can be used as a reliable calibration of fibril diameters in biological tissues with a sensitivity threshold of 30 nm for fibril diameter [264]. Polarization-resolved SHG has the potential to characterize liver fibrosis based on quantitative analysis of collagen types, namely collagens I and III, and fibril orientation [265]. The method is sensitive enough to differentiate collagen I from collagens I/III gel mixtures, and the data are consistent with the fact that collagens I and III can comingle within the same fibrils [237]. The ratio of the tensor elements exhibited two distinct peaks that could estimate the collagen I ad III concentration in human skin [233]. Altogether, SHG microscopy is a major technique for quantifying and qualifying fibrous collagen *in vivo* and *in vitro*. This high resolution and quantitative microscopic approach, easy to handle, should be more often used to help pathologists in the diagnostic and follow-up of patients in association with routine histological techniques,



## 4. COLLAGEN REMODELING IN THE COURSE OF FIBROSIS

Fibrogenesis results from an imbalance between ECM biosynthesis and degradation, especially of fibrillar collagens that mainly involves matrix metalloproteases and serine proteases [266]. Collagenase activities increase in the early stage of fibrosis and decrease in the advanced stage. Other proteases involved in ECM remodeling include adamalysins, cathepsins and meprins.

### 4.1 Collagen-degrading enzymes

Remodeling regulates ECM composition, the release and bioavailability of bioactive fragments and of growth factors sequestered in the ECM. Metalloproteinases play a major role in ECM remodeling and their various functions has been recently reviewed [267].

#### 4.1.1 Matrix Metalloproteases

Classification of MMPs is commonly based on their domain organization and substrate preference, and they are divided into collagenases, stromelysins, matrilysins, gelatinases and membrane type-MMP. While the expression and activity of MMPs have been widely documented, an integrative view of their individual contribution to the fibrotic process is missing [268]. MMP1, MMP8, and MMP13 play a critical role in fibrosis because of their ability to cleave native collagens I, II and III. This cleavage takes place at the Gly-Ile bond of the  $\alpha 1(I)$  chains and Gly-Leu bond of the  $\alpha 2(I)$  chain, leading to the release of characteristic 3/4 N-terminal and 1/4 C-terminal fragments. The denatured collagens are degraded by the so-called gelatinases MMP2 and MMP9. However, MMP2 has been shown to digest also native interstitial collagen, as well as the membrane MMP14 [269]. While proteolytic activity is upregulated in early events of fibrosis, the effects of degradation may be counterbalanced by an increased expression of tissue inhibitors of metalloprotease inhibitors. Depending on their substrates, MMP activities lead to pro- or anti-

fibrotic effects, and determine whether the TIMPs act as inhibitors or activators of ECM proteolysis [270]

#### **4.1.2 Adamalysins.**

Members of the ADAM (A Disintegrin And Metalloprotease) family share a multi-domain organization that includes metalloprotease, disintegrin, cystein, transmembrane and cytoplasmic domains and have been implicated in highly diverse biological processes, such as spermatogenesis/fertilization, neurogenesis, inflammatory responses and cancer [271]. Among the 21 human ADAMs, only four have the ability to degrade the ECM, including ADAM9 [272–274], ADAM10 [275], ADAM12 [276] and ADAM15 ([277] and none affect directly interstitial collagens.

Unlike the mammalian ADAMs which are, with few exceptions (e.g. variant forms of ADAM-9, -12 and -28), membrane proteins, the ADAMTSs (A Disintegrin And Metalloprotease with ThromboSpondin motifs) and ADAMTSL (ADAMTS-like molecules that lack proteolytic activity) are secreted proteins, characterized by an ancillary domain containing one or more thrombospondin type 1 repeats [278]. ADAMTS activities have been associated with development, angiogenesis, cardiovascular diseases, cancer, and arthritis [279,280]. The implication of ADAMTS proteases in ECM proteolysis is mainly mediated by their catalytic activity on the proteoglycans versican and aggrecan but also on gelatin [281]. According to such activities, ADAMTS display anti-fibrotic functions. Consistent with the protective effect of ADAMTS, ADAMTS-5, -9, -15, and -20 expression increases during the resolution of liver fibrosis in parallel with a decrease in versican [282]. However, ADAMTS5 deficiency leads to accumulation of aggrecan, which prevents the activation of fibroblasts and reduces dermal repair responses suggesting it has pro-fibrotic effects [283].

