UNIVERSITA' POLITECNICA DELLE MARCHE
FACOLTA' DI MEDICINA E CHIRURGIA

Corso di Dottorato in Salute dell'Uomo
Ciclo XXXI

Ageing-related expression of Twinfilin-1 regulates cholangiocyte biological response to injury

Ph.D. dissertation of:
Debora Maria Giordano

Advisor:
Prof. Antonio Benedetti
Co-advisor:
Prof. Marco Marzioni

Academic year 2017-18

INDEX

1. INTRODUCTION 1

1.1 Biliary tree 1

1.1.1 Architecture 1

1.1.2 Vascularization 2

1.1.3 Innervation 3

1.2 Cholangiocyte 4

1.2.1 Morphological and functional heterogeneity 5

1.2.2 Functional role in health state: bile production 6

1.2.3 Functional role in disease state 9

1.2.3.1 Proliferative response 11

1.2.3.2 Ductopenia 11

1.2.3.4 Fibrosis 13

1.2.3.5 Releasing of proinflammatory mediators 13

1.2.3.6 Upregulation of adhesion molecules 16

1.2.3.7 Immunoglobulins and peptides releasing 16

1.3 Cholangiopathies 17

1.3.1 Primary sclerosing cholangitis and Primary biliary cholangitis: an overview 19

1.3.1.1 Therapy 21

1.4 Aging 22

1.4.1 The hallmarks of aging 23

1.4.1.1 Primary hallmarks 23

1.4.1.2 Antagonistic hallmarks 24

1.4.1.3 Integrative hallmarks 27

1.5 Aging, senescence and biliary tree disorders 28

2. AIM OF THE THESIS 31

3. MATERIALS AND METHODS 32

3.1 Materials 32

3.2 Methods 33

3.2.1 MicroRNAs selection 33

3.2.2 MicroRNAs testing in cholangiocyte: isolation, reverse transcription and real-time PCR 33

3.2.3 In silico analysis of microRNAs putative targets and intracellular pathways **34**

3.2.4 Expression of Twf1 in cholangiocyte 34

3.2.5 Cell lines and in vitro experiments 35

3.2.5.1 *Evaluation of Twf1 role in cholangiocytes proliferation* 35

3.2.5.2 *Evaluation of Twf1 role in cholangiocyte senescence* 36

3.2.6 Animal models **37**

3.2.6.1 SAMR1 and SAMP8 **38**

3.2.6.2 Twf1+/+ and Twf1-/- **40**

3.2.7 RNA extraction, reverse transcription and real-time PCR **42**

3.2.8 ELISA 44

3.2.9 Western Blotting **44**

3.2.10 Immunohistochemistry and histochemistry analysis **46**

3.2.11 Liver hydroxyproline content quantification **47**

3.2.12 Statistical analysis 47

4. RESULTS **48**

4.1 Age-related miRs are upregulated in diseased cholangiocytes **48**

4.2 Twf1 is the molecular target of upregulated miRs **50**

4.3 Twf1 and related miRs are upregulated in old and diseased cholangiocytes **52**

4.4 Evaluation of TWF1 expression in human samples **54**

4.5 Twf1 is involved in cholangiocytes proliferation **56**

4.6 Twf1 is involved in cholangiocytes senescence **58**

4.7 Accelerated aging exacerbates biliary injury in vivo **64**

4.8 The absence of Twf1 reduces biliary response to injury in vivo **67**

5. CONCLUSIONS **69**

6. BIBLIOGRAPHY **76**

7. TABLES INDEX **96**

8. FIGURES INDEX **97**

ABSTRACT

Cholangiocytes are the epithelial cells which line the bile ducts and represent the targets of a heterogeneous group of chronic biliary diseases termed cholangiopathies, with different aetiology. The two most common cholangiopathies are the Primary sclerosing cholangitis (PSC) and the Primary biliary cholangitis (PBC) whose pathogenesis have to be fully elucidated. Despite several lines of evidence suggest the potential link between ageing and hepatobiliary diseases onset and progression, it is not yet known whether ageing process modulates cholangiocyte biology during bile duct injury. The aim of the current study is to unveil molecular pathways related to ageing process and to evaluate their possible effect in the pathophysiology of cholangiocytes. A panel of microRNAs (miRs) known to be involved in ageing process, was evaluated in cholangiocytes isolated by young and old mice (2-month and 22-month old, respectively) subjected or not to DDC diet, as a model of sclerosing cholangitis. By *in silico* analysis, it was possible to identify Twinfilin-1 (*Twf1),* an actin - binding protein involved in motile and morphological cellular processes, as putative molecular target commonly regulated by more than one miR taken into account. TWF1 expression was evaluated in human samples collected by PSC and PBC patients. *In vitro*, cholangiocyte proliferation and senescence were evaluated in NRC silenced for *Twf1* expression. *In vivo,* bile ducts proliferation and collagen deposition were evaluated in a mouse model of accelerated senescence (SAMP8) and in *Twf1* $^{-/-}$ mice. *Twf1* lack of expression reduces cell proliferation *in vitro.* Knock-down of *Twf1* increased senescence and SASP markers expression upon pro-proliferative stimulation as well as in a model of cholangiocyte senescence. *In vivo*, *Samp8* mice showed increased

