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CCN proteins in normal and injured liver

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1. ABSTRACT

CCN proteins are small secreted cysteine-rich proteins containing up to four individual structural modules including an insulin-like growth factor binding domain, a von Willebrand Factor type C motif, a thrombospondin type I module and a carboxyl-terminal cystine knot. Actually, there is a large body of evidence suggesting that members of the CCN protein family encompass an expansive repertoire of functions in crucial areas including control of development, cell fate, angiogenesis, tumorigenesis, osteogenesis, cell adhesion, mitogenesis, migration, chemotaxis, and cell survival. Moreover, this family is supposed to modulate signalling of integrins, transforming growth factor-betas, bone morphogenetic proteins, vascular endothelial growth factor, Notch and factors that mediate signals *via* the canonical Wingless-type MMTV integration site family. However, several of these properties are not substantiated by experimental data but were deduced from proteins sharing one or more of the structural modules with these proteins. In this review, the actual knowledge of biological activities and molecular involvement of CCN proteins in maintenance of liver health and in initiation and progression of hepatic diseases is summarized and discussed.

2. INTRODUCTION

The CCN (CYR61, CTGF, NOV) protein family contains six individual members (see Table I), namely CCN1/CYR61, CCN2/CTGF, CCN3/NOV, CCN4/WISP1, CCN5/WISP2, and CCN6/WISP3 which have a modular architecture (Figure 1) and contain up to four distinct modules (1) that include an insulin-like growth factor-binding (IGFBP) domain, a von Willebrand factor type C (vWFc) domain, a thrombospondin type I homology (TSP-1) domain, and a C-terminal cystine knot (CTCK). All six proteins further contain an N-terminal leader sequence of 20 to 40 amino acids that characterize them as secreted proteins.

The first member of this protein family, originally termed 3CH61 and later renamed cysteine-rich protein 61 (CYR61) or CCN1, was isolated as an immediate-early gene that became transcriptionally activated within 5 min when serum or purified platelet-derived growth factor (PDGF) was added to quiescent mouse BALB/c 3T3 fibroblasts (2, 3). The chicken orthologue of this gene was isolated somewhat later in chicken embryo fibroblasts as one of twelve genes (i.e. CEF-1 to CEF-12) that were induced after infection with a temperature-sensitive mutant

Table 1. The CCN family of proteins in human

Member	Synonyms	Chromosomal localization*	Accession no./ Gene structure/ mRNA details	First Isolation (Refs)
CCN1	cysteine-rich, angiogenic inducer 61 (CYR61), cysteine-rich protein 61, homolog of insulin-like growth factor-binding protein 10 (IGFBP10), GIG1, 3CH61 (a name of a specific cDNA clone that was original used for isolation of *cyr61*), betaIG-M1, chicken embryo fibroblast (CEF)-10	1p22.3	NM_001554 / mRNA: 2295 nt cds: nt 225- nt 1370 exon 1: nt 1- nt 287 exon 2: nt 288- nt 501 exon 3: nt 502- nt 858 exon 4: nt 859- nt 1067 exon 5: nt 1068- nt 2295	2
CCN2	connective tissue growth factor (CTGF), insulin-like growth factor-binding protein 8 (IGFBP8), Fisp12, betaIG-M2, hypertrophic chondrocyte-specific protein 24 (Hcs24), heparin binding growth factor (HBGF)-0.8, ecogenin, IGFBP-rP3	6q23.1	NM_001901 / mRNA: 2368 nt cds: nt 207- nt 1256 exon 1: nt 1- nt 272 exon 2: nt 273- nt 495 exon 3: nt 496- nt 747 exon 4: nt 748- nt 959 exon 5: nt 960- nt 2344	7
CCN3	Nephroblastoma-overexpressed gene (NOV), oncogene NOV, insulin-like growth factor-binding protein 9 (IGFBP9)	8q24.1	NM-002514 / mRNA: 2601 nt cds: nt 222- nt 1295 exon 1: nt 1- nt 305 exon 2: nt 306- nt 531 exon 3: nt 532- nt 783 exon 4: nt 784- nt 998 exon 5: nt 999- nt 2601	9, 10
CCN4	WNT1-inducible signalling pathway protein 1 (WISP1), WISP1c, WISP1i, WISP1tc, expressed in low-metastatic cells (Elm)-1	8q24.1-q24.3	v1: NM_002514 / mRNA: 2819 nt cds: nt 77- nt 1180 exon 1: nt 1- nt 145 exon 2: nt 146- nt 425 exon 3: nt 426- nt 686 exon 4: nt 687- nt 880 exon 5: nt 881- nt 2798 v2: NM_080838 / mRNA: 1035 nt cds: nt 77- nt 919 exon 1: nt 1- nt 145 exon 2: nt 146- nt 425 exon 3: nt 426- nt 619 exon 4: nt 620- nt 1019	11
CCN5	WNT1-inducible signalling pathway protein 2 (WISP2), rat caspase recruitment domain (CARD) only protein (rCop-1), connective tissue growth factor-related protein 58 (CT58), CRGR4 (old gene symbol), connective tissue growth factor-like (CTGF-L)	20q12-q13	NM_003881 / mRNA:1433 nt cds: nt 148- nt 900 exon 1: nt 1- nt 207 exon 2: nt 208- nt 424 exon 3: nt 425- nt 679 exon 4: nt 680- nt 1404	11
CCN6	WNT1-inducible signalling pathway protein 3 (WISP3), progressive pseudorheumatoid dysplasia (PPD) gene, CCN6, lost in inflammatory breast cancer (LIBC), progressive pseudorheumatoid athropathy of childhood (PPAC) gene	6q22-q23	v1: NM_003880 / mRNA: 1252 nt cds: nt 111- nt 1175 exon 1: nt 1- nt 87 exon 2: nt 88- nt 158 exon 3: nt 159- nt 456 exon 4: nt 457- nt 699 exon 5: nt 700- nt 893 exon 6 : nt 894- nt 1241 v2: NM_198239 / mRNA: 1332 nt cds: nt 137- nt 1255 exon 1: nt 1- nt 238 exon 2: nt 239- nt 536 exon 3: nt 537- nt 779 exon 4: nt 780- nt 973 exon 5: nt 974- nt 1321	11

***** Chromosomal localizations are given to the Online Mendelian Inheritance in Man (OMIM) database that can be found at http://www.ncbi.nlm.nih.gov/omim

of the Rous sarcoma virus (4). By use of RNase protection assay, it was later shown in mouse that the respective *cyr61* mRNA was highest expressed in lung, while the expression was low in kidney, adrenal gland, testes, brain and ovary and moderate in heart, uterus, and skeletal muscle (5). Interestingly, the authors found no expression in normal liver using this sensitive detection method. However, subsequent Northern blot analysis that was performed in the course of chromosomal assignment of the human *cyr61* gene revealed that respective mRNA is rather widely expressed and found in many fetal as well as adult human tissues (6), possibly reflecting that there exists an inter-species differences between the murine and the human orthologous.

The second member of the CCN protein family was first described in human umbilical vein endothelial cells as a novel 349-amino acid mitogen with biological activities similar to PDGF and termed connective tissue growth factor (CTGF) (7). Sequence comparison revealed that CTGF belongs to the group of immediate-early genes, which are expressed after induction by certain growth factors or oncogene products. The respective 2.4 kb messenger RNA was found to be expressed at high levels in

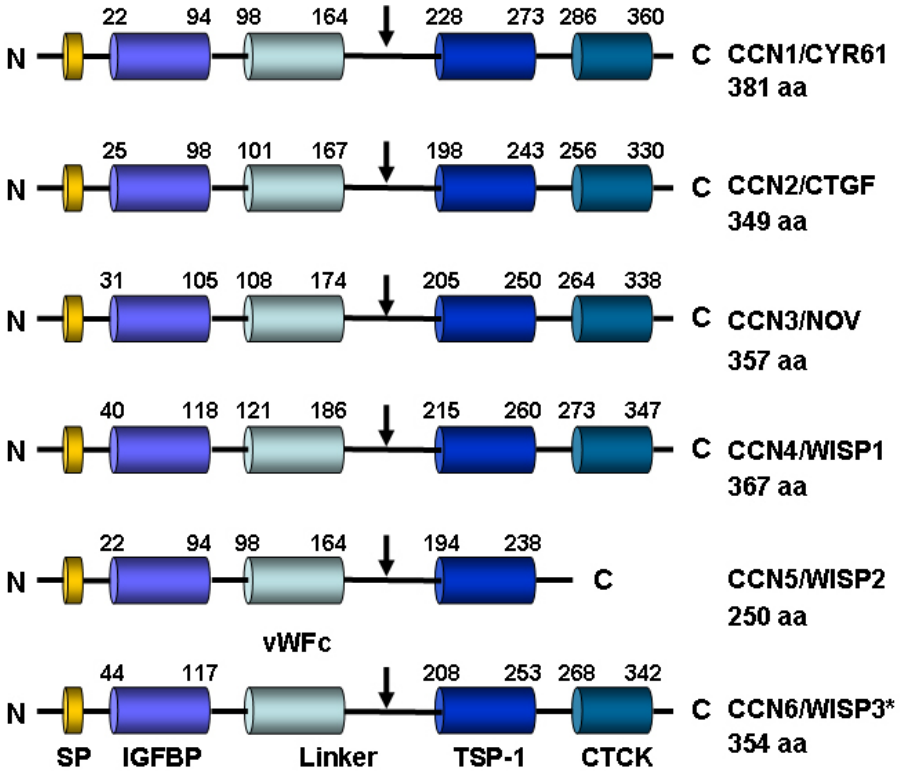

Figure 1. Structure of human CCN proteins. In the schematic representation the six human CCN proteins are depicted. Indicated are the locations of individual domains. Abbreviations used are: SP, signal peptide; IGFBP, insulin-like growth factor-binding domain; vWFc, von Willebrand factor type C domain; TSP-1, thrombospondin type-1 repeat; CTCK, C-terminal cystine knot domain. The identification of individual domains was performed using the PROSITE software (release 20.66) that can be found at the ExPASy Proteomics Server (http://expasy.org/prosite/) using the default settings and the deposited GenBank protein entries for human CCN1/CYR61 (acc. no. CAG38757.1), CCN2/CTGF (acc. no. CAG46559), CCN3/NOV (acc. no. CAA65403.1), CCN4/WISP1 (acc. no. NP_003873.1), CCN5/WISP2 (acc. no. CAB94788.1) and CCN6/WISP3 (acc. no. CAI42331.1), respectively. * Please note, that the PROSITE software does not recogize a vWFc domain in human CCN6/WISP. However, according to Kutz and colleagues (62) a vWFc is located at amino acid position 117-179. Arrows indicate the location of the linker regions that are sensitive for proteolytic cleavage.

spleen, ovary, gastrointestinal tract, prostate, heart and testis but was virtually absent in normal liver samples (8).

