

# Role of Chemokines in the Biology of Cholangiocarcinoma

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**Abstract:** Cholangiocarcinoma (CCA), a heterogeneous tumor with poor prognosis, can arise at any level in the biliary tree. It may derive from epithelial cells in the biliary tracts and peribiliary glands and possibly from progenitor cells or even hepatocytes. Several risk factors are responsible for CCA onset, however an inflammatory milieu nearby the biliary tree represents the most common condition favoring CCA development. Chemokines play a key role in driving the immunological response upon liver injury and may sustain tumor initiation and development. Chemokine receptor-dependent pathways influence the interplay among various cellular components, resulting in remodeling of the hepatic microenvironment towards a pro-inflammatory, pro-fibrogenic, pro-angiogenic and pre-neoplastic setting. Moreover, once tumor develops, chemokine signaling may influence its progression. Here we review the role of chemokines in the regulation of CCA development and progression, and the modulation of angiogenesis, metastasis and immune control. The potential role of chemokines and their receptors as possible biomarkers and/or therapeutic targets for hepatobiliary cancer is also discussed.

**Keywords:** cholangiocarcinoma; chemokines; cancer associated fibroblasts

## 1. Introduction

Cholangiocarcinoma (CCA) comprises a heterogeneous group of biliary cancers, which can originate from cholangiocytes located at any portion of biliary tree [1]. Based on the anatomical position, this tumor can be classified in intrahepatic (iCCA) and extrahepatic (eCCA) CCA, this latter further divided into perihilar (pCCA) and distal CCA (dCCA), depending on the site within the biliary system [1,2].

CCA represents the second most frequent hepatic malignancy, accounting for 10–20% of all primary liver cancers [2,3] and its incidence is increasing dramatically [3]; accordingly, CCA mortality has increased worldwide in the last decades [4–8].

CCA is commonly asymptomatic at early stages and is often diagnosed when the disease is disseminated [9,10]. This limits the effectiveness of the current therapeutic strategies, which are preferably based on surgical resection, because antitumor drugs have only limited effects, in part owing to the high chemoresistance of this tumor [9,10]. As a result, CCA prognosis is dismal, with a 5-year survival lower than 20% [9,10].

Although the resistance to drugs is an intrinsic feature of malignant cholangiocytes [11], an additional role is played by the extensive desmoplastic microenvironment wherein neoplastic cells are embedded. Recent data support the concept that the desmoplastic stroma, that is a main feature of CCA, contributes to the decreased sensitivity of this tumor to drug-cytotoxicity, hampering responses to chemotherapy and resulting in a poor clinical outcome [1,2,4].

Although most CCAs are diagnosed *de novo* without an apparent liver disease background, there are well-established risk factors indicating that the appearance of CCA is favored in the context of chronic inflammatory conditions of the biliary tree (such as primary sclerosing cholangitis (PSC)) [1]. In this setting several autocrine, paracrine and endocrine signals concur to modify the environment in which tumors eventually develop: a spectrum of soluble factors (growth factors, cytokines, chemokines and proteases), released in a dysregulated fashion, sustain the inflammatory response and induce the ECM remodeling, promoting CCA initiation and progress [12]. Among these, chemokines are emerging as key factors in the complex network of events involved in development, invasiveness and immune evasion of several malignancies, including CCA.

In this review we will summarize the most recent findings on the role played by chemokine-induced signals in driving CCA malignancy. We will also focus on the possible mechanisms responsible for CCA drug resistance involving chemokine systems. Finally, we will highlight recent developments on the role of chemokines and their receptors as possible predictive and prognostic biomarkers and their potential employment as therapeutic targets for hepatobiliary cancers, also discussing the current limitations of this approach.

## 2. CCA and CCA-Associated Tumor Microenvironment

Stromal desmoplasia is a prominent histopathological hallmark of CCA that profoundly affects neoplastic ducts, contributing to CCA pathogenesis [5,6].

The highly reactive microenvironment is a dynamic and sophisticated compartment consisting of activated fibroblasts (cancer-associated fibroblasts, (CAFs)), endothelial and immune cells (tumor-associated macrophages (TAMs), neutrophils, natural killer (NK) cells, T and B lymphocytes) embedded in a non-physiological, fibrillar ECM [1].

CAFs, the major cellular components of desmoplastic stroma, play a critical part in biliary carcinogenesis, from neoplastic transformation to tumor dissemination. Their activation is due to a wide range of soluble mediators produced by tumor cells, as well as by the multiple inflammatory cells populating the desmoplastic stroma. By secreting growth factors, cytokines and chemokines (CXCL2, CXCL12, CXCL14), CAFs recruit inflammatory and endothelial cells, sustaining neoangiogenesis and lymphangiogenesis [7]. Moreover, CAFs elicit ECM structural changes, further supporting desmoplastic stroma and promoting cancer invasiveness [7].

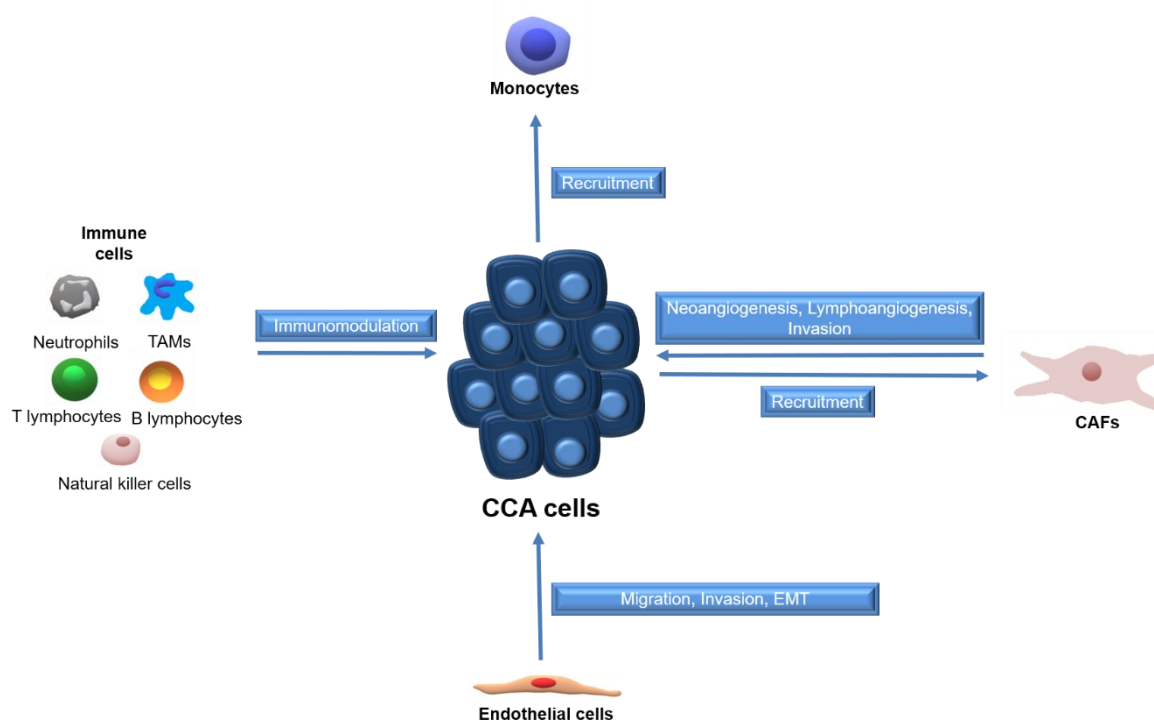
Among the immune cell types infiltrating the desmoplastic stroma, TAMs play a crucial role in regulating angiogenesis, lymphangiogenesis, tumor proliferation and modulating ECM changes [1], through the release of inflammatory mediators [8].