biliary proliferation and fibrosis, whereas *Twf1*$^{-/-}$ had a tendency to reduce biliary proliferation and collagen deposition upon DDC administration compared to control animals. In conclusion, it was possible to verify the role of *Twf1* in cholangiocyte adaptation to injury, in terms of proliferation and senescence establishment. We identified *Twf1* as a potential mediator of cholangiocyte adaption to injury and ageing process. Our findings suggest that cholangiopathies and aging might share common molecular pathways. A deeper understanding of intracellular pathways involved in the modulation of cholangiocyte biology (proliferation, senescence or apoptosis, releasing of proinflammatory mediators and cross-talk with other liver cells) is essential to devise novel effective therapies, for cholangiopathies treatment which are currently lacking. Taken together our findings allow the identification of a possible molecular target which is able to modulate both *in vitro* and *in vivo*, different aspects of cholangiocyte pathophysiology.

1. INTRODUCTION

1.1 Biliary tree

1.1.1 Architecture

The biliary tree is a network of ducts arising from the canals of Hering up to the terminus of common bile duct that progressively increase in size. The biliary system drains the hepatic-derived bile from the bile canaliculi into the gallbladder or directly into the lumen of intestine. It includes intrahepatic and extrahepatic ducts which are encircled by epithelial cells, the cholangiocytes [1], [2]. According to biliary lumen diameter and proximity, human intrahepatic bile ducts (IHBDs) could be divided into ductules (diameter < 15 µm) arising from the canals of Hering, interlobular ducts (diameter comprises between 15-100 µm) that originate from the convergence of ductules, septal ducts (diameter comprises between 100-300 µm) consisting of at least two interlobular ducts, area ducts (diameter comprises between 300-400 µm) which converge to form the segmental ducts (diameter comprises between 400 -800 µm) and the hepatic ducts (diameter > 800 µm) which underlie the passage towards the extrahepatic bile ducts. Extrahepatic bile ducts (EHBDs) include the common hepatic duct, originating from the union of right and left hepatic ducts, the cystic duct, the gallbladder and the common bile duct which transports the bile directly into the duodenum [3] (Figure 1). In cross section, small intrahepatic bile ducts are lined by 4-5 cholangiocytes while about 40 cholangiocytes could be found in large intrahepatic bile ducts [4],[5].

In rodents, the architecture of the biliary tree is more simplified. It could be divided by size into small (diameter < 15 μm) and large (diameter > 15 μm) intrahepatic bile ducts. Small bile ducts are lined by 4 to 5 cholangiocytes, as observed in humans, while large bile ducts are constituted by 8 up to 15 cholangiocytes [4], [6], [7].

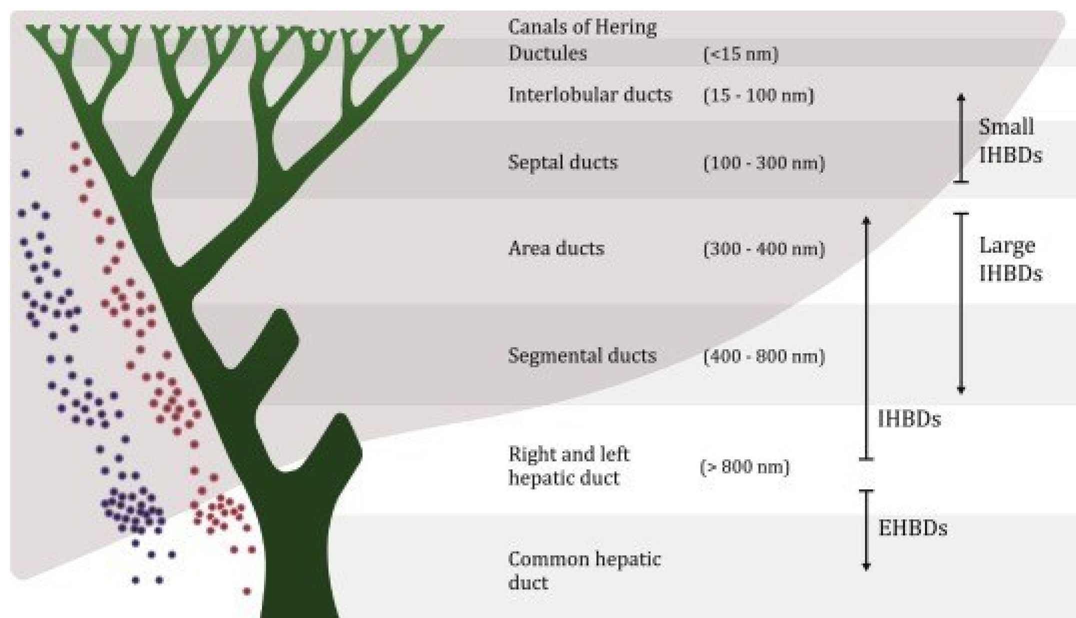


Figure 1. *Biliary tree architecture* (From de Jong *et al.* 2018 [8]).