Searching for further proteases involved in ECM remodeling occurring in liver fibrosis and cancer, we demonstrated that ADAM12 and ADAMTS1 are involved in ECM remodeling and fibrosis through the modulation of TGF- $\beta$  signaling. We first identified ADAM12 as a new actor in hepatic stellate cells [284,285]. We then demonstrated that ADAM12 binds to TGF- $\beta$  receptor II and promotes TGF- $\beta$  signaling and transcriptional activity by modulating TGF- $\beta$  receptor dynamics [286,287]. Deciphering molecular mechanisms involved in functional activity of ADAM12 led to the identification of the Protein kinase C receptor RACK1 [288], and Integrin Linked Kinase as new ADAM12 partners in hepatic stellate cells [289]. RACK1 allows the translocation of ADAM12 to the membrane in response to collagen-activated integrins, whereas ILK transduces ADAM12-dependent survival signal. Using global analysis of the degradome in patients with chronic liver diseases, we also identified ADAMTS1 as a new marker of hepatic stellate cell activation and fibrosis. Furthermore we demonstrated that ADAMTS1 interacts with and activates the latent form of TGF- $\beta$ , LAP-TGF- $\beta$ , regardless of its protease activity [290]. In agreement with the contribution of ADAMTS1 to a pro-fibrotic phenotype, silencing ADAMTS1 in myocytes inhibits ethanol-induced collagen expression [291]. By interfering with TGF- $\beta$  activity, both ADAM12 and ADAMTS1 regulate fibrosis and collagen expression in hepatic stellate cells through a retro-regulatory loop (**Figure 2**).

The bioavailability of TGF- $\beta$  depends on the storage of the large latent complex (LLC) associating LTBP proteins and LAP-TGF- $\beta$  within the extracellular matrix [292]. ADAMTSL2 interacts with LTBP1 [293] and numerous mutations in ADAMTS-TSL genes are associated with alteration of ECM remodeling in human genetic disorders [294,295].

ADAMTS-2, -3 and -14 are procollagen N-proteinases, which cleave the N-propeptide of fibrillar procollagens I-III and V [296], while the C-propeptide is mainly cleaved by C-proteinases of the tolloid family [297]. ADAMTS-2 inactivation attenuates liver fibrosis *via* a decrease in collagen deposition [298]. Further substrates of ADAMTS-2, -3, and -14, including ECM proteins and

TGF- $\beta$ -related proteins (latent TGF- $\beta$  binding protein 1 and TGF- $\beta$  RIII), have been identified, strengthening their regulatory role in ECM deposition and remodeling and in TGF- $\beta$  signaling [299]. Bone morphogenetic protein-1 (BMP-1)/tolloid-like proteinases, which act as procollagen C-proteinases, activate TGF- $\beta$ 1 and contribute to collagen deposition [297]. Furthermore two substrates of BMP-1, betaglycan and CD109 (150 kDa TGF-beta-1-binding protein) control the activity of TGF- $\beta$  co-receptors [300].

#### 4.1.3 Other proteases and mechanisms of collagen degradation

The role of cysteine cathepsins in ECM remodeling has been recently reviewed [301,302]. Cathepsins are implicated in the degradation of proteoglycans and fibrous proteins including collagen I [303]. The degradation of matrix components by cathepsins occurs either in the extracellular space or through endocytosis pathways towards lysosome. Cathepsin K contributes to ECM remodeling by degrading collagen fibers. It binds to collagen-bound glycosaminoglycans in the gap region [304] and sulfated glycosaminoglycans protect collagen fibrils from degradation by cathepsin K [305]. TGF- $\beta$  [306] and MMP-9 [307] are putative substrates of cathepsin K. Conflicting results have been reported regarding the role of cathepsin B in fibrosis. Its inactivation was found to attenuate hepatic fibrosis during cholestasis [308], whereas it was shown to promote the activation and proliferation of hepatic stellate cells as did cathepsin D [309]. Furthermore cathepsin B participates in the differentiation of lung fibroblasts by TGF- $\beta$ 1, which in turn up-regulates the secretion of cystatin C, an inhibitor of extracellular cathepsins [310].