The third member (CCN3/NOV) that was eponymic for this protein family was isolated from myeloblastosis-associated virus (MAV)-1-induced avian nephroblastoma (9, 10) representing a well established animal model of the pediatric Wilms tumor. The authors found that each one of eight nephroblastomas tested expressed increased levels of this cellular gene and termed the gene therefore nephroblastoma-overexpressed gene (*ccn3*/*nov*). Northern blot analysis further revealed that *ccn3*/*nov* expression in embryonic tissue is found in normal chicken embryonic kidney, brain, heart, muscle and intestine while in adult tissues the expression is mainly found in brain, lung and spleen. Expression analysis from embryonic and adult livers demonstrated that *ccn3*/*nov* transcripts were virtually not present in liver (10). Based on its expression profile, the authors speculated that *ccn3*/*nov* is a novel proto-oncogene that is overexpressed in nephroblastoma while the expression is probably not transforming *per se* in all tissues.

The first two Wingless-type MMTV integration site family member-1 (WNT-1)-inducible signalling pathway proteins, i.e. WISP1 and WISP2, were originally identified in a subtractive hybridization approach for genes that were upregulated in a mouse mammary epithelial cell line transformed by the WNT-1, but not by the WNT-4 gene (11). In the same study expressed sequence tag (EST) databases were screened to search for related proteins of WISP1 and WISP2 resulting in the identification of a close homologue that was named WISP3 sharing 42% and 32% sequence identity with WISP1 and WISP2, respectively.

WNT-1 itself represents a cysteine-rich, glycosylated signalling protein that mediates diverse developmental processes, such as control of cell proliferation, adhesion, cell polarity, and establishment of cell fate. It was identified as an oncogene activated by the insertion of mouse mammary tumor virus in virus-induced mammary adenocarcinomas.

Based on the finding that *WISP1* and *WISP2* expression was mainly observed in the stromal cells that

surrounded the tumor cells in the WNT-1 transgenic mouse sections of breast tissue, it was suggested that both *WISP* genes are involved in paracrine signalling processes. Detailed analysis using a PCR strategy on adult and fetal tissue panels revealed that *WISP1* gene expression is mainly detectable in adult heart, kidney, lung, pancreas, placenta, ovary, small intestine, and spleen, while the expression in brain, liver, skeletal muscle, colon, peripheral blood leukocytes, prostate, testis and thymus is rather low or absent (11). In the same study it was reported that *WISP2* has a more restricted tissue expression and is predominantly expressed in adult skeletal muscle, colon, ovary and fetal lung. Highest expression of *WISP3* was noticed in adult kidney, testis and in fetal kidney and at lower levels in some other organs. Collectively, this fundamental study revealed that none of the three *WISP* genes is expressed in normal adult liver and that only the *WISP1* gene shows a weak hepatic expression during fetal development.

3. STRUCTURAL ASPECTS OF CCN PROTEINS

Five of the CCN proteins (i. e. CCN1, CCN2, CCN3, CCN4, and CCN6) contain four individual structural modules that include an IGFBP domain, a vWFc repeat, a TSP-1 domain, and a CTCK that is lacking in CCN5 (Figure 1). In addition, two splice variant forms for CCN4 were identified in scirrhous gastric carcinoma (12) and human hepatocellular carcinoma (HCC) cells (13). These lack either exon 3 (WISP-1va or WISP-1v) encoding the vWFC domain or exons 3 and 4 (WISP1Delta ex 3-4) resulting in an all-out truncated protein simply retaining the IGFBP domain (13). Although it was found that the *WISP1* variant lacking the vWFc domain had remarkable effects on cellular growth (12), it was later shown that it has overall similar effects on proliferation and osteogenic differentiation (14). Another potential CCN6 variant (WISP3VL) in which both the TSP-1 and the CTCK domains are missing was isolated with a reverse transcriptase polymerase chain reaction approach (13).

Based on the presence of four distinct structural modules covering nearly the whole molecule and the location of exons and introns within the CCN genes, it was suggested that CCN family members are genuine mosaic proteins. In such proteins each individual domain has typically its own function that is supposed to be similar or identical to other proteins encompassing the same structural module. Moreover, in such proteins the function of the complete protein is thought to be roughly the total sum of biological activities and functions of the individual domains. In regard to CCN proteins the assumption that the action of a CCN protein is wholly based upon the action of its particular domains has totally changed during the last years because the large variety of studies dealing with CCN proteins have clearly shown that each CCN protein have a sheer endless functional repertoire of specific activities that are often opposing to other CCN molecules (see below in chapter 4.4.). Moreover, several experimental findings suggest that some of their biological functions require cooperation between two or more modules. The actual knowledge of key features of the individual domains of the CCN proteins using structure prediction algorithms and their deduced structure-function relationships were already recently summarized in detail elsewhere (15, 16). However, the exact role of each domain in the context of CCN proteins is not fully understood yet. In the following a brief introduction into the four structural motifs of the CCN protein family is given.

3.1. The insulin-like growth factor binding protein domain

The six different IGFBPs known today are 24 to 45 kDa proteins that control the bioavailability, activity, and distribution of insulin-like growth factor (IGF)-1 and -2 by forming high-affinity IGFBP/IGF complexes (17). They share approximately 50% homology with each other and encompass a typically IGF-binding site (Figure 2A). The complete functionality in IGF binding requires, however, cooperative actions of both amino- and carboxyl-terminal domains within the complete IGFBPs (18). Under normal conditions, most of the biologically active forms of IGF-1 and IGF-2 are bound to one of the six known binding proteins. It is generally accepted that this binding lengthens the half-life of circulating IGFs and modulates the activity for endogenous receptors in all tissues. Therefore, the individual IGFBPs are thought to enhance or attenuate IGF signalling depending on the physiological context in which they appear (i.e. cell type, IGFBP concentration, ligand concentration, receptor density).

The IGFBP domain of the CCN proteins typically contains 11 of the 12 conserved cysteine residues that are also found in the amino-terminus of IGFBP-1 to -5 suggesting that this sequence might account for an IGF-binding activity (19). In particular, the characteristic conserved N-terminal cysteine pattern of the IGFBPs (GC(G/S)CCXXCAXXXXXXC) is included in all human CCNs. In line with this hypothesis, affinity-labeling and ligand-blotting studies demonstrated weak IGF-binding affinity for at least two members of the CCN family, i.e. CCN2/CTGF (8) and CCN3/NOV (20). However, a chimeric protein in which the amino-terminal domain of IGFBP-3 was substituted with those of human CCN3/NOV bounded IGFs only weakly, similar to but not better than CCN3/NOV itself (21). This result indicates that IGFBP domain of a CCN family protein cannot completely fulfill the IGF-binding function of an IGFBP and argues against a direct role of CCN3/NOV in the modulation of IGF activity and signalling (21).

3.2. The von willebrand factor type C domain

The von Willebrand factor (vWF) is a large, adhesive, multimeric plasma glycoprotein that is encoded by 52 exons covering around 178 kbp of DNA on human chromosome 12. It is one of the key factors involved in blood clotting and hemostasis. The vWF precursor is composed of four different repetitions of subdomains (22, 23) that are also found in many other proteins including various plasma proteins, complement factors, integrins, various collagens (types VI, VII, XII and XIV) and a multitude of other extracellular proteins (24, 25). Although the majority of these proteins that include these segments are obvious extracellular, the most ancient ones present in

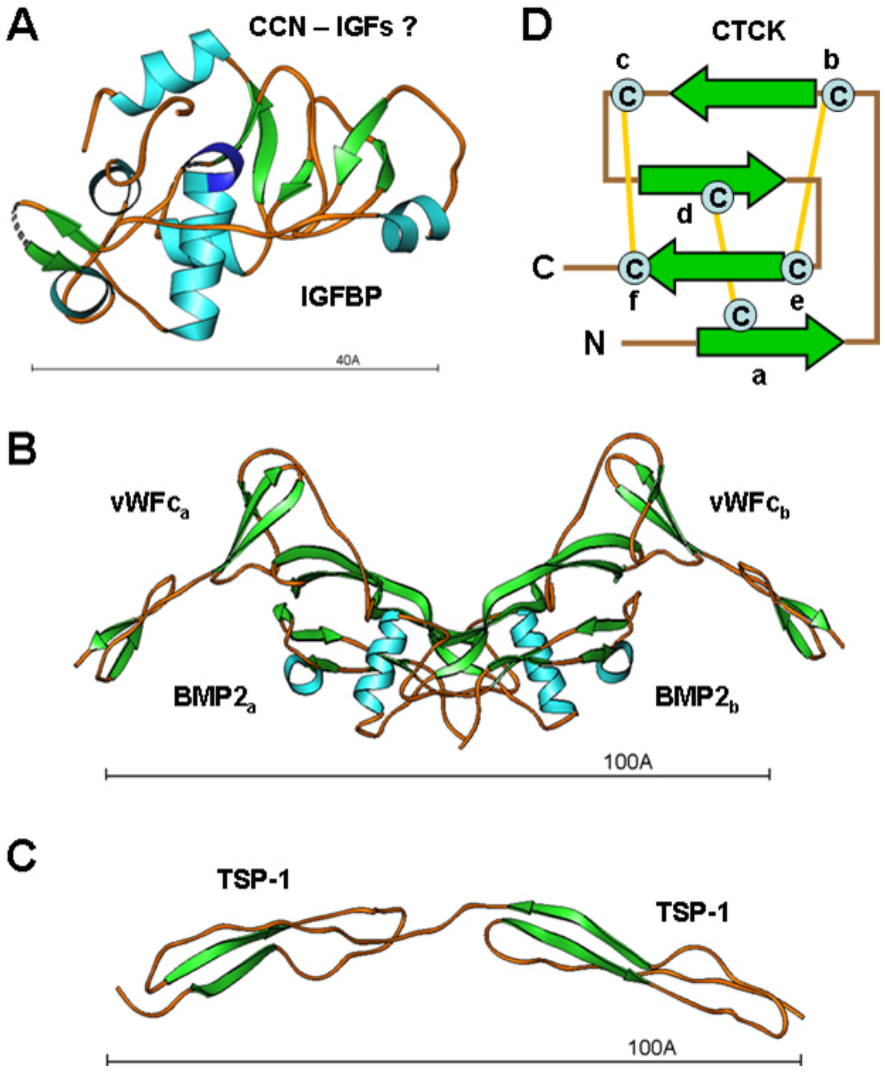

Figure 2. Modules found in CCN proteins. **(A)** The insulin-like growth factor-binding protein (IGFBP) domain is a conserved module with capacity to form high-affinity IGFBP/IGF complexes. The depicted structure is based on coordinates that are deposited under entry 2DSP in the Protein Data Bank (www.pdb.org/). Presently, it is under discussion if the IGFBP domains of CCN proteins are able to bind IGFs. (B) The von Willebrand factor type C (vWFc) domain is a common feature that is found in many proteins involved in multiprotein complexes. In the depicted ribbon representation, the complex of BMP2 with two vWFc domains (vWFc$_a$ and vWFc$_b$) of crossveinless 2, representing a member of the Chordin family that contains five closely spaced vWFc domains at its N-terminal domain, is shown. The coordinates were taken from entry 3BK3 of the Brookhaven Protein Data Bank (26). (C) The thrombospondin type I (TSP-1) homology domain is found in many matricellular glycoproteins that bring together various protein constituents. The ribbon diagram of two neighboured TSP-1 domains from human recombinant thrombospondin-1 is depicted. Each domain represents a long, thin, spiralling, anti-parallel, three-stranded domain. The coordinates were taken from the PDB Protein Data Bank (acc. code 1LSL). (D) Schematic diagrams of the C-terminal cystine knot (CTCK) found in CCN proteins. In the fold, the individual four beta-strands are drawn as arrows, the six cysteine residues that form the different disulfide bonds (yellow lines) are labelled (a-f). In this motif that is also found in many other growth factors two disulfide bridges (b-e and c-f) form a loop through which a third, penetrating disulfide bond (a-d) passes through. Half-cystine residues that are involved in structure formation are indicated by light blue open circles containing a C. Panels A-C were generated using Ribbons XP version 3 (131) and coordinates of respective PDB entries.