1.1.2 Vascularization

Blood supply of biliary tree is ensured by intrahepatic and extrahepatic biliary plexus (PBP). It is a vascular system which originates from hepatic artery branches and flows either directly (lobular branch) or indirectly, through the portal vein (prelobular branch), into the hepatic sinusoids. The PBP supports the transport of bile substances reabsorbed from

cholangiocytes to hepatocytes [9]. PBP encircles large bile ducts, becoming less ramified and thinner around the small bile ducts.

The importance of PBP has been evidenced in animal models subjected to bile duct ligation (BDL) as a model of liver fibrosis, taken into account when it is intended to study cholangiocytes proliferation. Therefore, BDL is associated to cholangiocytes proliferation, noticeable since the 2 days after treatment. The microvasculature arrangement follows cholangiocyte proliferation to support newly formed bile ducts in terms of nutrients. In particular, between PBP and bile duct expansion there is a lag time suggesting that, in the first phases of biliary obstruction, cholangiocytes are more susceptible to ischemic and toxic reabsorbed substances-related damage [9].

1.1.3 Innervation

The liver is characterized by two autonomic nervous fibers. The sympathetic fibers which originate from the celiac ganglion and the parasympathetic fibers which originate from the vagus nerve [10]. These fibers extend from the hilar of the hepatic portal and then run along with the hepatic arteries and portal veins. The nervous fibers could release a number of neuropetides such as catecholamines and acetylcholine (Ach) but also other neurotransmitters (e.g. neuropeptide Y), calcitonin gene-related peptide or CGRP and somatostatin). These molecules regulate cholangiocytes absorptive/secretory activities and proliferation through the expression of relative specific receptors on cholangiocytes membrane. An elegant review of Marzioni and his colleagues focused on the neuropeptides/hormonal regulation in cholangiocytes adaptive response to injury [11].

1.2 Cholangiocytes

Cholangiocytes are the epithelial cells which line the bile ducts and are the central target of a group of diseases with different aetiology, termed cholangiopathies. The main physiological function of cholangiocytes is to modify the canalicular bile volume and composition through both absorptive and secretory processes, tightly regulated by molecules of different nature (e.g. neurotransmitters, hormones or peptides) [12]. Cholangiocytes are polarized cells which possess an apical or luminal membrane and a basolateral membrane. At the apical membrane level, biliary epithelial cells are supplied by tight junctions that join adjacent cells [13] and maintain cell polarity [14]. Cell-cell communication between adjacent cells is also ensured by the presence of gap junctions [15]. Along the apical membrane, cholangiocytes possess a number of microvilli protruding in the bile duct lumen, which increase of 5-fold cell surface area [6]. Moreover, biliary cells are provided with a primary cilium which exerts sensory functions, transmitting extracellular-derived informations inside the cells thus modulating different intracellular pathways involved in cellular biological functions (e.g. differentiation, proliferation or secretion) [16]. The primary cilium is a non-motile structure composed by a microtubule-based core surrounded by axoneme, which extends from the basal body up to cell membrane [3], [16]. At the primary cilium level it is possible to find a number of proteins and receptors whose activation results in the upregulation or downregulation of different signalling pathways [16].

As well as other cell types, cholangiocytes' plasma, possess an actin-cytoskeleton which plays a key role as structural support of cell membrane, in conferring and maintaining cell polarity, in vesicle-trafficking and in the modulation of protein distribution [17]. Cholangiocytes exocytic capabilities lie in multivesicular bodies (MVBs) and exosomes releasing, processes that take place in the apical domain of plasma membrane [18]. MVBs are intracellular organelles, also known as late endosomes, which have a diameter > 1 μm and are part of the lysosomal system. Some MBVs are fused with lysosomes for protein recycling others instead, are released as exosomes in the extracellular space following MVBs membrane and cell plasma membrane fusion [19]. On the other hand, the exosomes are small cell-derived vesicles (diameter comprises between 30–100 nm) that seem to be involved in physiological biological processes. They deliver several macromolecules (e.g. lipids and proteins) and nucleic acids (e.g. mRNAs and miRs) to closer or even distant cells, activating molecular pathways in the target cells [20].

1.2.1 Morphological and functional heterogeneity

According to morphology, cholangiocytes could be divided into small and large cholangiocytes which line small and large intrahepatic bile ducts, respectively. Small cholangiocytes possess a cuboidal shape and a high nucleus to cytoplasm ratio. Thus small cholangiocytes are likely able to modify their biology in pathological conditions, due to a less cellular differentiation (functional plasticity). They possess a rich Golgi while the endoplasmatic reticulum is not abundant and although it appears to be increased in large cholangiocytes, its increasing is only slight [6].

Unlike small cholangiocytes, large cholangiocytes have a columnar morphology and a small nucleus to cytoplasm ratio [6].

The morphological heterogeneity of small and large cholangiocytes reflects the functional heterogeneity existing between the two cells populations. Large cholangiocytes lining interlobular, septal and larger bile ducts actively participate to modification of bile trough secretory and absorptive mechanisms, due to the presence of proper ion transport systems and hormone receptors at the plasma membrane and supported by the presence of microvilli in the apical domain of cell membrane [1].