Tumor-infiltrating lymphocytes (TILs) represent a highly heterogeneous populations [9,10] that comprise CD8<sup>+</sup> cytotoxic T cells, CD4<sup>+</sup> T helper cells, Tregs and B lymphocytes. Whereas high levels of CD4<sup>+</sup> and CD8<sup>+</sup> within CCA microenvironment have been associated with better prognosis [10–12], low numbers of CD8<sup>+</sup> TILs are correlated with poor overall survival [13]. Regarding B cells, no data on their pathogenic role in CCA are available, even if high densities of CD20<sup>+</sup> B cells have been observed in low-grade tumors and associated with a favorable overall survival [9,10]. Little is known regarding the pathogenic role of NK cells in CCA, although, according to recent studies, these cells seem to inhibit CCA growth and reduce tumor chemoresistance [11,12].

The role of neutrophils in CCA is still indefinite, even a significant commitment of infiltrated tumor-associated neutrophils (TANs) in CCA tissues has been reported [14].

Tumors employ several mechanisms to establish a functional vascular system comprised of both blood and lymphatic vessels, to sustain cell growth [15,16]. CCA cells promote neo-vascularization by enhanced expression of angiogenic growth factors, whereas endothelial cells can release inflammatory chemokines to attract leukocytes and establish a pro-fibrotic and pro-angiogenic

milieu, which in turn support migration, invasion and EMT [17]. The paracrine effects between CCA cells and surrounding stromal cells are summarized in Figure 1.



**Figure 1.** Schematic representation of paracrine effects between stromal cells and CCA cells. Tumor associated macrophages (TAMs), cancer-associated fibroblasts (CAFs), cholangiocarcinoma (CCA).

### 3. Chemokines Ligands and Receptors

Chemokines are a family of highly conserved small (8–12 kD) proteins, sharing the ability to chemoattract leukocytes. In humans, 48 chemokines have been identified, classified in four groups, according to the position of the first cysteine residues in their N-terminal sequence: XCL, CCL, CXCL and CX3CL, where X represents any other amino acid [18,19]. Chemokine signaling is transduced by G protein-coupled receptors, also divided in four groups (XCR, CCR, CXCR and CX3CR). Among the 19 receptors identified, most can bind to different chemokines, generally belonging to the same subfamily, with variable affinity and different functions [20]. Similarly, some chemokines bind and activate more than one receptor. As an important consequence of this promiscuity, chemokine-receptor interaction and the resulting signaling cascade are finely modulated, in concert with the modifications of the microenvironment.

Chemokines can also bind and activate a different category of receptors, named atypical receptors (ACKR1-6) [21], which show extreme ligand promiscuity. Although their functions are not fully elucidated, most of them appear to act as decoy receptors, negatively modulating the activation of “main” chemokine receptors [22]. ACKRs lack a G protein activation motif and ligand-receptor interaction leads to  $\beta$ -arrestin recruitment and subsequent internalization and degradation/recycling of the ligand-receptor complex, thus serving as a chemokine reservoir or scavenger [21,23,24]. Deregulation of ACKR expression has been reported in many tumors and appears to correlate with the metastatic process [25].

Chemokines were firstly reported as key effectors of immune and inflammatory reactions, driving recruitment and homing of leukocytes into infected or injured tissues [26]. Subsequently, an essential role in several pathophysiological processes, including organ development, tissue homeostasis, angiogenesis and cancer has been recognized [27]. According to their biological functions, chemokines can be distinguished in homeostatic, which are constitutively expressed in specific cell types and contribute to immune homeostasis, and inducible, whose expression is related to certain conditions, such as inflammatory responses. Concomitantly, leukocytes express a broad

spectrum of receptors, making them susceptible to many chemokine ligands [28]. In particular, CC ligands mainly act on monocytes/macrophages and T cells, while CXCL1–8 primarily exert their chemotactic action on neutrophils and CXCL9–11 on T-cells. Finally, a few chemokines display both homeostatic and pro-inflammatory functions, depending on the localization and the timing of expression [28,29].

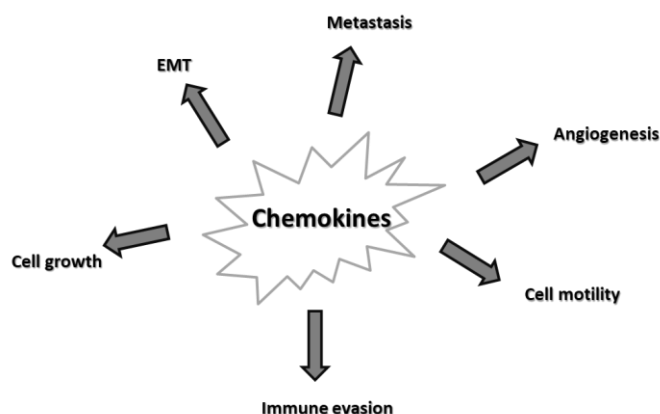
#### 4. Regulation of Chemokine Expression and Effects