all eukaryotes are all intracellularily localized and involved in transcription, DNA repair, ribosomal and membrane transport and proteasome functionality suggesting that several of the vWF domains appear to be associated with multiprotein complex formation. Therefore, proteins that contain such domains are suggested to have the capacity to participate in numerous different biological processes (e.g. cell adhesion, migration, homing, pattern formation, and signal transduction) that require modular features allowing interaction with a large array of different ligands.

The type C domain (vWFc) that is synonymously called Chordin-like domain has a typical tertiary fold (Figure 2B). In the vWF this domain is thought to participate in oligomerization, but not necessarily to be involved in the initial dimerization step (22). Interestingly, in several other proteins (e.g. noggin, follistatin, members of the Dan protein family, chordin-like proteins), this domain is known to bind several bone morphogenetic proteins (BMP) members (26).

3.3. The thrombospondin type i homology domain

The thrombospondins are a family of five matricellular glycoproteins that regulate extracellular matrix structure and cellular phenotype. They act by bringing together cytokines, growth factors, matrix components, membrane receptors, and extracellular proteases (27). Thrombospondin-1 is a trimeric 420-kDa protein in which each protomer is composed of multiple domains including N- and C-terminal globular domains, a procollagen-like domain, and three types of repeated sequence motifs that are designated type 1, 2, and 3 repeats.

Type 1 repeats have a typical three dimensional fold (Figure 2C) that is also found in many other proteins that mediate cell attachment, glycosaminoglycan binding, are involved in activation of transforming growth factor-beta (TGF-beta) and inhibition of matrix metalloproteinases. In the human genome, a total of 41 proteins harbouring 1 to 18 thrombospondin type 1 (TSP-1) repeats were identified (28). Typically, proteins of this class are secreted or are transmembrane proteins in which this motif is found in the extracellular portion.

3.4. The cystine knot

The cystine knot (CK) is a highly conserved three-dimensional fold found in several cytokines (e.g. nerve growth factor, TGF-betas, PDGFs) and hormones (e.g. luteinizing hormone, chorionic gonadotropin, thyroid-stimulating hormone, follicle stimulating hormone). In this unusual structural arrangement (Figure 2D), two disulfide bridges and their connecting backbone sections that are typically build out of two pairs of antiparallel beta-strands form a loop through which a third disulfide bond passes (29). It occurs in many peptides and proteins among divergent species and provides considerable structural stability. Most interestingly, this motif is especially found in many distinct families of dimeric proteins that appear to have little in common beyond their ability to induce a biological response by binding to a specific cell surface receptor kinase that oligomerize after ligand binding and initiate signal transduction (29). In contrast, the five proteins of the CCN family (CCN1/CYR61, CCN2/CTGF, CCN3/NOV, CCN4/WISP1, and CCN6/WISP3) that share this common CK are thought to act as monomers. Therefore, it is most likely that the C-terminal parts of CCN proteins adopt a similar three dimensional fold allowing binding similar receptors that are monomeric in nature or alternatively form upon binding non-covalent dimers that develop agonistic activities.

4. BIOLOGICAL ACTIVITIES OF CCN PROTEINS

The existence of up to four individual modules that share a high degree of homology raised fundamental questions about their biological activities within the context of a CCN protein. Early studies demonstrated the importance of the immediate-early gene product CCN1/CYR61 in fibroblast attachment, spreading, and chemotaxis (30). Later it was shown that CCN1/CYR61 further promotes proliferation responses to growth factor and cell adhesion through its interaction with cell surface integrins (31, 32). Based on their overall similarity (cf. Figure 1) and evolutionary conservation (e.g. Figure 3), it was initially speculated that all CCN protein may have identical or similar functions. However, in the last years this view has dramatically changed and CCN proteins are nowadays supposed to evolve common but also rather specific activities.

At the molecular and biochemical level CCN proteins have binding activities, intrinsic activities, modulator activities, and antagonistic activities. These should be discussed in the following four subparagraphs (4.1. to 4.4.).

4.1. Binding activities

After identification of CCN proteins as a novel protein family, several attempts have been made to identify possible receptors for CCN proteins. A first clue in this analysis was the finding that CCN1/CYR61 interacts with heparan sulphate proteoglycans (33) that are present on the plasma membrane of all animal cells studied so far (34). Some years later CCN1/CYR61 was identified as a novel ligand for $alpha_v beta_3$ indicating that the property to serve as a cellular adhesion protein is at least in part mediated through interaction with this integrin (31). The affinity for the $alpha_v beta_3$ integrin was also demonstrated one year later for CCN2/CTGF (35) suggesting that the affinity for integrins might be a general aspect in CCN biology and function (36). This assumption was confirmed in many subsequent studies showing that CCN members have affinity or interact with $alpha_6 beta_1$ (CCN1/CYR61, CCN2/CTGF, CCN3/NOV), $alpha_v beta_3$ (CCN1/CYR61, CCN2/CTGF, CCN3/NOV), $alpha_v beta_5$ (CCN1/CYR61, CCN3/NOV), $alpha_5 beta_1$ (CCN2/CTGF, CCN3/NOV), $alpha_2 beta_1$ (CCN1/CYR61), $alpha_{IIb} beta_3$ (CCN1/CYR61, CCN2/CTGF), $alpha_M beta_2$ (CCN1/CYR61, CCN2/CTGF), and $alpha_D beta_2$ (CCN1/CYR61) (for recent review on this topic see 37 and references therein). Of course this complex network involving CCNs and integrins offers a vast combinatorial variety for specific and common modalities by which extracellular and intracellular signals can be transduced. Interestingly, most of the studies that investigate these interactions report that these interactions influence the production and composition of the extracellular matrix suggesting that they are especially relevant in embryonic development, cell and tissue differentiation, wound healing, and metastasis.

In regard to liver, CCN2/CTGF has become to be of particular interest for many laboratories since several studies have shown that this CCN protein is one of the key modulators that regulates activity of TGF-beta, a cytokine

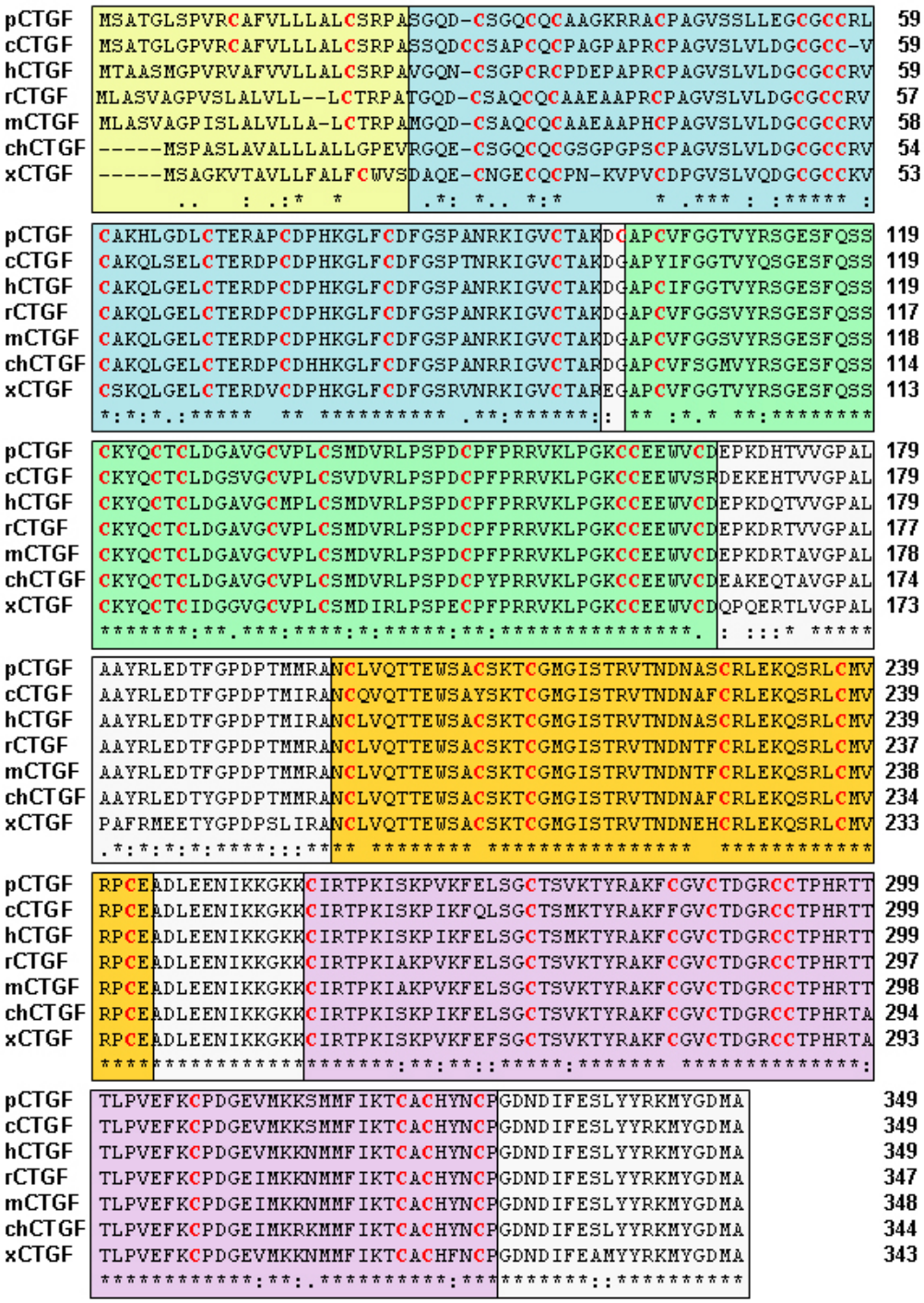

Figure 3. Multiple sequence alignment of CCN2/CTGF from different species. CCN2/CTGF from *Sus scrofa* (pig, p; AAD00174.1), *Bos taurus* (cattle, c; DAA26141.1), *Homo sapiens* (human, h, CAG46559), *Rattus norvegicus* (rat, r; NP_071602.1), *Mus musculus* (mouse, m; NP_034347.2), *Gallus gallus* (chicken, ch; NP_989605.1), and *Xenopus laevis* (frog, x; AAH94492.1) were aligned with the ClustalW2 multiple sequence alignment program of the European Bioinformatics Institute (http://www.ebi.ac.uk/) using the default settings. The cysteine residues are given in red colour. "*" means that the residues or nucleotides in that column are identical in all sequences in the alignment, ":" means that conserved substitutions have been observed, and "." means that semi-conserved substitutions in the aligned sequences are observed. The location of the individual domains are highlighted in yellow (signal sequence), light-blue (IGFBP), light-green (vWFc), orange (TSP-1) and purple (CTCK), respectively. The linker and intervening regions are highlighted in light-gray. Positions of amino acids are marked on the right margin.

that is involved in hepatic injury during which overshooting extracellular matrix-synthesis and -remodelling is one of the key characteristics (see below).