On the other hand, small cholangiocytes are able to modify their phenotype in response to exogenous/endogenous noxious stimuli thus participating to the inflammatory response during biliary tree damage, and serves as liver progenitor cells under certain conditions [21].

1.2.2 Functional role in health state: bile production

Physiologically, cholangiocytes are implicated in the modification of hepatic-derived bile. To accomplish this task cholangiocytes are equipped of different transporters and channels localized at both apical and basolateral cell membrane which ultimately lead to secretion into the bile of water, Cl^- and HCO_3^-, and extraction of glucose, bile acids and amino acids [22]. The releasing of HCO_3^- is functional to bicarbonate umbrella composition. This mechanism protects cholangiocytes against bile acids-dependent injury, by reducing the passive absorption of bile acids through cholangiocytes [3], [22]. Bile is an aqueous secretion produced by hepatocytes and composed of water for the 95% and for the remaining 5% by organic (e.g. bile acids), inorganic solutes and lipids [23].

The hepatic bile is transported by bile canaliculi, formed by the apical membrane of neighbour hepatocytes kept together by tight junctions (zona *occludens*), in the opposite direction to that of portal blood toward the canals of Hering in the bile ducts and, after modification by cholangiocytes, into the gallbladder or into the duodenum [23]. In the ileum the majority of bile acids (95%) is absorbed and then recycled in the liver via enterohepatic circulation [22]. Differently, through the cholehepatic shunt, a portion of bile acids (unconjugated) secreted by hepatocytes, after being passively absorbed by cholangiocytes, return to hepatocytes [3]. Subsequently, these unconjugated bile acids are secreted again by hepatocytes stimulating once again HCO_3^- secretion.

One of the most important molecule which regulates biliary secretion is the secretin (STC) hormone, whose specific receptor is expressed at the basolateral domain of large cholangiocytes membrane [24]. Briefly, in response to peptides and acidic pH produced after a meal, duodenal and jejunal S cells release the STC into the portal blood [3]. Once reached the liver via enterohepatic circulation, STC binds to secretin receptor (SR) thus initiating an intracellular molecular cascade culminating in 3′,5′-cyclic monophosphate (cAMP) production. The increased levels of cAMP in turns activate the protein kinase A (PKA) that catalyses the phosphorylation of cystic fibrosis transmembrane conductance regulator (CFTR). The CFTR is a chloride channel which upon activation determines the extrusion of Cl^- ions in the bile lumen [12]. The existing Cl^- gradient across the two sides of plasma membrane activates the anion exchanger 2 (AE2) and, as a consequence, the net releasing of HCO_3^- associated to the osmotic influx of H_2O through the aquaporin 1 (AQP1) [12], [25].

An alternate mechanism though which the efflux of Cl^- ions is ensured, depends on acetylcholine (ACh) binding to muscarinic receptor M3, expressed on the basolateral side of plasma membrane [26]. In response to ligand binding to its specific receptor, the levels of Inositol trisphosphate (IP3) increase and the release of Ca^{2+} evidenced following IP3 binding to its receptors, results in the apical secretion of Cl^- [27]. Besides the well-studied secretory functions of cholangiocytes mainly dependent on STC/SR axis, other channels and proteins located in the apical membrane as well as at the basolateral level, are involved in physiological secretory functions of biliary epithelial cells (e.g. Na^+-independent Cl^-/HCO_3^- exchanger, K^+ channel (SK2)) [3]. The modification of bile is achieved not only by secretory functions but also by absorptive capabilities of cholangiocytes which rely on the expression of transporters on both apical and basolateral cellular side (e.g. Na^+-dependent bile acid transporter or ASBT, sodium-dependent glucose cotransporter 1, water channel or AQP1) [3]. As mentioned above, bile contains organic cations excreted by hepatocytes which could be noxious for cholangiocytes [23]. The passive diffusion and accumulation of such molecules inside cholangiocytes could lead to deleterious effects. With this regard, cholangiocytes possess different protective mechanisms. As an example, the multidrug resistance 1 (MDR1) is an ATP-dependent transmembrane efflux pump localized in the apical pole, which is able to excrete again into the bile, exogenous or endogenous lipophilic compounds [27],[28]. At the basolateral pole of cholangiocytes membrane, it is possible to find transporters which allow organic anions to be released from cholangiocytes into the PBP (e.g. multidrug-associated protein 3 or MPR3 and MRP4) [29].