A variety of mechanisms have developed to control chemokine expression and/or activity, in order to ensure a proper cell trafficking and homing during innate and adaptive immune response. A number of polymorphisms have been identified in genes encoding chemokine and chemokine receptors, resulting in an altered expression/stability or improper ligand-receptor interaction. For instance, nine SNPs have been described in *CCR2* gene, associated with various disorders, including cancer [22]. Alternative splicing of precursor mRNAs has been observed for chemokine receptors or their ligands. Splice variants can exhibit different functions and be implicated in pathological conditions, such as cancer. Interaction with glycosaminoglycans (GAGs) is essential to maintain high chemokine levels in the site of release and altered chemokine binding to GAGs can result in impaired leukocytes extravasation [30]. GAG-chemokine interactions also influence the chemokine pattern and, consequently, the leukocyte populations recruited in specific areas. Finally, GAGs promote chemokine oligomerization preserving them by proteolytic cleavage and modulating chemokine-receptor linking [31]. Proteolytic cleavage can occur at either N- or C-terminal region by several proteases [32], including metalloproteases [33], dipeptidyl peptidase 4 (DPP4) [34] or cathepsin B [35]. Cleaved chemokines can display either reduced or increased activity, or different receptor selectivity. Inactivation of chemokines through proteolytic cleavage may be an efficient mechanism adopted by cancer cells to evade immune response [36], as recently demonstrated for CXCL9–11 and CX3CL1 [37]. Other post-translational modifications of chemokines and their receptors include O- and N-glycosylation, citrullination, ubiquitination, sulfation, nitration and nitrosylation [22]. These processes have been shown to affect protein localization, stability and clearance, as well as their chemotactic properties [22].

#### 5. Chemokines and Cancer

Aberrant expression of chemokine ligands and receptors has been observed in several tumors, concurring to altered chemokine functions that contribute to tumorigenesis, sustained by inactivation of tumor suppressor genes, constitutive activation of transcription factors or deregulation of oncogenes regulating chemokines [38]. Indeed, in many tumors a constitutive activation of nuclear factor- $\kappa$ B (NF- $\kappa$ B) is associated with expression of chemokines that promote carcinogenesis [39]. Hypoxic conditions frequently occurring in tumor microenvironment lead to overexpression of chemokine ligands and receptors, both in cancer and stromal cells [40,41]. Cancer metabolism represents an additional element in chemokine regulation. Aerobic glycolysis and lactic acid were reported to induce NF- $\kappa$ B activity, and to increase CXCL8 expression and angiogenesis in breast and colon cancer [42]; ROS release has been associated with overexpression of CXCL14 and enhanced invasion and motility [43].

Alterations in the chemokine system are implicated in many aspects of tumorigenesis, as depicted in Figure 2 and listed below.



**Figure 2.** Major effects of chemokines on cancer. Epithelial-mesenchymal transition (EMT).

### 5.1. Tumor Growth

Most chemokine/GPCRs sustain cancer cell survival and proliferation, which are mainly mediated by activation of mitogen-activated protein kinases (MAPKs) [44,45] and PI3K/Akt [46] pathways. In contrast, some chemokine systems can transduce inhibitory signals, e.g., CCR1 [47] or CCR5 [48].

### 5.2. Epithelial-Mesenchymal Transition (EMT)

The CXCL8/CXCR1 system has been frequently associated to EMT [49–51]. Among atypical receptors, CXCR7 (ACKR3) has been reported to induce EMT and sustain tumor development in bladder cancer [46].

### 5.3. Angiogenesis

A high variety of chemokines directly or indirectly affect angiogenesis, with positive or negative actions. Angiogenic effects have been reported for CXCL1–3, CXCL5–6, CXCL8, CXCL12, CCL2, CCL11 and CCL16 [52,53]. In general, chemokines displaying the ELR motif, which allows leukocytes to roll on activated endothelium and migrate to the site of injury, are angiogenic [54]. Angiogenesis can be mediated by the expression of pro-angiogenic factors (such as vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF) and others), or directly promoting endothelial cell recruitment and proliferation. Alternatively, these chemokines can recruit immune cells, as neutrophils, dendritic cells (DCs), myeloid derived suppressor cells (MDSCs) and TAMs [55–57] able to secrete angiogenic factors [39,58,59]. MDSCs and TAMs can even adopt endothelial cell features, contributing to vessel formation [60].

### 5.4. Metastasis

Changes induced by chemokines and their receptors on endothelium are crucial for cancer cell migration, invasion and metastasis. Chemokines released by the tumor microenvironment (TME) increase vessel permeability, promoting intra/extravasation and migration of malignant cells expressing the appropriate receptors and driving them to distant organs [61]. A primary role is played by TAMs, whose recruitment/activation is mainly mediated by CCL2, although other proteins, as CCL3, CCL5, CCL8 [62] or CCL18 can be also effective. Some of these molecules, e.g., CCL3, CCL8, CCL22, further sustain chemokine secretion, thus favoring the accumulation of pro-metastatic immune cells [63].

### 5.5. Immune Evasion

As mentioned above, many tumors express proteinases able to process and inactivate chemokines, thus impairing leukocyte recruitment and host defense [37]. Cancer cells, as well as the

diverse cell types of the surrounding stroma, produce cytokines and chemokines, such as CXCL5 and CXCL8 that induce neutrophil recruitment and phenotypical transition into pro-tumorigenic MDSCs [64–66]. In the TME, MDSCs exert pro-cancer actions secreting soluble factors able to suppress TIL trafficking and anti-cancer activity [67,68].

## 6. Chemokines and CCA

Molecular mechanisms favoring the development of a tumor reactive stroma (TRS) are crucial in the progression of CCA. Gene expression profiling of human CCA tissues identified a number of stromal-specific dysregulated genes correlated with poor clinical outcome, including genes encoding chemokines or chemokine receptors (CXCR4, CCR7, CCL2, CCL19, CCL21) [69]. CAFs have been identified as major contributors of soluble mediators with pro-tumorigenic functions, and CCA cells co-cultured with CAFs or exposed to CAF conditioned medium exhibit increased survival, proliferation and motility [70–72]. In addition, immunodeficient mice co-inoculated with CCA cells and myofibroblastic hepatic stellate cells (HSCs) showed higher tumor development respect to animals only injected with CCA cells [73,74]. Moreover, CAF depletion in TRS reduced tumor growth in a rat model of CCA [75]. Thus, chemokines involved in the cross-talk between tumor and stroma can modulate the biological activities of cancer cells, as growth and invasiveness, acting in autocrine or paracrine fashion [64]. Soluble factors secreted by CAFs also recruit and activate inflammatory and endothelial cells, providing additional mechanisms to sustain tumor progression and metastasis [76].