Another direct molecular interaction of CCN2/CTGF with fibronectin-1 was recently found in a yeast-two-hybrid using full-length and truncated versions of CCN2/CTGF as baits (38). Interestingly, CCN2/CTGF specifically enhances $alpha_5beta_1$ integrin-dependent cell adhesion to fibronectin through its CTCK domain (38). Since fibronectin is one of the most widespread adhesive extracellular glycoproteins with numerous functions in the control of cellular activities (cell adhesion, cell motility, control of cell shape, proliferation, differentiation) and also involved in numerous physiological and pathological processes (growth and development, wound healing, cancerogenesis, inflammation, tissue repair), it is comprehensible that this affinity might offer further pathways for CCN signalling and fine tuning of its biological activities.

Moreover, a common feature of CCN proteins is their ability to interact with members of the low density lipoprotein receptor-related protein (LRP) gene family encoding a total of twelve distinct (LRP1-LRP12) but structurally closely related surface receptors that fulfil diverse biological functions in different organs, tissues, and cell types (for a recent review see 39). In general these scavengers bind and internalize ligands *via* receptor-mediated endocytosis. Several of these receptors (e.g. LRP5, LRP6) are known to transduce signals by means of the canonical WNT signalling pathway that increases the amount of nuclear beta-Catenin that subsequently interacts with the T-cell-specific transcription factor/lymphoid enhancer binding factor (TCF/LEF) family of transcription factors to control gene expression (39, 40). The functionality of this signalling route was consistently shown for CCN2/CTGF that binds through its CTCK to LRP1 and LRP6 thereby modulating WNT signalling (41-43).

Furthermore, it was demonstrated that the CCN proteins have capacity to bind a wide repertoire of different growth factors and cytokines belonging to the TGF-beta, BMP, and the vascular endothelial growth factor (VEGF) families (see also 4.3.). Paradigmatic for such kind of action is the finding that CCN proteins can regulate the cell surface localization and modulate the manner of receptor interaction of individual members of the BMP family (44-46). However, the precise formation of the predicted complexes and the mechanisms of this potential interaction and their impact on cellular signalling are not yet fully addressed and understood. Likewise it was demonstrated that the binding of CCN2/CTGF to VEGF inactivates its angiogenic properties (47). Based on the fact that this masking effect on VEGF is abolished by matrix metalloproteinase-2 (MMP-2) mediated proteolysis of CCN2/CTGF, it was speculated that CCN2/CTGF is a temporary sequestering factor for VEGF activity and might especially release large quantities of VEGF during wound healing processes when the activity of MMP-2 is rather high (48).

4.2. Intrinsic activities

As mentioned above, CCN2/CTGF was originally identified as a 36-kDa immunoreactive peptide that was bound to a site in the cell surface of murine fibroblasts (NIH3T3) that could be competed by increasing concentrations of recombinant PDGF-BB (7). Based on this finding it was initially suggested that CCN2/CTGF has a biological activity similar to PDGF and may bind to one of the PDGF receptors or structurally related recognition sites. Subsequent studies on a human chondrosarcoma-derived chondrocytic cell line (i.e. HCS-2/8), however, demonstrated that the binding site for CCN2/CTGF was different than those recognized by PDGF-BB (49). In regard to liver biology, Gao and coworkers reported that the stimulation with recombinant CCN2/CTGF (100 ng/ml) promoted phosphorylation of the *ets* oncogene family member Elk-1 and the extracellular signal-regulated kinases ERK1 and ERK2, increased the expression of c-*fos* and cellular proliferation in primary hepatic stellate cells (HSC) (50). All these findings demonstrate that CCN2/CTGF either has intrinsic activities on its own or has capacity to modulate the activity of special cytokines involved in regulation of afore mentioned processes during ongoing hepatic fibrogenesis.

Similar intrinsic activities were reported for the CCN3/NOV protein. In particular it was found that the stimulation of 3T3 cells with recombinant CCN3/NOV resulted in a dose-dependent increase of cellular proliferation (51). Moreover, in the same study it was demonstrated that the stimulation with 10 nM recombinant human CCN3/NOV protein for 30 min strongly stimulated tyrosine phosphorylation of a 221 kDa protein and to a lesser extent of proteins in the size range of 135, 98, and 75-80 kDa suggesting that CCN3/NOV can bind to distinct receptors and evolve growth factor activities (51). Although these CCN3/NOV-sensitive phosphorylation sites were not further investigated, these findings are indicative of the fact that CCN proteins are part of a complex network in which they bind to specific surface receptors or affinity sites.

4.3. Modulator activities

Clear evidence that CCN proteins are capable to modulate cellular signalling by binding to cytokines was reported for CCN2/CTGF. A direct physical interaction of full length CCN2/CTGF with BMP4 that was competed by excess BMP2 or TGF-beta1 but not by IGF-1 was demonstrated in immunoprecipitation and cross-linking experiments (44). Fine mapping further revealed that this interaction was mediated *via* the CTCK of CCN2/CTGF. Complementary kinetic measurements using surface plasmon resonance spectroscopy yielded dissociation constants of 5 nM for BMP4 and a lower affinity of 30 nM for TGF-beta1 (44). Most importantly, the sequestering of BMP4 by purified CCN2/CTGF caused an overall reduced biological activity of BMP signalling and CCN2/CTGF was able to antagonize BMP4 binding to an artificial recombinant type Ia BMP-receptor-Fc fusion protein indicating that CCN2/CTGF is a sequestering factor for this cytokine. On the contrary, binding of CCN2/CTGF to TGF-beta1 enhanced the binding of this cytokine to endogenous surface receptors resulting in enhanced TGF-beta signalling.

Possibly, CCN2/CTGF might act as a chaperone to modify the conformation or solubility of TGF-beta1 and facilitate presentation to its cognate receptors (44). Prototypically all these findings indicate that members of the CCN family of proteins might be important regulators that critically modulate the activity of cytokines and their triggered intracellular signalling pathways.

A similar modulator role in BMP signalling was reported several years later for CCN6/WISP3 (52). In this study that was conducted in zebrafish the over-expression of the wild type CCN6/WISP3 protein inhibited BMP signalling by binding to the ligand and blocking WNT signalling, again demonstrating the effect of CCN proteins on intracellular signalling pathways.

Interestingly, an amino-terminal truncated 31/32kDa form of the CCN3/NOV protein that was mainly located in the nucleus was found in human cancer cell line 143 and HeLa cells (53). Although the biological significance of this finding is still unclear, the fact that the CCN3/NOV has intrinsic activity to bind to the subunit G of the RNA polymerase II (i.e. rpb7) might further point towards the possibility that CCN proteins module processes involved in regulation of gene expression (53). In this context it is noteworthy that also CCN2/CTGF and CCN5/WISP2 were found in the nucleus of many cells (54).

Another interesting finding that was made in a yeast-two-hybrid screen several years ago was the demonstration that full-length CCN3/NOV protein can physically interact with Fibulin-1C, the single-pass transmembrane receptor Notch and with the S100 calcium binding protein A4 (S100A4). Fibulin-1C contains nine calcium-binding type II EGF-like modules that allow interaction with the extracellular domain of the heparin-binding EGF-like growth factor precursor. Likewise, the activities of Notch and S100A4 are strongly Ca^{2+}-dependent. The coupling of CCN proteins with pathways that become either directly activated after calcium influx resulting from activation of specific ion channels or indirectly after stimulation of receptors involved in signal transduction pathways such as G protein-coupled receptors further demonstrate the high capacity of CCNs to modulate cellular functions in coordinating extra- and intracellular signals (for detailed review on this topic see 55).

Moreover, there is more and more evidence that CCN2/CTGF has modulatory activity on the organization of the cytoskeleton (56) or might be a key regulatory factor relevant for some extracellular matrix remodelling components (57).

4.4. Antgonistic activities (yin yang)

The concept of Yin and Yang was originally gathered from Chinese philosophy and is used to describe how polar or seemingly contrary forces are interconnected and interdependent in the natural world. This concept was recently transferred to the biological function of CCN3/NOV and CCN2/CTGF (58). In this pioneering study, the authors showed that the exposure of kidney mesangial cells to exogenous CCN3/NOV resulted in a dose-dependent attenuation or blockade of the TGF-beta stimulated increase in CCN2/CTGF transcript and an overall reduction in TGF-beta stimulated synthesis, secretion and redistribution of intracellular collagen type I (58). Moreover, TGF-beta reduced CCN3/NOV expression but increased CCN2/CTGF expression in these cells. Collectively these data supported the notion that endogenous CCN3/NOV activity plays a role in extracellular matrix metabolism in mesenchymal cells, and that CCN2/CTGF and CCN3/NOV are opposing factors in regulating collagen promoter activity and secretion of this extracellular matrix protein. Although the underlying mechanisms how CCN3/NOV regulates CCN2/CTGF activity were not identified in this study, the results clearly indicate that different combinations of CCN proteins can affect each other and might mediate divergent biological effects.