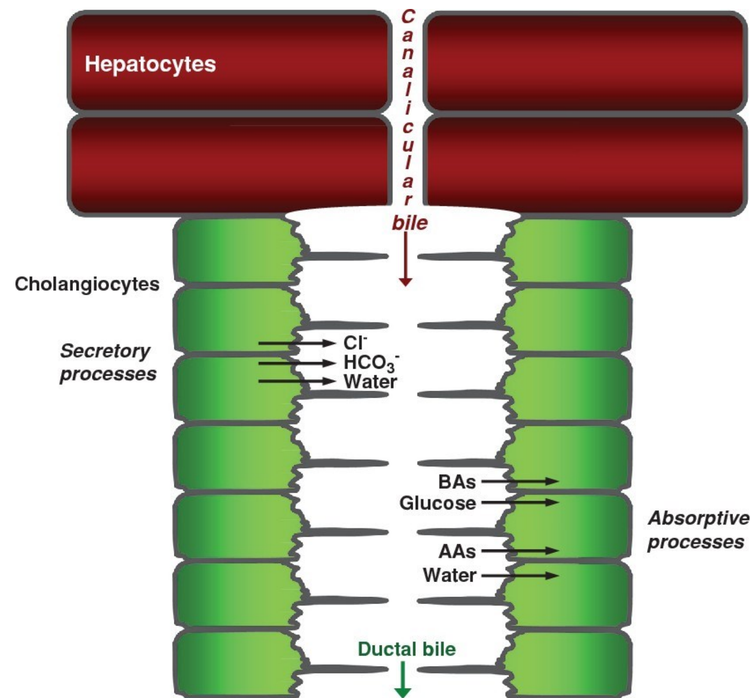


Figure 2. *Hepatic bile production and modification, an overview.* Hepatocytes synthetize the canalicular or primary bile which, as it flows into the bile duct lumen, is modified by cholangiocytes through either secretory (Cl-, HCO3- and water extrusion) or absorptive processes (glucose, bile acids, water and amino acids absorption). BAs: bile acids; AAs: amino acids (From Tabibian *et al.* [3]).

1.2.3 Functional role in disease state

In physiological conditions cholangiocytes are mitotically dormant, until biliary tree damage occurs. In response to exogenous/endogenous stimuli (e.g. microorganisms, toxins/drugs or hormones), cholangiocytes become reactive and orchestrate a compensatory response. This includes the releasing of a wide range of proinflammatory and fibrogenic mediators which act in autocrine/paracrine fashion mediating: 1) cholangiocyte proliferation [30] so as to overcome biliary mass loss and to preserve the secretory/absorptive activities; 2) senescence and apoptosis processes which lead to ductopenia when the balance between proliferation/cell death come less; 3) immune cells chemotaxis and mesenchymal cells activation to repair

the injured tissue and remodel the biliary tree. Thus, cholangiocyte response to injury is not merely characterized by enhanced proliferation, but it also relies on the activation of immune response through the secretion of proinflammatory and chemotactic mediators, on the upregulation of adhesion molecules expression and on antimicrobial molecules releasing through which cholangiocytes directly exert host defence activities (Figure 3).

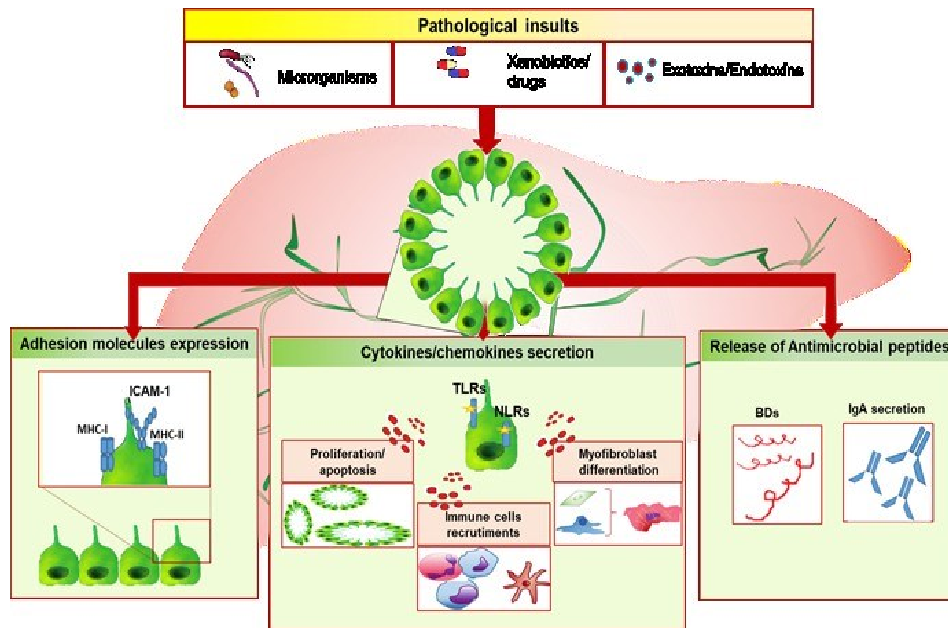


Figure 3. *Overview of cholangiocytes immune biolo*gy. Once activated by noxious stimuli of different nature (microorganisms, xenobiotics, drugs or exotoxins/endotoxins), reactive cholangiocytes respond by releasing cytokines and chemokines which in turns determine cholangiocytes proliferation and apoptosis, when the balance between cell growth/date come less, immune cell recruitments and myofibroblast differentiation. Moreover, activated cholangiocytes upregulate the expression of surface proteins such as MHC-I, MHC-II and ICAM-1) and start to release antimicrobial molecules such as beta-defensins (BDs) or IgA, following epithelial transcytosis. If the biliary damage persists, these compensatory processes may lead to chronic inflammation and fibrosis establishment. PF, portal fibroblast; HSC, hepatic stellate cell. (From Giordano *et al*. [31]).