In addition, chemotactic factors released by both tumor and stromal cells contribute to recruitment and activation of TAMs [77]. Soluble mediators also induce the switch toward the M2 macrophage phenotype, although M1 and M2 features can often coexist [78]. Recently, Raggi et al. [19] have shown that CCA stem-like cells (CSC) can be involved in recruitment of circulating monocytes and their differentiation into TAMs. These CSC-associated TAMs co-express M1- (CXCL9 and CXCL10) and M2-related (CCL17 and CCL18) chemokines, suggesting that diverse TAM subpopulations can coexist in the TRS, displaying different phenotype and functions, depending on the components of the stromal milieu.

Under the chemotactic action of tumor-secreted chemokines, mesenchymal stem cells (MSCs) can be also recruited into the primary tumors. MSCs release soluble mediators that contribute to cancer progression, by promoting angiogenesis, impairing immune cell activity and increasing cancer invasiveness [79–81].

In the next sections, an in depth analysis of the main chemokine systems involved in biliary malignancies is presented. They are also summarized in Table 1.

**Table 1.** Major chemokines and their receptors involved in CCA biology.

Chemokine Family	Chemokine	Chemokine Receptor	Target Cells	Key Functions	References
CXCL	CXCL12	CXCR4	CAFs CCA cells	CCA cell survival, migration and invasion; EMT transition; metastasis; poor prognosis	[71,82–84]
		CXCR7	CCA cells	CCA cell adhesion, migration, invasion, growth and survival	[85]
	CXCL7	CXCR2	CCA cells Fibroblasts Immune cells	CCA cell proliferation and invasion; poor prognosis	[86]
	CXCL9	CXCR3	CCA cells Immune cells Fibroblasts	Inflammation	[87]
	CXCL5	CXCR2	CCA cells Neutrophils	CCA cell migration and invasion; poor prognosis; neutrophil infiltration	[88–91]
CCL	CCL2	CCR2	Monocytes, Macrophages	Immune cell migration; poor prognosis	[77,92]

MDCs					
	CCL5	CCR5	CCA cells Immune cells Stem cells	CCA cell migration and invasion	[93]
	CCL20	CCR6	CCA cells Immune cells	CCA cell migration; EMT transition	[94,95]
CX3CL	CX3CL1	CX3CR1	Mononuclear cells	Infiltration of immune cells; inflammation	[96,97]

## 7. CXCL12/CXCR4 and CXCL12/CXCR7

CXCL12, also known as stromal cell-derived factor-1 (SDF-1), is a member of the CXC chemokine subfamily expressed in many tissues and cell type [98]. CXCL12 binds to two different receptors: CXCR4 and CXCR7 [99]. In solid tumors, CXCL12 can be secreted both by cancer cells and CAFs [99], playing a role in paracrine or autocrine fashion, through either CXCR4 or CXCR7. CXCR4 is a highly conserved receptor expressed on various cell types, including several cancer cells [100]. In the circulatory system CXCR4 regulates hematopoietic stem cell homing to the bone marrow, and trafficking of hematopoietic cells and lymphocytes [101].

CXCR4/CXCL12 interaction triggers different downstream pathways in cancer cells or in several cells of TME, resulting in diverse biological responses, as angiogenesis, metastasis, proliferation and survival [102,103].

Upregulation of CXCR4 expression has been observed in different human cancers [104], and the CXCL12/CXCR4 axis is often considered a hallmark of cancer aggressiveness and correlates with tumor size, grade and recurrence [105–108], poor prognosis [106] and low survival [109]. In some tumors, it is also critical for drug-resistance [110,111].

CXCR7, also known as ACKR3 and previously considered only a scavenger receptor, binds to CXCL12 with higher affinity than CXCR4 [112]. Binding of CXCL12 to CXCR7 induces non-G protein-mediated  $\beta$ -arrestin accumulation and subsequent ERK activation [113].

CXCR7 and CXCR4 can form heterodimers, shifting the CXCL12-induced signaling from G-proteins-dependent to  $\beta$ -arrestin-dependent signals [114,115].

CXCR7 expression has been observed in fetal liver cells, activated endothelial cells and various tumor cells, as well as in malignancy associated blood vessels, where CXCR7 induces cell growth, survival and increased adhesion [116].

Increased expression of CXCR7 has been observed in pancreatic, prostate, liver, ovarian, kidney, colon, breast and lung cancer [116], and in general there is a positive correlation between CXCR7 expression and tumor malignancy [117]. In addition, high expression of CXCR7 confers a high risk of developing lymph node metastasis [118].

The role of CXCL12/CXCR4 axis in CCA has been extensively examined, mostly highlighting a paracrine function of this pathway. Ohira S. et al. [119] demonstrated that migration, but not proliferation, of iCCA cells is induced by CXCL12 released by WI-38 fibroblasts. Moreover, CXCR4 expression in cancer cells increased upon treatment with tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), released both by TAMs and tumor cells. In human iCCA tissues TNF- $\alpha$  is mainly expressed in infiltrating macrophages, while CXCR4 is present in cancer cells but not in non-neoplastic ducts and CXCL12 in stromal fibroblasts and, to a lesser extent, in tumor cells [119]. In another study, CXCL12 release by fibroblasts was downregulated by transforming growth factor- $\beta$  (TGF- $\beta$ ) secreted by iCCA cells, identifying a regulatory mechanism of CXCL12 secretion. In addition, immunohistochemistry analysis showed that the highest expression of CXCL12 in stromal fibroblasts and the least levels of TGF- $\beta$  in iCCA cells are found at the invasive front of the tumor, probably favoring CCA invasion [82]. On the other hand, CXCL12 is positively modulated by angiotensin II in HSCs, and in turn CXCL12 increases activation and proliferation of HSCs, acting in autocrine fashion. In cancer cells, this chemokine induces EMT transition, invasion and migration. Notably, in human iCCA, CXCR4 is also expressed in fibroblast-like stromal cells [83]. The effect of the autocrine action of CXCL12 in CCA was recently highlighted in a study where high CXCL12 expression was associated with

metastasis and poor prognosis, and CXCL12 knockdown in CCA cells reduced their migration and invasion, but not proliferation [84].

CXCL12/CXCR4 signaling in CCA cells has also been explored. ERK1/2 and PI3K are involved in CXCL12-induced invasion in pCCA cell lines [120]. More recently, CXCR4 was found upregulated in cancer cells expressing CD24, a cell surface protein associated with adverse prognosis of CCA, together with p-ERK1/2 [120]. Our research group reported that, upon CXCR4 activation, Akt and ERK show an oscillatory pattern of phosphorylation, similarly to that observed in other cancer cells. Moreover, we showed that CXCR4 expression was remarkably higher in iCCA human tissues, compared to non-neoplastic tissues and detected CXCL12 in HSCs conditioned medium capable to promote migration of iCCA cells [71].