The concept that CCN2/CTGF and CCN3/NOV act under certain experimental conditions in a Yin Yang fashion is most likely not specific for kidney mesenchymal cells. Preliminary, unpublished results from our group indicate that the opposing effects of these two CCN proteins are also detectable in EA•hy 926 cells that represent a fusion of human umbilical vein endothelial cells with the permanent human cell line A549. Theoretically, it is possible that the ligand binding to one CCN member leads to sequestering and inactivation, the binding to another member might be associated with an enhanced (often adverse) biological activity. In the case of CCN2/CTGF and CCN3/NOV this scenario would result in inactivation of TGF-beta by CCN3/NOV and activation by CCN2/CTGF. Possibly under other conditions these activities might reverse. These attitudes again demonstrate the high complexity in CCN protein functionality.

5. CCN PROTEINS IN NORMAL LIVER

Generally it is assumed that the essential functions of a specific protein product in embryonic and organ development become obvious in models in which it is lacking. Presently, targeted gene disruptions in mice have been established for *ccn1/cyr61* (59), *ccn2/ctgf* (60), *ccn3/nov* (61), and *ccn6/wisp3* (62). The observed phenotypes of the different CCN disruptions are distinct and highly variable in regard to their phenotypes. While *ccn1*/*cyr61*-deficient mice are lethal and die because of vascular defects in the placenta (59), mice that are disrupted for the *ccn6/wisp3* gene appear quite normal (62). Based on the phenotype of the *ccn1/cyr61* nulls it is most likely that this gene has major functions in angiogenesis. Lack of functional *ccn2/ctgf* gene leads to decreased expression of VEGF, skeletal dysmorphisms as a result of impaired chondrocyte proliferation and extracellular matrix composition within the hypertrophic zone, and decreased expression of specific cartilage-specific components and matrix metalloproteinase-9 (60) demonstrating the fundamental role of CCN2/CTGF for cell proliferation and matrix remodelling. In mice lacking full-length CCN3/NOV the correct development of the appendicular and axial skeleton is strongly affected and respective animals have increased bone mineralization and severe

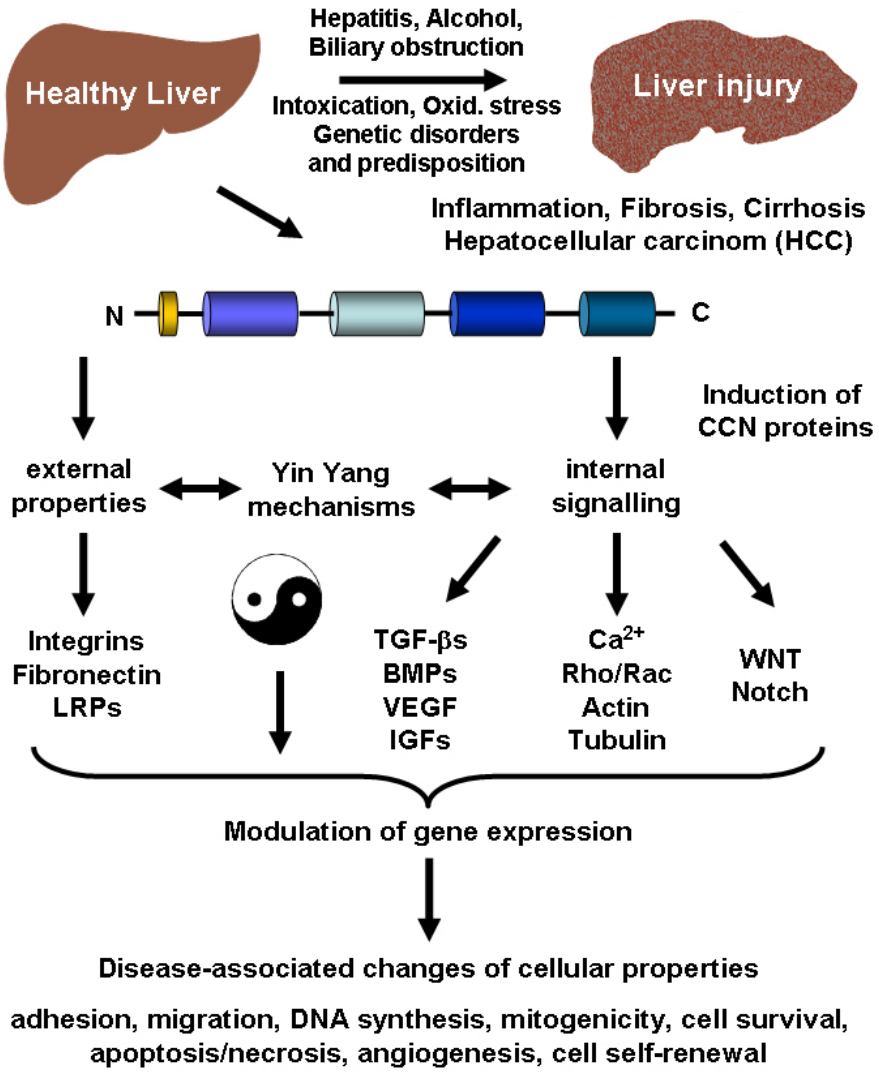

Figure 4. Summary of known biological activities of CCN proteins in injured liver. There are different factors (e.g. viral hepatitis, alcohol, biliary obstruction, intoxication, oxidative stress, genetic disorders and predispositions) that sensitize a healthy liver to inflammation, fibrosis, cirrhosis and HCC. There is now increasing evidence that various CCN proteins (i.e. CCN2/CTGF, CCN3/NOV, WISPs) become up-regulated during hepatic damage and change external properties and internal signalling pathways by binding to LRPs, integrins, fibronectin, cytokines (e.g. TGF-betas, BMPs, VEGF, IGFs), regulators of Ca^{2+} signalling, cytoskeletal components and by modulating Notch and WNT pathways. As a consequence, the expression of typical disease-associated marker proteins or overall modified cellular properties that affect adhesion, migration, DNA synthesis, mitogenicity, cell survival, apoptosis/necrosis, angiogenesis, and altered cell self-renewal.

joint malformations suggesting that CCN3/NOV is a regulator of skeletal and cardiac development (61).

Interestingly, in regard to liver development, no alterations were described (or addressed) in any of these models. With the further knowledge that none of the CCN genes is actively expressed in normal adult livers (5, 8, 10, 11), it is therefore frivolously tempting to speculate that CCNs might have no relevant function in liver development and maintenance of normal (adult) liver health. Surely, this view is somewhat provocative but fits quite well to the view that some (or all) CCNs become at first relevant under conditions of hepatic injury, disease and remodelling.

6. CCN PROTEINS IN INJURED AND DISEASED LIVER

As discussed in the paragraph before, it is tempting to speculate that in liver most of the biological activities of an individual CCN protein become foremost relevant during hepatic injury and remodelling. Nowadays, several biological activities of CCN proteins are known that might be essential for initiation and progression of hepatic disease (Figure 4). The cellular and tissue alterations that are induced by different disease-associated factors (e.g. viral hepatitis, alcohol, biliary obstruction, intoxication, oxidative stress, genetic disorders and predispositions)

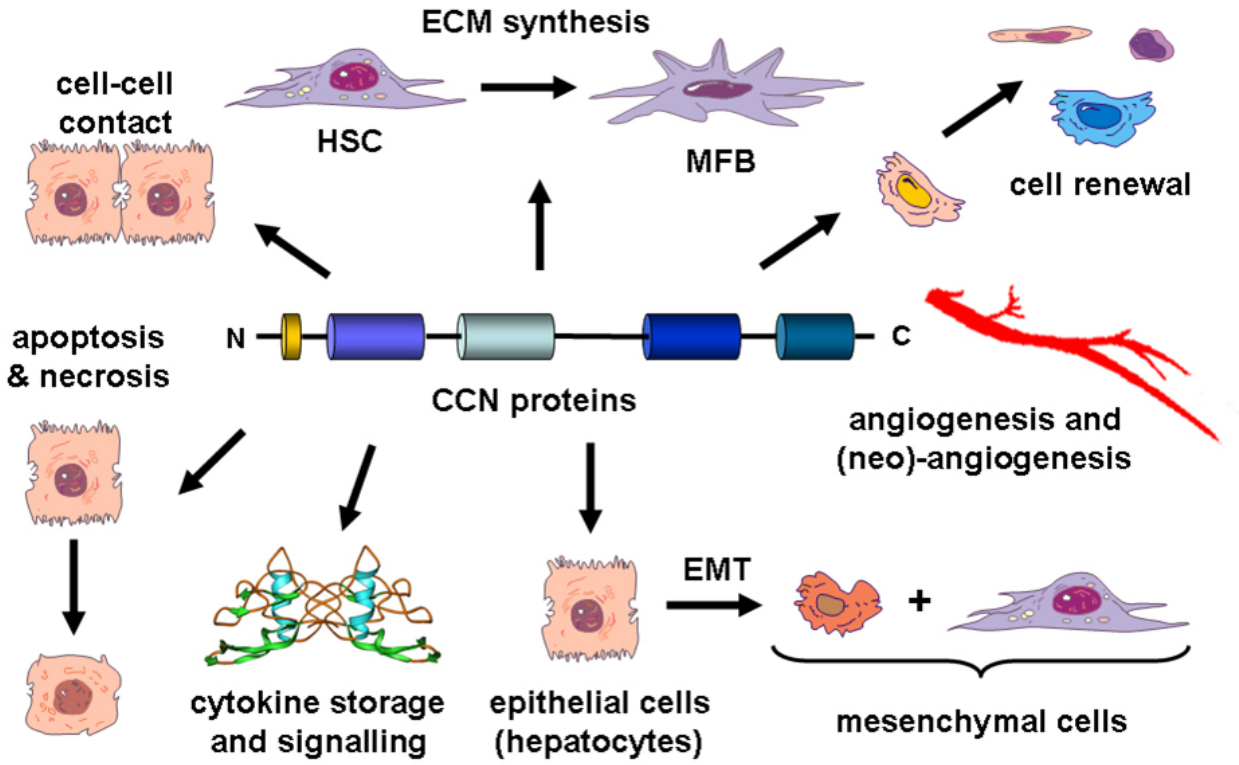

Figure 5. Proposed phenotypic liver cell-specific alterations in which CCN proteins are involved. The majority of biological effects of CCN proteins are visible during liver injury and affect the activation and transdifferentation of hepatic stellate cells (HSC) to matrix-producing myofibroblasts (MFB), cytokine signalling, regulation of apoptosis/necrosis, control of cell-cell contact, cell renewal, epithelial-to-mesenchymal transition (EMT), and angiogenesis and neo-angiogenesis.

include the activation and transdifferentiation of HSC to matrix-producing myofibroblasts (MFB), modulation of cytokine signalling, regulation of apoptosis/necrosis, control of cell-cell contact, cell renewal, epithelial-to-mesenchymal transition (EMT), and (neo)angiogenesis (Figure 5).