1.2.3.1 Proliferative response

The ability of cholangiocyte to undergo proliferation has been evidenced in both experimental studies and in different human pathological conditions [32], [33]. Cholangiocyte proliferation is the main contribution of the so called “ductular reaction”. This term has been coined by Popper in 1957 and refers to proliferation of either pre-existing ductules, progenitor cell activation and appearance of intermediate hepatocytes at the interface between biliary tree and hepatocytes [34].

Based on *in vivo* experimental studies, four types of ductular reaction have been identified and summarized in a comprehensive review of Svegliati-Baroni and his colleagues [35].

In course of biliary damage, cholangiocytes proliferation is achieved through interleukin-6 (IL-6) upregulation. It plays a crucial in biliary mass maintenance and in functional activities enhancement [11], [36]. The importance of IL-6 in cholangiocytes population support, has been confirmed by an *in vivo* study carried out on mice lacking IL-6 gene expression. Transgenic mice subjected to BDL showed a worsening of pathological conditions (e.g. increase of serum total bilirubin, development of advanced stage fibrosis and an high mortality rate) compared to controls [37].

1.2.3.2 Ductopenia

Ductopenia occurs in the setting of chronic biliary inflammation, when the balance between proliferation and cell death phenomena come less. In normal conditions, senescent or damaged cholangiocytes undergo programmed cell-death to be replaced by newly formed cholangiocytes [38].

In pathological conditions, the homeostasis between cholangiocyte proliferation and death is lost, and disease progresses toward ductopenia.

Apoptosis has been evaluated in several experimental studies [39], [40]. As an example an *in vivo* study carried out on BDL rats, has shown that isolated cholangiocytes incubated with both TNFα and Actinomycin D, undergo cell programmed death which in turns lead to loss of cholangiocytes secretion capabilities. These data suggest that in course of cholestasis, cholangiocytes are more susceptible to TNFα-mediated cell death [41].

The establishment of senescent phenotype leads to progressive ductopenia, as a result of impaired proliferative and regenerative compensatory processes, which make non-replicative cells prone to undergo subsequently injuries [42]. Interestingly, it has been shown that cholangiocytes of PSC patients upregulate both senescence markers ($p16^{INK4A}$ and $p21^{CIP/WAF1}$) and SASP secretome components [42]. These findings suggest a possible role of cholangiocytes in the modulation of both organ homeostasis and microenvironment [42], [43].

According to human data previously discussed, multi-drug resistance 2 knockout ($mdr2^{-/-}$), a genetic mouse model of sclerosing cholangitis [44], showed the accumulation of senescent cholangiocytes in the liver [42]. In line with these data, the prolonged exposure to LPS (10 days*)* determines a persistent TLR4 activation in cultured normal human cholangiocytes (NHC) and as a consequence the upregulation of proteins which regulate cell-cycle and SASP components [42].

1.2.3.4 Fibrosis

As a readout of chronic liver injury, it could be observed hepatic fibrosis development. It has been demonstrated that the bile ducts of fibrotic livers express high levels of transforming growth factor- β2 (TGF-β2) transcripts [45]. TGF-β acts by downregulating adhesion molecules expression on cell surfaces, by promoting the myofibroblastic differentiation (by activating portal fibroblast and hepatic stellate cells [46] and by inhibiting cholangiocytes proliferation [47].

On the other hand, TGF-β stimulates cholangiocytes secretion of endothelin-1 and modulates extracellular matrix protein deposition by mesenchymal liver cells [45].

Injured cholangiocytes are also able to secrete MCP-1, a well-known chemotactic factor, which could induce portal fibroblasts proliferation and transdifferentiation in myofibroblasts [48].

1.2.3.5 Releasing of proinflammatory mediators

Cholangiocytes represent the first line of defence of biliary system against potentially noxious stimuli of different nature (against microorganism, endotoxin/esotoxins, drugs) that reach the hepatic compartment through the portal blood circulation. For this purpose, cholangiocytes are endowed with pathogen recognition receptors (PRRs) that bind to either microbial components (e.g. bacterial DNA, product or cell wall components) identified as pathogen-associated molecular patterns (PAMPs) and molecules released from damaged cells known as damage- associated molecular patterns (DAMPs) [49].

Two main PRRs families exist. The Toll-like receptors (TLRs) family

includes several type 1 transmembrane glycoprotein receptors (10 members in human and 13 in mammalian cells) that upon recognition of conserved microbial components, activate a cascade of intracellular signals which culminate in the activation of nuclear factor kappa-b (Nf-kB) or activator protein-1 (AP-1) and, as a consequence in the biosynthesis of proinflammatory cytokines [50].

On the other hand, the nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) family consists of soluble PRRs, which could recognize intracellular pathogens and endogenous noxious stimuli through the DAMPs [51]. Depends on the triggering stimulus, cholangiocytes release a different pattern of soluble mediators (Figure 4).