Meprins, members of astacin family composed of alpha and beta subunits, are also able to degrade ECM, namely collagen IV, nidogen-1 and fibronectin but not collagen I [311]. In addition meprins  $\alpha$  and  $\beta$  are C- and N-procollagen proteinases and process procollagens I and III [312–314]. They are overexpressed under fibrotic conditions and their inhibition could decrease collagen deposition in fibrotic processes [312–314].

Plasmin, a serine proteinase activated by urokinase and tissue plasminogen activators, contributes to ECM remodeling. It acts as an anti-fibrotic agent both by targeting matrix components such as fibronectin and by activating proMMPs. Plasmin-mediated activation of MMPs is inhibited by the plasminogen activator inhibitor type-1, which has a pro-fibrotic activity. The inhibition of PAI-1 attenuates fibrosis, except in heart where it promotes myocardial fibrosis *via* enhanced enhanced multiple TGF- $\beta$  signaling elements [315].

Another mechanism of collagen degradation to take into account in fibrosis is mediated by the endocytic collagen receptor, uPARAP/Endo180. It is overexpressed in human cirrhotic liver and in a murine model of liver fibrosis, where it plays a protective role against liver fibrosis [316].

#### **4.2 Collagen matricryptins/matrikines in fibrosis**

Collagen fragments released by proteolytic remodeling of the ECM have biological activities of their own, and modulate cell signaling pathways mediated by ECM and growth factor receptors [317–320]. Some of them regulate tissue fibrosis.

A peptide of endostatin, the C-terminus fragment of collagen XVIII, exerts an anti-fibrotic activity. This peptide, with oral bioavailability [321], prevents TGF- $\beta$ -induced dermal and pulmonary fibrosis *in vivo* and decreases existing fibrosis [321–323]. Endostar, a derivative of endostatin, attenuates CCl<sub>4</sub>-induced liver fibrosis in mice [324,325]. It decreases the hepatic sinusoidal endothelial cell capillarization and the amount of VEGFR1 and VEGFR2 in liver. This suggests that the anti-fibrotic effect of endostatin could be mediated by the VEGF pathway, in agreement with the fact that endostatin bind to these receptors [319]. The inhibition of fibrosis by endostatin may also be mediated by the modulation of the PDGFR/ERK signal pathway [326]. Indeed endostatin decreases both PDGF-BB- and TGF- $\beta$ 1-induced over-expression of collagen I in human dermal fibroblasts, and inhibits the expression of PDGFR and p-ERK [326]. The expression of VEGF/VEGFR-2 and the activation of ERK1/2 are inhibited by endostatin, which

also decreases the levels of tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) and transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1) in a rat model of pulmonary fibrosis [322]. Furthermore soluble endostatin inhibits epithelial repair in idiopathic pulmonary fibrosis [327]. However the increased expression of endostatin in aging mice is associated with tubulointerstitial fibrosis, and endostatin might compromise anti-fibrotic mechanisms in aging [328]. Endostatin is indeed able to induce kidney fibrosis in transgenic mice overexpressing it and in mice receiving endostatin via minipumps implanted subcutaneously [329].

Endostatin concentrations in biological fluids have been measured in patients with fibrotic diseases in a number of studies. Elevated serum endostatin levels were observed in patients with systemic sclerosis accompanying pulmonary fibrosis where it is associated with vascular organ damage [330], and in patients with idiopathic pulmonary fibrosis [331]. Serum endostatin levels are correlated with liver regeneration capacity after hepatectomy in normal but not cirrhotic mice [332]. Last, elevated endostatin level in bronchoalveolar lavage fluid correlated with lung function in idiopathic pulmonary fibrosis [327].

Another collagen bioactive fragment, endotrophin, the C-terminus of the  $\alpha$ 3 chain of collagen VI, regulates fibrosis. The overexpression of endotrophin upregulates pro-inflammatory and pro-fibrotic genes in adipose tissues by potentializing TGF- $\beta$ -dependent signaling pathways [333,334]. Endotrophin triggers adipose tissue fibrosis and metabolic dysfunction [333,334]. Its serum level is independently associated with mortality in chronic kidney diseases [335].