6.1. Liver inflammation, fibrosis, cirrhosis, and failure

The liver is the largest gland in the body that consists in humans of two wedge-shaped lobes. This organ has major functions in regulation of blood sugar, lipids and amino acids, formation of bile and cholesterol, elimination of hormones and toxic substances, creation and removal of red blood cells, production of plasma proteins and heparin, as well as storage of vitamins. This multitude of functions requires a close cooperation between the hepatic parenchymal (hepatocytes) and the different non-parenchymal cell subpopulations (HSC, Kupffer cells, and sinusoidal endothelial cells).

Liver fibrosis, that is the excessive accumulation of extracellular matrix proteins, is the common sequel of chronic liver disease and is associated with high morbidity and mortality in affected patients. It is nowadays well accepted that hepatic fibrogenesis is an active wound-healing process in which many different biological mediators and residental and non-residental cells have pivotal roles in disease initiation, promotion, and progression. Mainly triggered by several cytokines (e. g. TGF-beta and PDGF) advanced liver fibrosis results in portal hypertension, liver failure and cirrhosis representing the end stage of the fibrogenic process. Strictly, fibrosis is a histological-based diagnosis that describes a several fold elevation of matrix proteins (e.g. collagens and elastin), structural (basement) glycoproteins, proteoglycans and pure carbohydrates (i.e. hyaluronan) (for review see 63). Formal pathogenesis of hepatic fibrosis is initiated by hepatocyte destruction (necrosis) due to multiple injurious agents (e.g. alcohol, chronic infection with hepatitis virus, fat, drugs) and mechanisms followed by inflammation. Subsequently, quiescent HSC become activated in a process that is termed transdifferentiation and start to express and secrete large quantities of matrix molecules, chemokines, MMPs and their respective inhibitors (TIMPs) resulting in the phenotypic appearance of fibrosis. Although the precise mechanisms that lead to development of fibrosis are strongly dependent on the injurious agent, the resulting phenotypically alterations are more or less common.

In the last two decades it become more and more evident that the expression and secretion of (some) CCN proteins might be directly or indirectly linked with the pathogenetic sequence responsible for the formation of various hepatic lesions. In the following the actual knowledge of CCN gene expression in diseased liver is summarized.

Table 2. CCN expression in experimental and human liver disease

Member	Findings and effects	Refs
CCN1	The expression of CYR61 mRNA in nodular HCC is significantly higher than the one in solitary HCC and small HCC suggesting that CYR61 may play an important role in hepatocellular carcinogenesis and correlate with recurrence and metastasis of HCC.	87
	CYR61 gene expression is significantly down regulated in the tumors of HCC patients. A fragile d(CA) dinucleotide repeat was identified in the CYR61 promoter in HCC patients that negatively regulates promoter activity. Loss of heterozygosity or somatic mosaicism in either the tumors, adjacent normal liver tissues or both was found in 32% of all HCC patients investigated.	65, 66
	CYR61 acts as a tumor suppressor in human HCC and is involved in DNA damage response.	
	The purgative resin emodin (6-methyl-1,3,8-trihydroxyanthraquinone) that has antiproliferative capacity increases expression of CYR61 in hepatoma cells (HuH-7, Hep3B, and HepG2).	64 112
CCN2	In experimental models of liver fibrosis (bile duct ligation, CCl_4), CCN2/CTGF mRNA and protein accumulated in parallel with the development of septal fibrosis and cirrhosis. Expression of CCN2/CTGF protein and mRNA was assigned to HSC. An increase of CCN2/CTGF immunostaining was associated with a higher score of fibrosis in specimen from patients suffering from chronic hepatitis C virus infection.	67
	CCN2/CTGF mRNA is consistently upregulated in human liver cirrhosis of various aetiologies as analysed by RT-PCR and ribonuclease protection assay.	113
	Overexpression of CCN2/CTGF in human liver cirrhosis was found in 16 patients, especially in fibroblasts/MFB and HSC.	114
	CCN2/CTGF overexpression and staining in extracellular matrix was observed in liver tissue of patients with Non-alcoholic steatohepatitis (NASH). CCN2/CTGF overexpression was found in obese fa/fa Zucker rats suggesting hyperglycemia and insulin as key factors in fibrosis progression.	115
	Significant increase of CCN2/CTGF mRNA expression in rats that were treated with dimethylnitrosamine (DMN) for 2 and 5 weeks.	116
	CCN2/CTGF expression was diffusely abundant in fibrous portal tracts/septa in human congenital hepatic fibrosis in mononuclear cells that were accentuated around of proliferating bile ducts and ductules.	117
	Targeted disruption of Smad3 resulted in marked increase of CCN2/CTGF expression in immunohistochemical analyses.	
	Ribonuclease protection assay revealed increasing CCN2/CTGF mRNA level 6 hours after injection of CCl_4, with peak levels after 72 hours. Not only HSC but activated bile duct epithelial cells are the main source of CCN2/CTGF.	118 119
	Abundant CCN2/CTGF mRNA was detected in the fibrotic area between cirrhotic nodules. Hepatocytes did not show any signals even when they became carcinomas. Normal livers showed little or no expression of CCN2/CTGF.	120
	CCN2/CTGF up-regulation might be a central pathway during rat primary HSC activation. Incubation of primary HSC with recombinant CCN2/CTGF induced a significant migratory and proliferative effect. Spontaneous activation of HSC on a plastic surface and stimulation by VEGF, lipid peroxidation products, acetaldehyde, and PDGF-BB significantly up-regulated CCN2/CTGF mRNA expression in HSC.	121
	CCN2/CTGF expression was suggested as a possible pathogenetic factor for collagen- and elastin-deposition in portal tracts of patients with idiopathic portal hypertension.	122
	In fibrotic liver, CCN2/CTGF mRNA and protein are produced by fibroblasts, MFB, HSC, endothelial cells, and bile duct epithelial cells. CCN2/CTGF is also produced at high levels in hepatocytes during cytochrome P-4502E1-mediated ethanol oxidation.	123
	CCN2/CTGF was found to be strongly expressed in HSC and hepatocytes in operative biopsy specimens from patients with biliary atresia. There was a significant correlation between CCN2/CTGF mRNA intensity and the amount of collagen type IV, which implies that CCN2/CTGF expression reflects prognosis.	124,
	The HCV core protein up-regulates CCN2/CTGF expression.	125,
	CCN2/CTGF may link steatosis and fibrosis *via* increased leptin levels.	95
	Caffeine suppresses TGF-beta-induced CCN2/CTGF expression in hepatocytes by stimulation of Smad2 degradation, inhibition of Smad3 phosphorylation and up-regulation of the PPARgamma-receptor.	126
	CCN2/CTGF acts as a Smad2-dependent sensitizer of TGF-beta actions that does not influence BMP7 signalling in hepatocytes.	111
	Production of elevated CCN2/CTGF levels in hepatocytes of transgenic mice *in vivo* does not cause hepatic injury or fibrosis *per se* but renders the livers more susceptible to the injurious actions of other fibrotic stimuli.	127 128
	The CCN2/CTGF single nucleotide polymorphism rs9402373 that lies in the close proximity to CCN2/CTGF gene is associated with severe hepatic fibrosis in Chinese, in Sudanese, and in Brazilians infected with either S*chistosoma japonicum* or *Schistosoma mansoni*.	75 107
CCN3	The prevalence of CCN3/NOV expression in HCC and metastatic tumors is higher than the one in surrounding non-tumor tissue suggesting that CCN3/NOV is associated with the development of tumors in the liver.	88
	There was no obvious correlation between CCN3/NOV mRNA and clinical-pathological features of HCC.	87
	CCN3/NOV is expressed during liver fibrogenesis and HSC are an important source of hepatic CCN3/NOV. TGF-beta1 stimulated CCN3/NOV protein expression in HSC without changing its mRNA level. Dexamethasone stimulated the expression of CCN3/NOV mRNA and protein.	86
CCN4	CCN4/WISP1 and WISP1v are expressed in the HCC cell lines HepG2, HuH-6, HuH-7, and HA22T/VGH suggesting that they may have a role in the development of HCC. The authors identified a new splice variant of CCN4/WISP1 (WISP1delta ex3-4).	13
	CCN4/WISP1 is overexpressed in transgenic mouse model (c-Myc/E2F1) of liver cancer.	129
CCN5	Presently, no findings or effects were reported for this CCN protein in human liver disease. However, first reports demonstrate that CCN5 has opposing effects to CCN2 in other organs.	130
CCN6	CCN6/WISP3 is expressed in the HCC cell lines HepG2, HuH-6, HuH-7, and HA22T/VGH suggesting that they may have a role in the development of HCC. The authors identified a new splice variant of CCN6/WISP3 (WISP3vL).	13

6.2. CCN1/CYR61 expression in diseased liver

There is only limited knowledge about the possible functional significance of CCN1/CYR61 expression in liver disease (Table 2). However, it was speculated that CCN1/CYR61 acts as a tumor suppressor in HCC and is involved in the control of DNA integrity (64). In the mentioned report it was shown that (i) CCN1/CYR61 mRNA expression is down-regulated in HCC tumors, (ii) the content of CCN1/CYR61 protein is reduced in several HCC cell lines, (iii) CCN1/CYR61 overexpression suppresses cell proliferation in monolayer and anchorage-independent growth in soft agar and increases adhesion activities of HepG2 cells, (iv) down-regulation of CCN1/CYR61 by siRNA increases the cell proliferation rate, (v) stably transfected HepG2-CYR61 cells show inhibited cell mobility and reduced invasiveness, and finally (vi) exposure to 5-Fluorouracil and UV irradiation resultes in a rapid induction of CCN1/CYR61 in several hepatic tumor cell lines (64).

In line with the assumption that CCN1/CYR61 has attributes of a tumor suppressor gene is the finding that CCN1/CYR61 gene expression is significantly down regulated in the tumors of HCC patients as independently

assessed in two studies by microarray analysis and qRT-PCR (65, 66). Interestingly, the overall activity of CCN1/CYR61 was found to be significantly modulated by a d(CA) dinucleotide repeat within the CCN1/CYR61 promoter that is potentially unstable in HCC patients (66). Together, all these results support the hypothesis that the CCN1/CYR61 gene might involve anti-carcinogenic effects in the liver.

6.3. CCN2/CTGF expression in diseased liver

Most of the actual knowledge about CCN function in experimental and human liver disease was raised in studies investigating CCN2/CTGF expression and function during various conditions of liver injury (Table 2). Research of the last decade has consistently shown that CCN2/CTGF acts as a profibrogenic factor. CCN2/CTGF expression in liver is found in hepatocytes, HSC, ductular epithelial cells, inflammatory cells, and sinusoidal as well as vascular endothelial cells in pathological conditions (Table 2). However, the participation of each cell type in CCN2/CTGF expression depends on various factors to which the organ is exposed. For example, in congenital hepatic fibrosis CCN2/CTGF is predominantly expressed by bile duct epithelial cells (67). In contrast, chronic alcohol intoxication results in a strong increase of hepatocellular CCN2/CTGF, whereas periductal mononuclear cells increase CCN2/CTGF expression in idiopathic portal hypertension (68). According to these reports, it is most likely that the hepatic target cell that is causatively involved in the formation of elevated hepatic CCN2/CTGF depends on the etiologic background of the respective hepatic lesion under investigation.