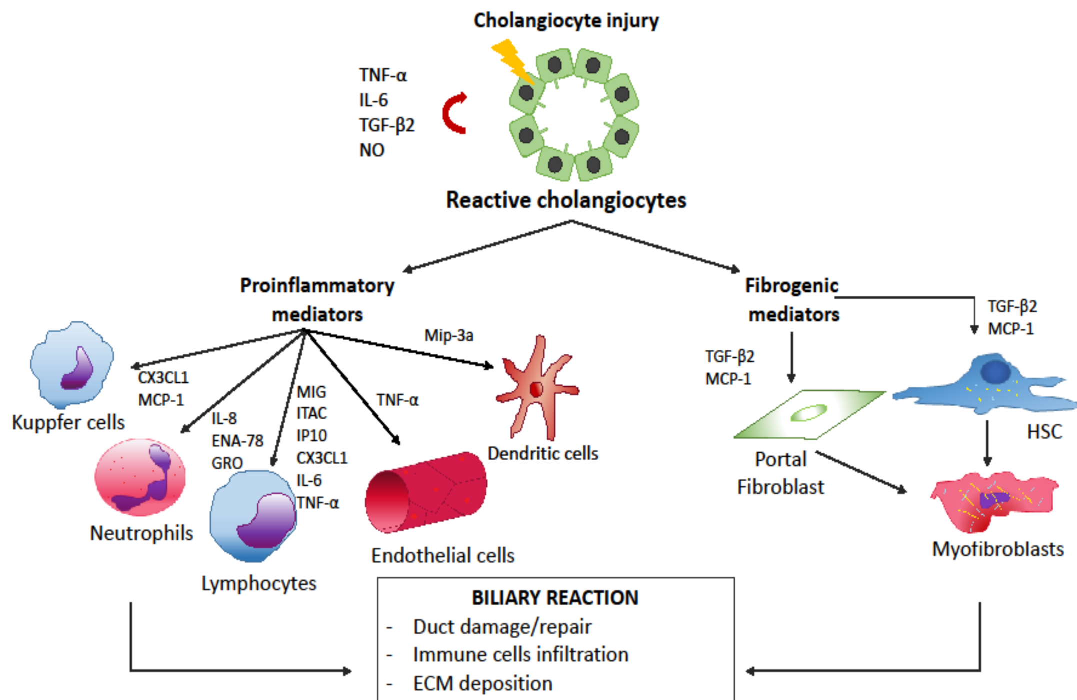


Figure 4. *Crosstalk between activated cholangiocytes and resident/non-resident cells in response to biliary injury*. Biliary epithelial cells are able to release a huge amount of proinflammatory cytokines and chemokines acting in autocrine/paracrine manner. The downstream events are cholangiocytes proliferation, immune cells recruitment and myofibroblast differentiation of portal fibroblast and HSC. HSC: hepatic stellate cell (From Pinto *et al*. [52]).

As an example, enteric-derived bacterial products (e.g. lipopolysaccharide or LPS, lipoteichoic acid), IL-1 and TNFα stimulus, trigger the release of IL-8, epithelial cell derived neutrophil activating protein (ENA-78) and growth-related oncogene (GRO), which are chemotactic factor acting on neutrophils [53]. On the contrary, INFy stimulus induces the releasing of a set of inflammatory mediators which characterize a chronic inflammatory process. With this regards, it has been shown that INFy treatment downregulates IL-8 releasing and, on the other hand, enhances the secretion of the monocyte chemoattractant protein-1 (MCP-1) and other mediators typical of a prolonged inflammatory process (e.g. monokine induced by INFy or Mig, interferon inducible T cell alpha chemoattractant or ITAC and interferon gamma inducible protein 10 or IP10) [53]. IL-1β, TNF-α, IL-17 stimuli or TLRs activation, culminate in cholangiocytes mediated-dendritic cells chemotaxis via macrophage inflammatory protein-3α (Mip-3a) releasing [54]. Among others, reactive cholangiocytes are able to release the fractalkine known also as chemokine (C-XC-C motif) ligand 1 (CX3CL1), that belongs to CX3C family. Fractalkine is a chemoattractant molecules acting on monocytes and T lymphocytes in its soluble form, and mediating leukocytes adhesion to CX3CR1 receptor-expressing cells in the cell-bound form [55]. High levels of fractalkine have been detected in sera of PBC-patients, suggesting its possible role in PBC pathogenesis. Moreover, patients affected by PBC showed an high infiltrate of CX3CR1-positive lymphocytes at the portal tracts and cholangiocytes level [56].

1.2.3.6 Upregulation of adhesion molecules

Several epithelial-derived inflammatory cytokines are able to modulate the expression of adhesion molecules (i.e. ICAM-1, MHC-I and MHC-II) on cholangiocytes surface, which are involved in cell-mediated immune response. *In vitro* experiments have shown that TNFα, IL-1 and INFγ drive the upregulation of the adhesion molecules MHC-I, MHC-II and ICAM-1 on cholangiocyte membrane [57], [58]. Conversely, TGFβ decreases the expression of these surface proteins [59]. Data collected from human specimens have shown the overexpression of MHC-II in the bile ducts of PBC- or PSC-patients [58], [60].