The collagen tripeptide N-acetyl proline-glycine-proline, a chemoattractant acting via CXC receptors, induces the recruitment of neutrophils and contributes to chronic inflammation associated with fibrotic diseases such as chronic obstructive pulmonary disease (COPD) and cystic fibrosis [336,337]. Fragments of other ECM proteins also have pro-fibrotic activity. The N-terminal fragment of osteopontin and its SVVYGLR sequence enhance the synthesis of collagen

III, induce Smad signal activation, and the expression of smooth muscle actin in myocardial fibrosis [338].

### 4.3 Collagen fragments as markers of fibrosis

The remodeling of ECM by proteases in the course of fibrogenesis generates soluble collagen fragments, which are referred to as matricryptins or matrikines when exhibiting biological activities of their own [16,317–320] or as neo-epitopes [16,317–320], which can be measured in biological fluids using ELISA-based techniques. The use ECM proteolytic fragments, including collagen fragments, as diagnostic and prognostic biomarkers of ECM diseases has been recently reviewed [339] with a focus on liver fibrosis [340], myocardial fibrosis [341] and collagen IV [21]. Neo-epitopes are generated by MMPs and by cathepsins as shown for collagen III. Collagen neo-epitopes frequently used to assess collagen turnover in fibrotic diseases are listed in Table 1. The turnover of collagens I, IV and V, reflected by the concentrations of their fragments in serum, vary significantly upon aging as shown in rats and this has to be taken into account for translational studies [342].

Collagen types	Process reflected	Collagen neo-epitopes	Fibrosis	Patients/models	References
I	Synthesis of collagen I	P1NP N-terminal propeptide of collagen I	Lung fibrosis	Rat	[343]
	Degradation of collagen I	C1M or CO1-764 Fragment of the $\alpha 1$ chain generated by MMP-2, -9, -13 [344]	Lung fibrosis	Rat	[343]
			Liver fibrosis (HBV, HCV)	Patients	[345]

		ICTP C-terminal cross-linked telopeptide of type I collagen	Liver fibrosis (HBV, HCV, alcoholic liver diseases, primary liver biliary cirrhosis)	Patients	[346]	
III	Synthesis of collagen III	Pro-C3 N-terminal type III collagen propeptide [347]	Liver fibrosis (HBV, HCV)	Patients	[345]	
			Liver fibrosis	Patients Clinical trials II and III	[348]	
			Liver fibrosis HCV/HIV co-infection	Patients	[349]	
		iP3NP An internal epitope of the N-terminal pro-peptide of collagen III	Lung fibrosis	Rat	[343]	
	Degradation of collagen III	C3M or CO3-610 Generated by MMP9 [350]	Liver fibrosis (CCl <sub>4</sub> )	Rat	[351]	
			Liver fibrosis (HBV, HCV)	Patients	[345,352]	
			Lung fibrosis	Rat	[343]	
		C3C Generated by cathepsins B, L, S and K	Lung fibrosis (COPD)	Patients	[353]	
	IV	Synthesis of collagen IV	P4NP7S 7S domain of collagen IV [354]	Liver fibrosis (HBV, HCV)	Patients	[345]
			CO4-MMP	Liver fibrosis (HBV, HCV)	Patients	[345]



	Degradation of collagen IV	Fragment of the $\alpha 1$ chain generated by MMP-9 [355]	Liver fibrosis (CCl <sub>4</sub> and bile-duct ligation)	Rat	[355]
VI	Degradation of collagen VI	C6M Fragment of the $\alpha 1$ chain generated by MMP-2 and MMP-9 [356]	Liver fibrosis (HBV, HCV)	Patients	[345]
VIII	Degradation of collagen VIII	C8-C C-terminal part of the $\alpha 1$ chain	Lung fibrosis (COPD, IPF)	Patients	[357]

**Table 1: Major collagen neo-epitopes used as serum biomarkers in fibrotic diseases** (COPD: Chronic Obstructive Pulmonary Disease, HBV: Hepatitis B virus, HCV: Hepatitis C virus, IDP: Idiopathic pulmonary fibrosis).