CXCL12/CXCR4 interaction can also induce activation of the canonical Wnt pathway that plays a key role in iCCA growth, metastasis and cancer susceptibility. Moreover, CXCR4 levels closely correlate with tumor progression, metastasis and lower overall survival. CXCR4-depleted cells showed reduced growth and reduced tumorigenesis in mice xenografts [121].

CXCR4 expression is also upregulated in eCCA human tissues, with positive correlation with lymph node metastasis and neural invasion [122]. The same group later reported an interaction between metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) and miR-204, which affects human eCCA growth and invasion directly targeting CXCR4. MALAT1 levels were higher in eCCA tissues than in surrounding non-tumor areas and negatively correlated with survival of eCCA patients [123].

A recent study reported an inverse correlation between the levels of osteopontin (OPN) and CXCR4. Low levels of circulating OPN were associated with aggressive characteristics, poor prognosis and low efficacy of chemotherapy in iCCA patients. OPN could inhibit the aggressiveness of cancer cells by negatively regulating metalloproteinase (MMP)1, MMP10, and CXCR4 [124].

A recent study, conducted by our group, has focused on the role of CXCL12/CXCR7 interaction in CCA. We found that CXCR7 was overexpressed in iCCA cells and analyzed its role using two CXCR7 antagonists and genetic interference. The CXCL12/CXCR7 axis was found to regulate adhesion, migration, invasion, survival and growth of iCCA cells. Noteworthy, the expression of CXCR7 was higher in CCA stem-like cells, and CXCR7 down-regulation reduced the ability to form stem-like cell enriched spheres, supporting the relevance of CXCR7 in tumor aggressiveness [85].

The above-illustrated data suggest the possible role of the CXCL12/CXCR4/CXCR7 axis as therapeutic target. Some inhibitors of CXCR4, like AMD3100, have been widely used in CCA studies [71,119,120]. They have been approved by the FDA and are clinically feasible [125]. Recently pharmacological inhibition of CXCL12/CXCR4 axis has been combined to anti-PD-L1 immunotherapy [126,127]. Along these lines, Xie et al. [128] developed PCX polymers from either AMD3100 or novel CXCR4-inhibiting monocyclam inhibitors that, besides affecting CXCR4/CXCL12 axis, introduce nucleic acids into the cancer cells to enhance the anticancer efficacy. The combined treatment, consisting of PCX and miR-200c, synergistically inhibited CCA cell migration, due to CXCR4 blockade and EMT prevention.

## 8. CCL2/CCR2

CCL2 exerts a key role in inflammatory reactions, promoting extravasation of a high variety of immune cells [129–131]. Several cells secrete CCL2, either constitutively or upon stimulation, including monocytes, smooth muscle cells, fibroblasts, epithelial and endothelial cells [131,132]. CCL2 acts through the CCR2 receptor [133], which exists in two alternative spliced forms, CCR2A and CCR2B, differing in the C-terminal tails [134]. CCR2A is expressed by mononuclear cells and vascular smooth muscle cells [135], whereas CCR2B is predominantly expressed in monocytes and activated NK cells [135].

CCL2 can also interact with the atypical chemokine receptors ACKR1 and ACKR2 [136], that are mainly expressed by non-leukocyte cell types [136,137] and regulate chemokine gradients [136,137].

As a mediator of inflammation, CCL2 recruits inflammatory cells, modifying the environment in which cancer eventually develops.

CCL2 plays a crucial role in the pathogenesis of liver disease, not only mediating inflammation but also favoring the activation of pro-fibrogenic cells [138]. In the chronically inflamed liver, the accumulation of monocyte-derived macrophages, recruited by CCL2, represents a critical event in the angiogenic and fibrogenic process that may lead to tumor development [139]. Current evidence shows that CCL2 is involved in tumor initiation and progression, by mediating monocyte recruitment/maturation into TAMs, as well as acting on stromal and tumor cells to modulate angiogenesis, metastasis and cancer cell proliferation [92]. Andersen et al. [69] found altered CCL2 expression in the microenvironment of human iCCA specimens, where a stromal signature characterized by upregulation of IL-6 and TGF- $\beta$ 3, in association with poor prognosis, was identified. Lin et al. [140] have shown that CAFs, isolated from samples of iCCA patients, are the major source for CCL2. Moreover, fibroblast activation protein (FAP), a serine protease selectively expressed by CAFs in solid tumors [141], was also expressed in CAFs from iCCA patients, where induced STAT3 activation and CCL2 secretion, greatly reduced by FAP knockdown [140]. In vitro studies revealed that FAP has a critical role in CAFs isolated from iCCA to mediate migration of MDSCs via CCL2 [140]. Finally, in iCCA xenograft model, CCL2 stimulated cell proliferation, induced angiogenic factors (VEGFA, MMP2, MMP9, MMP12, Angiopoietin II) and mediated migration of MDSCs, macrophages [140] and other immune cells, promoting cancer growth [142].

## 9. CCL5/CCR5

CCL5 is widely established as an inflammatory chemokine. CCL5 represents a target gene of NF- $\kappa$ B and is expressed by many different cells, including certain types of tumor cells [143]. CCL5 activity is mediated by CCR1, CCR3 and mainly CCR5 [144]. Upon ligand binding, CCR5 stimulates cell proliferation and survival, glycolysis, immune cell differentiation and growth of progenitor and stem cells [145].

CCL5 recruits several leukocyte subsets towards the injured site, including T cells, macrophages, eosinophils and basophils. In association with cytokines released by T cells, such as IL-2 and IFN- $\gamma$ , CCL5 induces activation and proliferation of specific NK cells, known as CC chemokine-activated killer cells [143]. Nonetheless, its precise role in tumor development is still unclear. It has been reported that the CCL5/CCR5 axis influences cancer cell proliferation, invasion and the establishment of an immunosuppressive microenvironment [146]. In certain cancer cells CCL5 has been found to promote angiogenesis, through down-regulation of miR-200b via the PI3K/Akt pathway [93]. In bone-marrow-derived human mesenchymal stem cells under inflammatory state (MSC-TI) CCL5 expression was upregulated and increased expression of CCR1, CCR3 and CCR5 was found in CCA cells treated with MSC-TI conditioned medium [147]. Moreover, when either Maraviroc, a CCR5 antagonist [148], or anti-CCL5 antibodies were added to conditioned medium, CCA cell motility was inhibited, suggesting that CCL5 may represent a key factor in influencing CCA biology. Accordingly, when CCA cells were stimulated with CCL5, the chemokine promoted cell migration and invasion, by inducing phosphorylation of Akt together with an increase in MMP2 and MMP9 expression.