However, there is no doubt about the fact that CCN2/CTGF expression in liver tissue correlates well with the extent of liver injury and that the biological inactivation of CCN2/CTGF by antisense nucleotides (69) small interfering RNA (70-72), short hairpin RNA (73) or hammerhead ribozymes (74) is sufficient to abrogate the process of ongoing hepatic fibrogenesis. *Vice versa*, transgenic FVB mice carrying the human CCN2/CTGF gene under transcriptional control of the hepatocyte-specific albumin enhancer promoter showed after chronic administration of carbon tetrachloride increased expression and deposition of collagen and elevated hepatic expression of alpha-smooth muscle actin (75). Interestingly, the liver histology and function were unaffected in these animals without exposure to carbon tetrachloride demonstrating that the production of elevated concentrations of CCN2/CTGF alone does not cause hepatic injury or fibrosis *per se* (75). On the other hand this result further indicates that CCN2/CTGF is an important prechallenging factor that requires additive factors for initiation and promotion of liver injury. In this regard it is noteworthy that CCN2/CTGF binds to and increases the biological activity of TGF-beta for the type II TGF-beta receptor complex (44). Since TGF-beta is also the main profibrogenic factor in hepatic fibrogenesis (63) and strategies targeting TGF-beta in experimental models of hepatic injury are effective in reducing the susceptibility to liver fibrosis (for review see 76), it is most likely that CCN2/CTGF acts in concert with TGF-beta. Based on the potential implication of CCN2/CTGF for disease initiation and its close biological cooperation with TGF-beta, the direct measurement of CCN2/CTGF in hepatology research and diagnostic has become a potential new modality that might provide information about the severity or outcome of liver disease (see paragraph 7.1.).

A possible functional involvement of CCN2/CTGF in the initiation phase of liver regeneration was found in a serial analysis of gene expression using the well-established model of 70% partial hepatectomy (77). In this hepatic regeneration model, CCN2/CTGF mRNA was up-regulated very early at 4 and 8 hours before the hepatocyte proliferation was observed that started around at 16 hours while the protein was strongly induced at 4 hours after the surgery, while its expression was not detectable at later regeneration time points (77). Based on this observation it was coherently speculated that CCN2/CTGF could act synergistically together with cytokines and other factors and initiate G_0/G_1 transition during the priming phase of liver regeneration (77). Again, one of the cytokines that might be relevant in this process is TGF-beta that itself effectively induces CCN2/CTGF in hepatocytes *via* the activin receptor-like kinase 5-pathway (78, 79).

Because CCN2/CTGF is both a positive binding protein (trap) that facilitates the binding of TGF-betato its cognitive receptor and a negative trap for BMP7, this CCN protein is potentially a key control factor that regulates the biological activity of these two opposing cytokines that are for example mostly relevant in epithelial-to-mesenchymal transition (EMT). This process describes an orchestrated series of molecular events in which epithelial cells loose their contact to their surrounding tissue, reorganize their cytoskeleton and initiate a completely new transcriptional programme ending in a mesenchymal phenotype (80). During the last years this emerging concept attracted much interest because it explains several important cellular changes that are observed in fibrosis and cancer in a non-developmental context. While the occurrence of EMT in kidney and other organs is already well accepted and important intracellular signal transduction pathways (e.g. TGF-beta/Smad, integrin-linked kinase (ILK), WNT/beta-catenin signalling) that drive EMT in these organs are well described (81), the existence of EMT in liver (i. e. the transition of adult hepatocytes into activated fibroblasts) and its impact on liver health and disease is presently highly controversially discussed (82-84). In this regard it should be mentioned that CCN2/CTGF was recently found to direct fibroblast differentiation from human bone marrow mesenchymal stem/stromal cells in rodents (85) clearly demonstrating that CTGF is a direct molecular mediator involved in EMT. However, the derived fibroblasts were overwhelmingly alpha-smooth muscle negative but subsequent induction with TGF-beta induced differentiation into alpha-smooth muscle positive myofibroblasts which are the cell type which is most relevant in insults such as organ fibrosis (85). Again these results indicate that CTGF and TGF-beta are closely linked and act synergistically in driving the formation of MFB and modulating aspects of ongoing fibrogenesis.

Table 3. Alterations of CTGF serum levels during liver injury

Aetiology	Correlation	Refs
Hepatitis C infection	Although independent of the underlying aetiology, serum CCN2/CTGF was most powerful in indicating fibrosis/advanced disease states in HCV-related disorders	92
Hepatitis B infection	The levels of CCN2/CTGF in the sera of patients with hepatitis B were strongly associated with the stages of hepatic fibrosis	93
Chronic hepatitis infection	Serum CCN2/CTGF was significantly correlated with the stage of liver fibrosis	94
Idiopathic portal hypertension	Overexpression of CCN2/CTGF is one of the most important features of idiopathic portal hypertension	68
Chronic inflammatory liver diseases	The mean concentration of CCN2/CTGF was highest in the fibrosis group and in the chronic viral hepatitis group but lower in those patients with fully developed cirrhosis	91
Biliary atresia	CCN2/CTGF is potentially a useful parameter for monitoring certain types of fibrotic disorders	95

6.4. CCN3/NOV expression in diseased liver

In regard to CCN3/NOV it was demonstrated that its expression is up-regulated in both *in vitro* activated HSC and *in vivo* models of experimentally-induced liver fibrosis (86). Immunhistochemistry further indicated that the expression of the CCN3/NOV protein in fibrotic rat and human livers is predominantly found in areas of ductular proliferation and HSC of the fibrous septa (86). Furthermore, the stimulation with TGF-beta and dexamethasone that was previously shown to induce the expression of CCN3/NOV, CCN2/CTGF and CCN1/CYR61 in the human glioma cell line U87 (51) resulted in strong induction of CCN3/NOV in culture-activated HSC (86). Bile acids including cholic acid, chenodeoxycholic acid and ursodeoxycholic acid that are a frequent counterpart of liver injury were also recognized to modulate *ccn3*/*nov* mRNA expression in HSC suggesting that this CCN protein has a pathogenic role in liver fibrogenesis. However, the analysis of *ccn3*/*nov* gene expression in diseased human liver is somewhat conflicting and still matter of debate (Table 2). While in one study the expression in resected specimen of thirty-one patients suffering from small, nodular or solitary large HCC revealed no significant expression differences (87), the prevalence of *ccn3*/*nov* expression in tumor tissue was found to be higher than the one in surrounding para-cancerous tumor tissue in another cohort that contained twenty-three patients with HCC and six patients with metastatic liver tumors (88). Therefore, the significance of *ccn3*/*nov* gene expression in HCC needs to be further investigated before definitive statements on CCN3/NOV functionality in HCC development can be made.

6.5. CCN4, CCN5, and CCN6 gene expression in diseased liver

There is only limited information available about the pathogenetic involvement of *WISP* genes in liver disease (Table 2). In a preliminary study using RT-PCR-based techniques it was demonstrated that WISP1, WISP1v, and WISP3 are expressed in four different HCC cell lines (i.e. HepG2, HuH-6, HuH-7, and HA22T/VGH) (13). In line with a potential involvement of *WISP* genes in the development or maintenance of HCC, it was shown that the hepatitis C virus (HCV) core protein that is implicated in the development of human HCC is able to increase expression of both WNT-1 and its downstream target gene *CCN5/WISP2* thereby inducing cell proliferation, DNA synthesis, and cell cycle progression in HuH-7 cells (89).

7. CCN PROTEINS IN DIAGNOSTIC OF LIVER DISEASE

Based on the multitude of reproduced findings that demonstrated elevated CCN2/CTGF levels in various fibroproliferative diseases, this protein was the first prototypic candidate of the CCN protein family that was supposed to be a surrogate marker of fibroproliferative diseases (90). Moreover, the fact that the suppression of CCN2/CTGF abrogated many different aspects of ongoing hepatic fibrogenesis in many experimental settings and cellular models (69-74) further supported the notion that CCN2/CTGF and maybe the five other CCN members are relevant for fibroproliferative activities in liver. Therefore, there is currently an overwhelming interest in translational biomarker research to introduce CCN2/CTGF as a disease indicator (biomarker) and to establish novel test systems for quantitative measurement of CCN2/CTGF (and other CCNs). In addition, genetic variants that introduce mutations, give rise to altered gene expression or even represent traits that are associated with a specific disease or predisposition for a disease are in the focus of intensive research. However, the present knowledge in this particular field is still limited. The relevance of CCN protein measurements in plasma, serum and urine as well the significance of gene polymorphisms in risk assessment of liver diseases will be discussed in the following two chapters.

7.1. Measurement of ccn proteins in plasma, serum, and urine

There is now a plenitude of literature consistently reporting that the measurement of CCN2/CTGF is appropriate to estimate the disease severity and/or progression of chronic inflammatory liver disease (91), ongoing fibrosis during hepatitis C and B infection (92-94), idiopathic portal hypertension (68), and biliary atresia (95) suggesting that this CCN protein is theoretically a potential new surrogate biomarker and mediator of hepatic disease that increases regardless of the insult that causes tissue injury (Table 3).

However, the reliable measurement and clinical validity of CCN2/CTGF in diagnosis of liver disease is somewhat hampered because CCN2/CTGF upregulation is also seen during insults of kidney (96), heart (97), lung (98), brain (99) and many other organs (Figure 6). Based on this close correlation of CCN2/CTGF levels with nearly all kinds of fibroproliferative responses and the incapability to discriminate between the different sources of CCN2/CTGF, it is reasonable that the usage of CCN2/CTGF as a diagnostic marker for hepatic diseases is therefore only useful when other concurrent organ lesions can be ruled out.