C3C level is significantly elevated in patients with COPD compared to healthy controls [353]. Elevated Pro-C3 levels are indicative of active fibrogenesis and can identify patients, who are most likely to benefit from anti-metabolic and anti-fibrotic treatments. Serum Pro-C3 may thus ease patient selection [348]. Pro-C3-levels in patients with HIV/HCV-co-infection reflect fibrosis stage and degree of portal hypertension [349]. A model combining Pro-C3 and C4M along with patient's age, body mass index and gender increases the diagnostic power for identifying clinically significant fibrosis [345]. The most reliable markers for the diagnosis of idiopathic pulmonary fibrosis are C1M and C3M [354].

Collagen neo-epitopes may help discriminating fibrotic diseases targeting the same tissue. The concentration of the C-terminal part of the  $\alpha 1$  chain of collagen VIII, C8-C, is increased in serum from patients with chronic obstructive pulmonary disease but not in patients with idiopathic pulmonary fibrosis [357]. Chronic hepatitis B induces a higher basement membrane turnover than chronic hepatitis C, suggesting that molecular signatures in liver fibrosis may be related to the etiology of fibrosis [358]. The concentration of the C-terminal cross-linked telopeptide of collagen

I (ICTP) is increased in sera from patients with liver diseases, except in patients with chronic active hepatitis of unknown origin and in patients with acute hepatitis A. It is correlated with the extent of liver fibrosis but not with inflammation and necrosis [346]. Collagen neo-epitope levels in serum may vary according to the stage of fibrosis as shown in an experimental model of liver fibrosis [359], and may reflect liver expression of the pro-fibrotic cytokines TGF- $\beta$ 1 and TGF- $\beta$ 2, and of liver MMP-2 activity in rats with severe fibrosis [359].

Collagen biomarkers may also be helpful to follow the efficiency of anti-fibrotic drugs. The N-terminal peptide of procollagen III associated with other ECM biomarkers, hyaluronic acid and tissue inhibitor of matrix metalloproteinase-1, is a predictor of the outcome of anti-fibrotic therapy in patients with chronic hepatitis C [360]. Markers of the ECM remodeling reflect the effect of anti-fibrotic therapy in a rat model of liver fibrosis induced by bile-duct ligation [359]. The effect of potential drugs such as 3-isobutyl-1-methylxanthine, a phosphodiesterase inhibitor, and GM6001 (a pan MMP inhibitor) on collagens I and III turnover has been monitored in a bleomycin *ex-vivo* model of lung fibrosis [343].

Procollagen C-Proteinase Enhancer-1 (PCPE-1), which enhances the C-terminal processing of human fibrillar procollagens by Bone-Morphogenetic Protein-1 [361], is also of interest to assess fibrosis. Indeed, PCPE-1 plasma levels are associated with the severity of liver fibrosis in mice, and correlates with the liver content of collagen I and PCPE-1 [362].

Collagen fragments and neo-epitopes have also been measured in urine. Urinary levels of collagen degradation markers C1M and C3M are correlated with deposition of collagens I and III in rat models of chronic kidney diseases and fibrosis [363]. They both reflect and predict tubulointerstitial fibrogenesis in a proteinuria-driven rat model of renal fibrosis.

## CONCLUSION

The ultimate goal of fibrosis studies is to design anti-fibrotic drugs. A screening method based on fluorescence polarization has been set to screen chemical compounds that inhibit LARP6-dependent collagen I synthesis by disrupting LARP6 binding to 5' stem-loop RNA [364]. The screening of ~50,000 chemical compounds using this approach has led to the identification of a compound inhibiting the biosynthesis of collagen I at nanomolar concentrations [365] offering new perspectives for the treatment of fibrosis. Targeting the NF- $\kappa$ B pathway by {2-Amino-6-[2-(cyclopropylmethoxy)-6-hydroxyphenyl]-4-(4-piperidinyl)-3 pyridinecarbonitrile}, an inhibitor of the I $\kappa$ B kinase, inhibits TGF- $\beta$ 1-induced myofibroblast formation, collagen I synthesis and the expression of lysyl hydrolases 1, 2 and 3. This inhibitor has thus anti-fibrotic properties [366]. Another potential therapeutic target is collagen degradation [367]. An anti-MMP9 antibody reduces fibrosis in a heterotopic xenograft model of intestinal fibrosis [368]. The inhibition of collagen fibril formation by preventing collagen-collagen interaction may be a target of anti-fibrotic therapy [369,370]. A single-chain Fv antibody directed against the  $\alpha$ 2 C-terminal telopeptide of collagen I inhibits excessive formation of fibrotic deposits formed around a tendon in response to trauma [371]. Collagen adhesion receptors also impact collagen biosynthesis and/or deposition in the ECM as shown *in vitro* and/or *in vivo* for integrins, discoidin domain receptors and GP556 [372]. The targeting of these receptors may be a further anti-fibrotic therapeutic approach.