## 10. CXCL7/CXCR2

CXCL7 (also known as NAP-2) is a platelet-derived chemokine. It is mainly expressed in platelets as an inactive precursor and its active form is generated at the injured site. CXCL7 belongs to a subset of seven chemokines characterized by a N-terminal ELR motif, which are agonists for CXCR2 [149]. Interacting with CXCR2, CXCL7 functions as a potent chemoattractant and activator of neutrophils [150].

This chemokine participates in diverse cellular processes, such as DNA synthesis, mitosis and glycolysis, and has been implicated in tumor growth [151,152]. It is also implied in different aspects of fibroblast metabolism, such as synthesis of matrix components (hyaluronic acid and glycosaminoglycans) and increase of glucose transporter GLUT1 expression, with consequent glucose uptake [153].

In CCA, CXCL7 has been found to be mostly expressed in tumor tissues in respect to adjacent non-tumor areas, and its overexpression has been associated with a poor prognosis [86]. Both CXCL7

and its receptor are upregulated in intra- and extrahepatic CCA cell lines and sustain cell proliferation and invasion, via Akt. In addition, exposure of CCA cells to CXCL7 enhanced their malignant phenotype. Similar effects were obtained by treating CCA cells with conditioned medium of CXCL7-overexpressing cells, suggesting that this chemokine could modulate CCA features in both autocrine and paracrine manner [86].

## 11. CXCL9/CXCR3

CXCL9 is an ELR-motif negative-CXC chemokine, induced by IFN- $\gamma$ . It is secreted by different cells, such as macrophages, endothelial cells, hepatocytes and cancer cells [154]. The transcriptional regulation of CXCL9 is a multistep process involving several transcription factors, including STAT1 and NF- $\kappa$ B [155]. CXCL9 is the ligand for CXCR3, through which it mainly acts as chemoattractant for activated immune cells, as T lymphocytes and NK cells. CXCR3 is highly expressed on several immune cells as well as endothelial, epithelial cells and fibroblasts [156,157]. Several studies have demonstrated that abnormal CXCR3 expression is involved in inflammation, angiogenesis, tumor appearance and immune response [158–161]. In humans three differential spliced forms of CXCR3 have been reported: CXCR3-A, CXCR3-B and CXCR3-alt. CXCR3-A stimulates chemotaxis, proliferation and metastasis, whereas CXCR3-B blocks angiogenesis, migration, cell growth and increases apoptosis, and CXCR3-alt mainly acts as a decoy receptor [162]. Generally, CXCR3 controls many signaling pathways, including MAPK, PLC and PI3K [163–165].

Several studies have reported a role for CXCL9 in regulating tumor biology, with controversial results. In a study by Gorbachev et al. [166], CXCL9-deficient fibrosarcoma cells showed higher malignant phenotype than CXCL9-sufficient counterparts. In other reports, CXCL9 promotes tumor growth [167]. The two splicing variants, CXCR3A and CXCR3B, appear to display opposite functions, with pro- and anti-tumor activity, respectively [167–170]. CXCL9 has been identified as a tumor suppressor, because in 70 iCCA resection specimens high levels of CXCL9 were associated to a favorable postoperative survival. High CXCL9 also correlated with remarkable abundance of tumor-infiltrating NK cells [87]. The protective role of CXCL9 was confirmed in a mouse model of iCCA, in which CXCL9 knockdown led to greater tumor masses, affecting NK cell recruitment into tumor areas. Fukuda et al. also investigated the impact of CXCL9 on tumor cell biological properties in vitro, demonstrating that CXCL9 was released in response to inflammatory stimuli by CCA cell lines. Moreover, exposure of CCA cells to CXCL9 did not modify either invasion or proliferation rate. These data suggest that boosting the CXCL9 system could represent a promising therapeutic approach, being involved in immunopotentialization [87].

## 12. CCL20/CCR6

CCL20, also known as liver activation regulated chemokine (LARC) or macrophage inflammatory protein-3 (MIP3A) is a small chemokine expressed in several immune cells, including NK [171], neutrophils [172], T helper (Th) 17 cells [173], B-cells [174] as well as in various organs and tissues [175]. As a rare feature among the CC chemokines, CCL20 has an exclusive known receptor, CCR6 [176,177]. CCR6 is expressed in various leukocyte subsets, including CD34<sup>+</sup> hematopoietic precursor-derived DCs [178], and memory T [179] and B cells [180]. It can function as an immune mediator, linking immature DCs (iDCs) to adaptive immune response. Once iDCs take up antigen, mature and activate, CCR6 expression is down-regulated and CCR7 up-regulated [181–183]. The presence of CCL20 in pro-inflammatory Th17 and regulatory Treg cells suggests that the CCL20/CCR6 axis may regulate both immune activation and suppression [94]. CCL20 exerts a strong chemotactic effect on lymphocytes and a weaker action on neutrophils.

CCL20 has been recently related to CCA. In order to identify shared transcriptional networks in CCA and potential therapeutic targets, Maung et al. [95] analyzed multiple microarray datasets, selecting from Gene Expression Omnibus (GEO) repository [184,185] a number of over-expressed genes, including CCL20, associated with cell cycle, motility and cytokine responsiveness [95]. Exposure of iCCA cells to CCL20 resulted in increased migration and enhanced expression of EMT markers, such as N-cadherin, whereas knockdown of CCR6 reduced CCA cell motility.

These data suggest that targeting CCL20/CCR6 axis could be a new approach for cancer treatment and GSK3050002, a CCL20 neutralizing has been used in a clinical trial to test its safety in human subjects [186].

### 13. CXCL5-CXCR2

CXCL5, also known as epithelial-derived neutrophil-activating peptide-78 (ENA-78) is secreted by inflammatory and endothelial cells of various organs in response to insults [2]. Binding to its receptors, such as CXCR2, which is expressed in neutrophils, monocytes, eosinophils, endothelial cells and others [88], CXCL5 participates in immune cell recruitment, promotes angiogenesis and is involved in tumor progression [187]. In tumors, the CXCL5/CXCR2 axis has been implicated in multiple processes, as angiogenesis, growth, metastasis and chemoresistance, acting on TME, cancer stem cells and immune checkpoints [188].

The biological functions and clinical relevance of CXCL5 in CCA were recently investigated [2], highlighting a role for this chemokine both in CCA cells and TRS. In a study aimed at identifying key proteins involved in the crosstalk between CCA and TME, Okabe et al. [189] employed HSC/iCCA cell co-culture, detecting higher levels of CXCL5 in co-culture medium than in monoculture. CXCL5 was mainly secreted by iCCA cells, on which it exerted a migratory and pro-invasive action, in autocrine manner. Noteworthy, the autocrine loop was predominantly induced by IL-1 $\beta$ -released by HSCs [189].