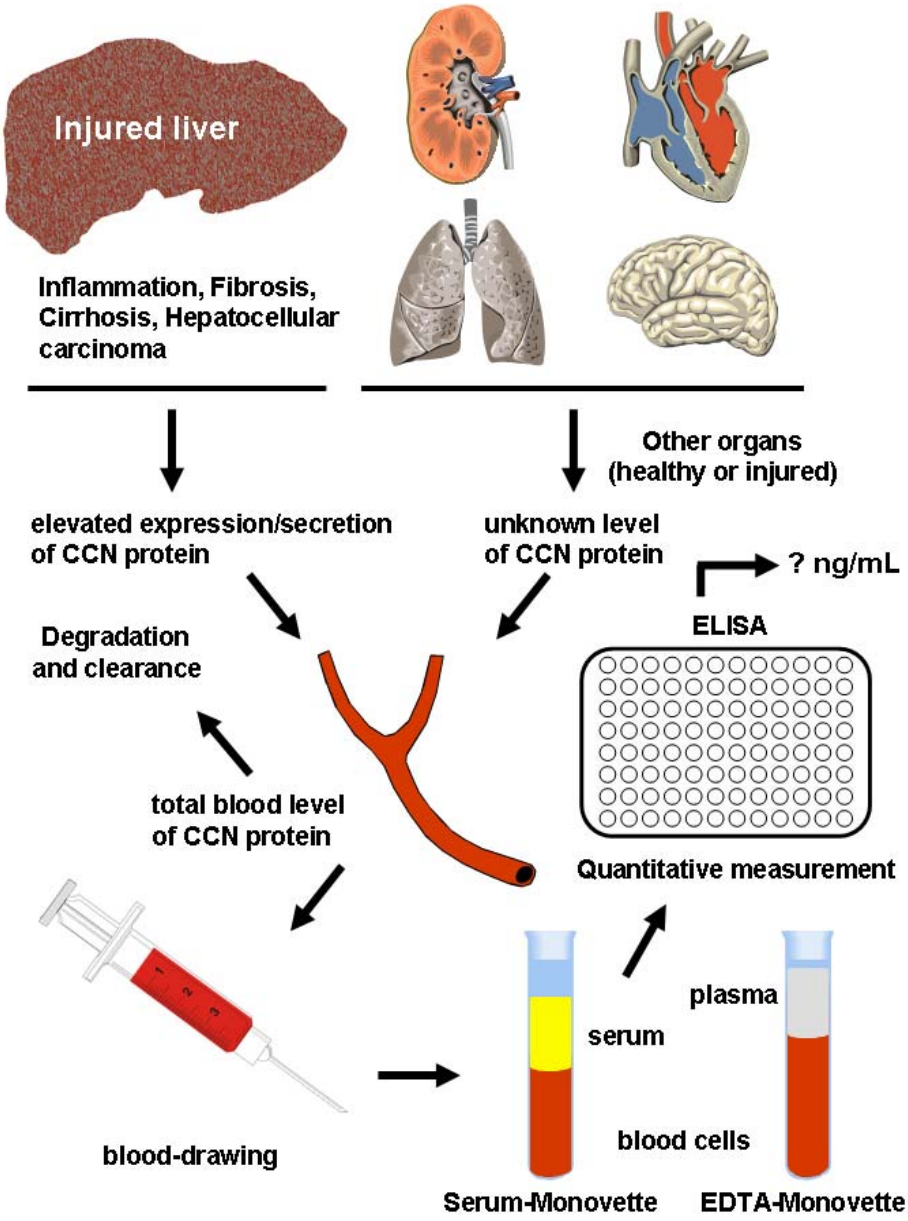

Figure 6. Diagnostic value of CCN serum/plasma analytics in diagnosis of hepatic diseases. Various liver insults (inflammation, fibrosis, cirrhosis, HCC) might result in an elevated expression of a specific CCN protein. After blood drawing the concentration of respective CCN protein representing the sum of those produced in liver and those synthesized by any other organ can be quantitatively measured from serum or plasma using for example ELISA techniques.

Chronic liver inflammation, fibrosis, cirrhosis and hepatic failure however, attend *per se* with many other complications including headache, spasms, tremors, fever, parasitic infestation, diarrhea, bleeding disorders and others that might have direct implication on CCN2/CTGF synthesis or secretion in other organs. Therefore, it is tempting to speculate that other already well-established and more specific serum marker tests (e.g. glutamic-pyruvic transaminase (GPT) = alanine aminotransferase (ALT); gamma-glutamyltransferase (GGT); alkaline phosphatase (AP); glutamic-oxaloacetic transaminase (GOT) = aspartate transaminase (AST)) and liver imaging tests (e.g. ultrasound, radioisotope imaging, computer tomography, magnetic resonance imaging) are favourable for establishing an initial diagnosis of liver diseases. Despite this documented difficulty, it might be worthwhile to routinely monitor CCN2/CTGF levels as a marker for fibrogenic activity during therapy and fulfil demands in the era of personalized medicine.

Because CCN2/CTGF is also a useful parameter for monitoring many other types of fibrotic disorders, it is not surprising that there are nowadays many commercial ELISA kits available allowing quantitative measurement of

CTGF/CCN2 concentrations. These test systems are not only appropriate to quantify CCN2/CTGF in serum and plasma samples but have been also successfully applied to detect and quantify urinary CCN2/CTGF (100, 101).

7.2. CCN gene polymorphysims in risk assessment of liver diseases

Presently, there is a controversial discussion if different gene variants and polymorphisms that are located within the coding, non-coding, or in flanking (intergenic) regions have impact on the biological activity of individual CCN proteins. Gene polymorphisms and mutations are unquestionable important genetic factors that might influence gene expression and function. Association studies that are often performed to define a possible genetic linkage between a DNA variant and a disease or predisposition for a disease are, however, extremely variable in terms of their experimental design and of the statistical power and methodology employed (102).

In the beginning most of the presented genetic association studies on CCN genes were simple case control studies that comparatively analyzed gene frequencies of one specific single nucleotide polymorphism (SNP) in a small cohort with respective controls. Thereby, several polymorphisms were identified within the human gene encoding CCN2/CTGF that might account for functional differences (103). One promising candidate identified is the G-945C substitution (rs6918698) within the promoter that alters the transcriptional activity of the CCN2/CTGF gene *in vitro* and was reported to be associated with the susceptibility to systemic sclerosis in patients from the United Kingdom (104). However, in subsequent studies this association could not be reproduced in other cohorts from North America (105) or in a large multicenter analysis study in which patients from Spain, France, Denmark, Germany, England, Sweden and North America were enrolled (106).

In regard to liver, the genotyping of six polymorphisms (rs6917644, rs9399005, rs6918698, rs9493150, rs2151532 and rs11966728) covering the entire CCN2/CTGF locus in 365 well-characterized patients suffering from chronic hepatitis C infection revealed that none of these polymorphisms showed a genotypic or allelic association with the severity of hepatic fibrosis (92). Another variant site (rs9402373) that is located in close proximity to the CCN2/CTGF gene was found to be associated with the outcome of hepatic failure during *Schistosoma japonicum* or *Schistosoma mansoni* infection in patients originating from China, Sudan, and Brazil (107). In the same study three other single nucleotide polymorphisms (rs1257705, rs12526196, rs6918698) were found to be associated with hepatic failure either in Chinese, Sudanese or Brazilian patients. However, if these results are reproducible in other (larger) cohorts or ethnicities has to be clarified in future studies.

An interesting association of two adjacent single nucleotide polymorphisms (rs3753794, 3753793) that are located upstream of the CCN1/CYR61 gene and plasma HDL-cholesterol levels was recently reported (108). In this unconfirmed study, the authors found that carriers of the A allele for rs3753794 were more likely to have high plasma HDL-cholesterol levels as compared with subjects homozygote for G at this variant site and that carriers homozygote for A at rs3753793 were more likely to exhibit low plasma HDL-cholesterol levels. These genetic findings are well interesting because epidemiology data suggest that low plasma HDL-cholesterol levels are inversely related to the risk of cardiovascular disease (109). Moreover, HDL cholesterol particles (commonly known as the "good cholesterol") prevent atherosclerosis by extracting cholesterol from the artery walls and disposing of them in the liver. On the other side, the atherogenic serum lipid profile in subjects with hepatic steatosis (commonly called fatty liver) is a factor that is strongly associated with the severity of liver injury in fatty liver disease (110) and accompanied by progressive inflammation (steatohepatitis) and fibrosis. In this regard it is further interesting that leptin induces expression of CCN2/CTGF in HSC suggesting that CCN2/CTGF resembles CCN1/CYR61 in its functional relevance in regulation of fat metabolism (111). However, additional investigations are surely necessary to estimate the pathogenetic implication of both CCN1/CYR61 and CCN2/CTGF in hepatic fat metabolism and the clinical validity of CCN1/CYR61 polymorphisms for disease assessment and risk stratification in steatohepatitis.

8. CONCLUSIONS AND PERSPECTIVES

There is no doubt that the six different CCN proteins are important for embryonic development, cellular commitment, tissue differentiation (angiogenesis, osteogenesis), and matrix remodelling as well as wound healing. Interestingly, none of the CCN genes is really actively expressed in normal adult liver and the four characterized murine gene disruption models for *ccn1/cyr61*, *ccn2/ctgf*, *ccn3/nov*, and *ccn6/wisp3* revealed no outstanding hepatic function for any member of this protein family. In sharp contrast, the expression of the CCN genes is drastically induced during any kind of liver damage (inflammation, fibrosis, cirrhosis, HCC, hepatic failure) suggesting that these proteins are pivotal in hepatic wound healing and regeneration. In line with this hypothesis is the fact that the blockade of CCN2/CTGF expression abrogates the hepatic wound healing process (fibrogenesis) in many different cellular and (rodent) injury models. Furthermore, it was shown that CCN2/CTGF and CCN3/NOV influence the proliferative capacity of HSC representing the main fibrogenic target cell in liver. They modulate cytokine actions, interact with the complex network of integrins and are involved in the extracellular matrix remodelling during hepatic fibrogenesis. Although the observed disease-associated expression changes and biochemical attributes of CCN proteins in these processes are most likely not really specific for the organ liver, first reports have clearly illustrated that CCN proteins (and in particular CCN2/CTGF) might be a new non-invasive candidate biomarker for identication and monitoring inflammatory responses or to estimate patient outcome during various hepatic diseases. It will be interesting to follow up how the increasing knowledge of CCN functions will improve disease management in hepatology and how

this group of proteins serves as aid in the diagnosis and prediction of fibrogenic and non-fibrogenic disorders in humans.

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Abbreviations: BMP(s), bone morphogenetic protein(s); CK, cystine knot; CTCK, C-terminal cystine knot; CTGF, connective tissue growth factor; CYR61, cysteine-rich, angiogenic inducer 61; EMT, epithelial-to-mesenchymal transition; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor-binding protein; LRP, low density lipoprotein receptor-related protein; MFB, myofibroblast(s); MMP, matrix metalloproteinase; NOV, nephroblastoma overexpressed gene; PDGF, platelet-derived growth factor; TGF-beta, transforming growth factor-beta; TSP-1, thrombospondin type I homology domain; VEGF, vascular endothelial growth factor; vWFc, von Willebrand factor type C domain; WISP1-3, WNT1-inducible signalling pathway protein 1-3; WNT, wingless-type MMTV integration site.

Key Words: CCN, Liver Disease, Fibrosis, Inflammation, Hepatocellular Carcinoma, Insulin-Like Growth Factor, Von Willebrand Factor, Thrombospondin, Cystine Knot, TGF-beta, Review

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