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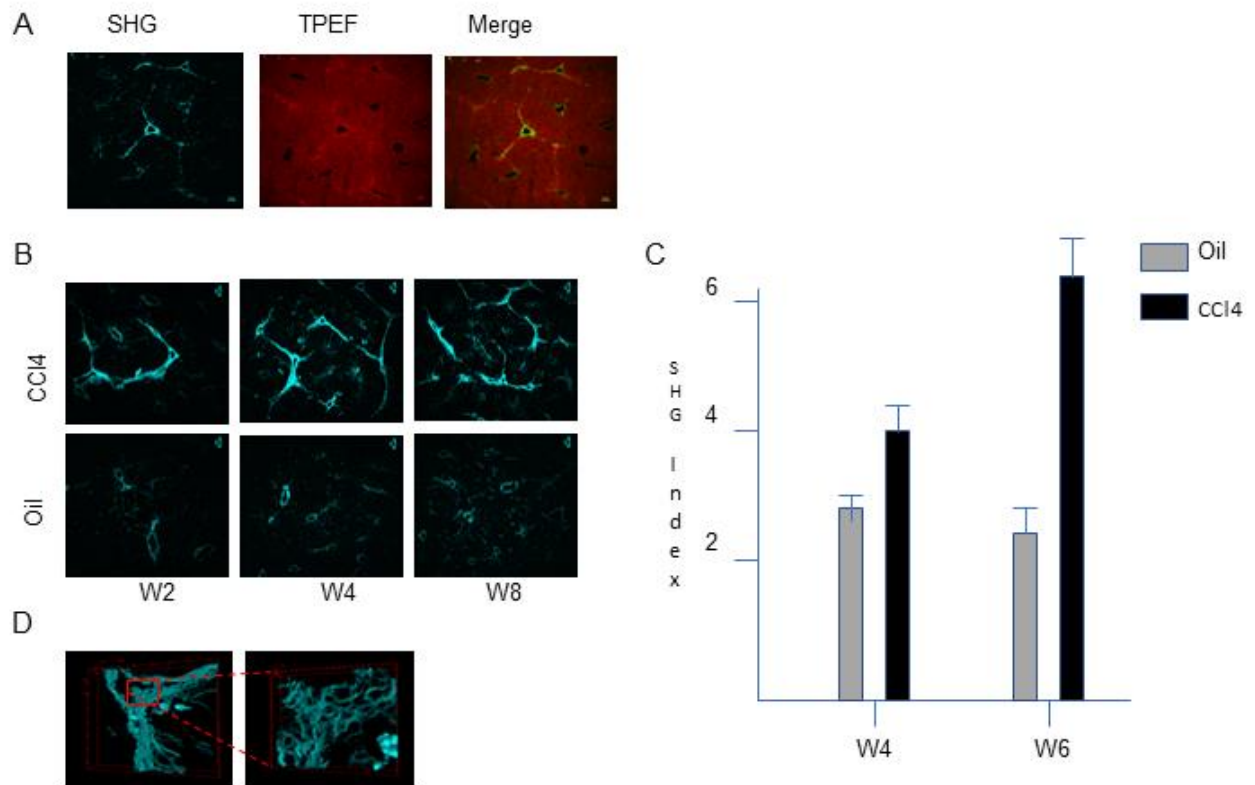
**FIGURE LEGENDS**

**Figure 1: TPEF and SHG images of mice fibrotic livers. A.** SHG, (blue), TPEF (green) and merge images obtained with the 10X objective focus at mid-depth (100  $\mu$ m) of the tissue. **B.** Typical SHG images representing the characteristic evolution of fibrillar collagen deposits of fibrotic and control livers after intraperitoneal injection of CCl<sub>4</sub> (0.5  $\mu$ g/ mouse/week) or oil at the indicated times in weeks (W) after the first injection. **C.** Evolution of SHG-index quantifying fibrillar collagen deposits as a function of time from fibrotic (CCl<sub>4</sub>-injected) or oil control (oil-injected) livers. **D.** High magnification 3D reconstruction of SHG and TPEF/SHG merge images.