Immunohistochemistry on human iCCA tissues revealed up-regulated expression of CXCL5 in neoplastic cells, associated with increased expression of  $\alpha$ -SMA, a reliable marker of HSCs, and high number of CD66b-expressing neutrophils. Importantly, high levels of CXCL5 correlated with poor overall survival [89]. On the other hand, in xenograft models, injection of CXCL5-depleted CCA cells induced smaller tumors, lesser neutrophil infiltration and lower pulmonary metastasis, than control cells. Indeed, CXCL5 acts as potent chemotactic stimulus for neutrophils in vitro and induces tumoral neutrophil infiltration in vivo through PI3K/Akt and ERK1/2 activation [89].

A recent meta-analysis aimed to evaluate the prognostic significance of CXCL5 in CCA patients, revealed that CXCL5 overexpression was inversely correlated with overall survival [90]. Another recent study, analyzing serum chemokine profiles in CCA, reported that CXCL5 was higher in CCA patients, compared to healthy subjects, and CXCL5 levels correlated with poor prognosis [91]. These reports clearly point at this chemokine as an important player in tumor progression and a possible novel prognostic marker for CCA.

### 14. CX3CL1/CX3CR1

CX3CL1 (also known as fractalkine) [190] is a transmembrane chemokine that mediates leukocyte activation by presenting its chemokine domain (CD) to the cognate receptor, CX3CR1 [191,192]. CX3CL1 can be cleaved by metalloproteinases, such as a disintegrin and metalloproteinase domain-containing protein (ADAM)10 and ADAM17, into a soluble molecule with potent chemoattractant properties [193,194].

Activation of CX3CR1 induces survival pathways in both normal [195] and cancer cells [196]. Moreover, CX3CL1/CX3CR1 signaling triggers a rapid mobilization and accumulation of immune cells to the sites of injury and is involved in several inflammatory diseases [197], such as primary biliary cholangitis (PBC). As a chronic inflammatory milieu represents a common risk factor for the appearance of CCA [198], it is conceivable that this pathway contributes to processes driving bile duct cancer. In a study investigating the role of CX3CL1/CX3CR1 axis in PBC, a condition prompting to CCA [199], CX3CL1 was found over-expressed in injured bile ducts, whereas the number of CX3CR1+ mononuclear cells infiltrated into portal tracts was highly increased [96]. In addition, soluble CX3CL1 was also found to recruit lymphocytes into injured bile ducts in PBC patients [199].

Cellular senescence is known to be involved in the pathophysiology of multiple chronic liver diseases, including hepatocarcinoma (HCC) [97,200]. Senescent cells affect the tissue microenvironment producing senescence-associated secretory phenotypes (SASP), comprising cytokines and chemokines, such as CX3CL1. Senescent BECs of PBC-damaged bile ducts released

increased levels of CX3CL1, promoting infiltration of CX3CR1-expressing cells, that further exacerbated bile duct inflammation [201].

Overall, these experimental lines of evidence highlight the possible role of CX3CL1/CX3CR1 axis in the pathogenesis of CCA, through the modulation of inflammatory response. Interestingly, in an *in vitro* study by Haga et al. [79], CX3CL1 was detected in the secretome of MSCs exposed to CCA cell-derived extravesicles (EVs), although untreated MSCs were unable to release this factor, further sustaining a contribution of this chemokine in CCA.

## 15. Microvesicles

CCA cells also interact with TME through secreted membrane vesicles, small heterogeneous spherical structures containing cytoplasmic components, mRNAs and microRNAs [202], acting as mediators of intercellular communication [203,204]. According to their origin, they are classified in microvesicles and ectosomes, when derive from budding or shedding plasma membranes, and EVs, exosomes and exosome-like vesicles, when originate in intracellular compartments and are secreted by fusion with plasma membrane [205].

These vesicles can interact with local or distant cells by EV-cell membrane contact, fusion or internalization [206], contributing to the development of several diseases, including cancer [207,208]. In tumors, EVs participate in the cross-talk between tumor and stroma, stimulating key processes, as angiogenesis [209], invasion [210], inflammation [79], chemoresistance [211,212] and immune escape [213]. By vesicle release, tumor cells can transfer genetic information, modulating recipient cell behavior [214]. Baj-Krzyworzeka et al. [215] showed that, carrying both proteins and mRNA, tumor cell-derived EVs activate monocytes, increasing the expression of human leukocyte antigen-DR isotype, IL-8, CCL2 and CCL4, production of ROS and secretion of TNF, IL-10 and IL-12p40. In a different study, the same authors reported that, in NOD-SCID mice, Evs, by delivering chemokines, induced angiogenesis and activated monocytes [216].

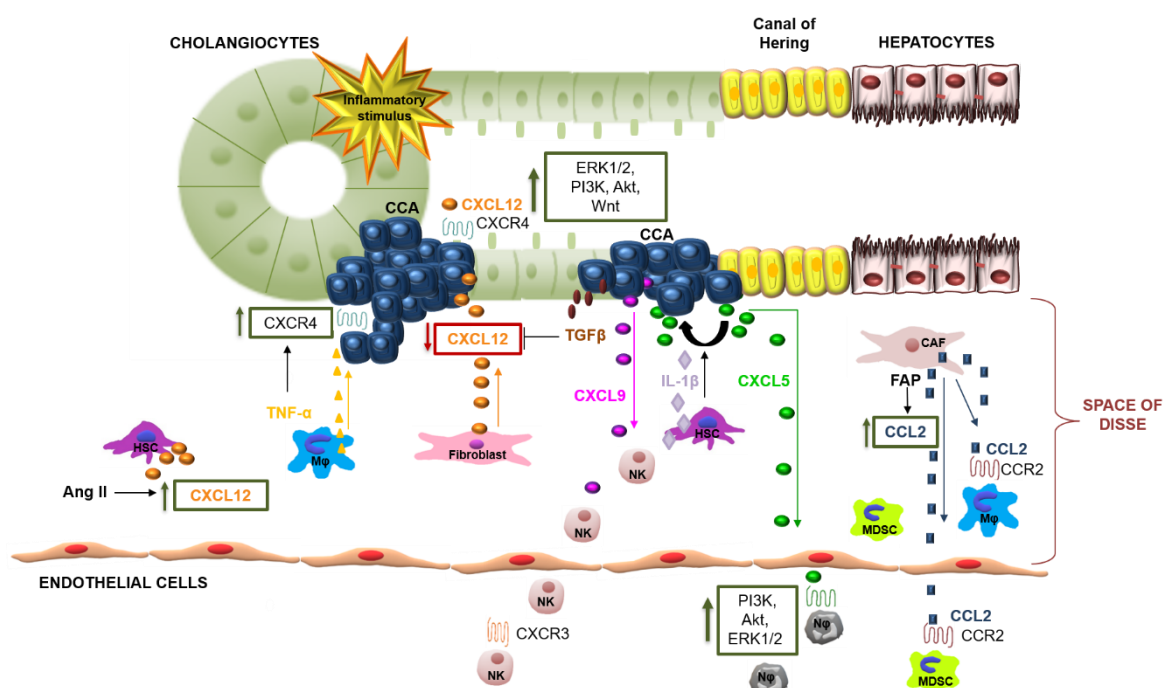
The involvement of EVs in biliary pathobiology and CCA carcinogenesis was recently described [217]. Haga et al. [79] showed that CCA cell-derived EVs induce the differentiation of bone marrow-derived MSCs into fibroblasts releasing cytokines and chemokines (IL-6, CXCL1 and CCL2), that stimulate cell proliferation via IL-6/STAT3 pathways. Dutta et al. [218] reported that KKKU-M213 exosomes promote migration and invasion of H69 cells. Moreover, integrin  $\beta 4$ , whose role in CCA is well-established, was recently recognized as an EV component driving future metastatic sites [219].