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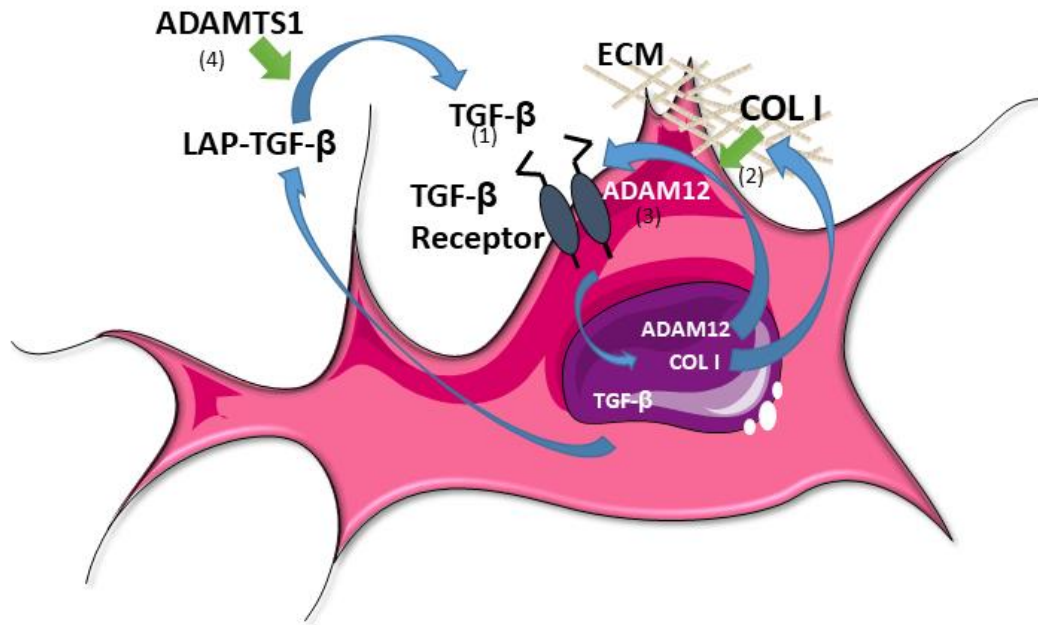
**Figure 2: Retro-regulation of TGF- $\beta$  signaling by ADAM12 and ADAMTS1 in hepatic stellate cells.** During liver fibrosis, (1) HSC are activated and adopt a myofibroblast-like phenotype. TGF- $\beta$  accelerates HSC activation, and induces collagen Type I expression. Increased expression of ADAM12 [284] and ADAMTS1 [290] is associated with activation of HSC. (2) Newly synthesized ADAM12 is translocated to membrane upon collagen stimulation and activation of PKC-dependent pathway [288]. (3) ADAM12 interacts with TGF- $\beta$ R2 and promotes TGF- $\beta$  transcriptional activity leading to upregulation of TGF- $\beta$  target genes including TGFB1, COL1 and ADAMTS12 [286]. (4) ADAMTS1 interacts with LAP-TGF- $\beta$ 1 and induces its conversion into active TGF- $\beta$  [290].

FIGURE 1



ACCEPTED

FIGURE 2



ACCEPTED M



**HIGHLIGHTS**

- Expression of collagens in fibrosis is mostly driven by TGF- $\beta$ , which cross-talks with other signaling pathways
- Excessive deposition of collagens in fibrosis results from an intricate interplay between processing and degradation enzymes mediated by growth factors and cell adhesion receptors
- Collagen covalent cross-linking mediated by lysyl oxidase and peroxidasin increases in fibrosis
- Collagen signature in fibrotic tissues can be assessed by Second Harmonic Generation microscopy

ACCEPTED MANUSCRIPT