Along these lines, altered EVs in serum and bile were proposed as diagnostic biomarkers and therapeutic target for CCA [220]. Early diagnosis, non-invasive diagnostic and treatment of CCA is still far from being achievable. The presence of EVs in biological fluids is rendering them an interesting issue in the study of hepatobiliary diseases. Indeed, emerging evidence demonstrated that EVs are key mediators of cancer biology, being also involved in chemoresistance and immune response, suggesting a potential therapeutic application to boost the responsiveness of chemo- and immunotherapy. However, present knowledge of EVs and EV-related chemokines is limited. It is mandatory to in depth study the intrinsic role of EVs in tumor onset, in order to evaluate novel therapeutic strategies for various cancer types, including CCA [221,222].

## 16. Conclusions and Future Perspectives

The highly aggressive behavior of CCA, together with its resistance to conventional therapies, mainly accounts for its poor prognosis and the scarce therapeutic options. Chemokines and their receptors are part of a well-orchestrated network, aimed at maintaining host health and to respond to conditions of perturbed environment. Dysregulation of this system results in deleterious responses involved in a variety of diseases, including cancer. Despite extensive knowledge of chemokines in tumor biology (Figure 3), several questions concerning the specific role played by the diverse chemokines in relation of tumor stage during cancer development are still uncertain. A deep understanding of chemokine-induced molecular cascades implicated in CCA biology could be helpful to develop novel approaches to complement surgery and chemotherapy. Moreover, by interfering with pro-inflammatory, -angiogenic and -fibrogenic chemokine pathways, chemokine-

based treatments might even contribute to reduce the risk to develop CCA in patients with chronic liver diseases. Understanding the roles of CCA chemokine associated molecular mechanisms could be crucial to identify predictive and prognostic biomarkers.



**Figure 3.** Modulation of CCA development by chemokine networks. Chemokines produced by CCA cells and by intratumor stromal cells, such as CAFs, activate signaling transduction pathways and attract different immune cell types into the CCA TME. CXCL12 released by fibroblast acts on the biological activities of CCA cells. CXCL12 released in CCA microenvironment is negatively modulated by TGF- $\beta$  and positively by Ang II. TNF- $\alpha$  released by tumor-infiltrating macrophages, promotes the increase of CXCR4 expression in CCA cells. CXCL12/CXCR4 interaction induces activation of PI3K, ERK, Akt and the canonical Wnt signaling in CCA cells. CCL2 secretion, which is induced by FAP in CAFs, mediates migration of MDSCs and macrophages in CCA. CXCL5, secreted by CCA cells and induced by IL-1 $\beta$  released by HSCs, promotes cell migration and invasion through an autocrine loop. In addition, CXCL5 acts as potent chemotactic stimulus for neutrophils inducing neutrophil infiltration through PI3K/Akt and ERK1/2 activation. CXCL9 released by CCA cells correlates with an increase of tumor-infiltrating NK cells. The figure represents a selection among various chemokines involved in CCA progression. Cholangiocarcinoma (CCA), Tumor microenvironment (TME), transforming growth factor- $\beta$  (TGF- $\beta$ ), angiotensin II (Ang II), tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), phosphoinositide 3-kinase (PI3K), metalloproteinase (MMP), hepatic stellate cell (HSC), cancer-associated fibroblast (CAF), fibroblast activation protein (FAP), myeloid-derived suppressor cell (MDSC), Interleukin-1  $\beta$  (IL-1 $\beta$ ), natural killer cell (NK).

Recently some chemokines or their receptor antagonists, such as for CXCR4 or CXCR2, have been approved for clinical trials [223,224]; hopefully in the future these studies could be extended also to cholangiocarcinoma.

Indeed, since drugs that selectively induce apoptosis or cytotoxicity in CAFs or TAMs are of great interest, targeting CCA stromal population for therapeutic purposes has been recently proposed [75,225]. Pharmaceutical approaches targeting chemokine pathways might be a strategy to treat CCA in a complementary way with conventional surgery and chemotherapy. Thus, pharmacological agents able to reduce cancer cell aggressiveness could be part of neoadjuvant strategies, to halt tumor dissemination before surgery, or even during palliative treatments to slow the development of the tumor.

However, CCA therapeutic approaches have given to date unsatisfactory results, due to the high tumor heterogeneity that underlies activation of different signaling cascades. Thus, more accurate pharmacological treatments should be selectively designated to improve patient's outcome.

In addition, CCA preclinical studies performed so far are limited due to poor animal models capable to mirror clinical and genetic features of the human disease. Indeed, besides xenograft model that are not specific for CCA, there are several transgenic mouse models, which can be eligible to study CCA developmental mechanisms. However, these models own technical difficulties and are very expensive. Therefore, innovative and well-defined molecular models of CCA are required for preclinical intervention trials.

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## Abbreviations

cancer-associated fibroblasts (CAFs); cholangiocarcinoma (CCA); extracellular matrix (ECM); hepatic stellate cells (HSCs); tumor-associated macrophages (TAMs); tumor microenvironment (TME)

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