1.2.3.7 Immunoglobulins and peptides releasing

Cholangiocyte immune response is also achieved through secretory immunoglobulin A (sIgA) transcytosis into the bile to preserve mucosal integrity during pathogens infection and prevent microbial attachment to cholangiocyte surface [61].

Cholangiocytes are able to synthesize and release antimicrobial peptides, in particular Defensins. They are small proteins rich in cysteine able to bind to microbial surface and induce membrane disruption, intracellular ion release and ultimately cell death. The family of defensins consists of two submfamilies, α and β-defensins. The latter plays a role in mucosal defense in course of local infections. Six isoforms (Hbd-1 to Hbd-6) have been identified in humans. The isoform Hbd1 is expressed in the cytoplasm of cholangiocytes, lining intrahepatic bile ducts, constitutively. By contrast, cultured biliary epithelial cells subjected to LPS stimulus, to *E.coli* infection [62] or stimulated with inflammatory cytokines whose levels are increased in

cholangiophies [63] (i.e. TNFα and IL-1β), show the upregulation of hbd2 expression.

1.3 Cholangiopathies

Cholangiopathies are a group of progressive liver diseases targeting cholangiocytes, which evolve into end-stage liver disease, with limited therapeutic strategies. Nowadays, due to the high rate of mortality and morbidity, the need for liver transplantation and the overall cost to society, cholangiopathies represent an important moiety of liver diseases.

Cholangiopathies possess a unique presentation and course but they share cholangiocytes-mediated processes involved in disease pathogenesis (e.g. proinflammatory cascade activation, innate immune responses, cholangiocyte proliferation and tissue repair).

According to aetiology, cholangiopathies could be classified into immune-mediated, infectious, genetic, idiopathic, malignant, and secondary sclerosing cholangitis [64].

Besides local cholangiocytes-associated events, other factors could determine cholangiopathies onset and perpetuation. These factors include genetic variants, epigenetic mechanisms, post-transcriptional events and protein expression modulation by miRs (Figure 5). They are short non- coding RNAs which have been recently described as important regulators of gene expression. However, the regulatory circuits of miRs are complex and still unknown. Thus, the knowledge of the relation between miR and its targets is not simple. The function of miRs rely on negative regulation of target or it could be executed thorough other different mechanisms.

The influence of genetic factors in the pathophysiology of cholangiopathies is not yet fully elucidated and the environmental factors contributing to disease perpetuation are largely unknown, thus limiting our current understanding. Taken together these variables influence disease progression or resolution.

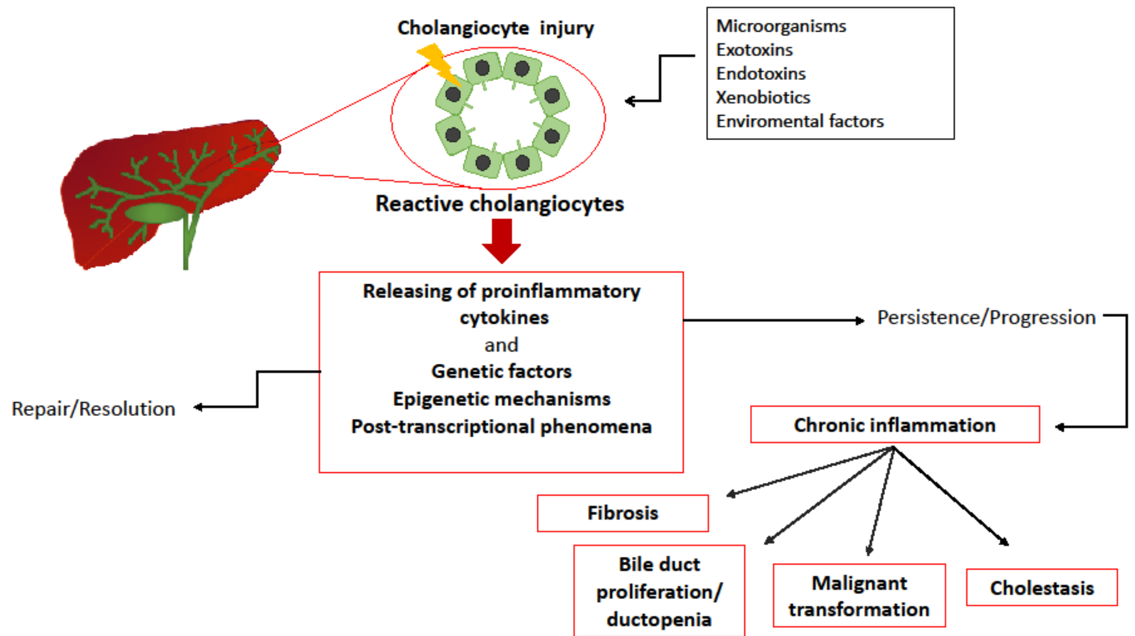


Figure 5. *Schematic representation of cholangiopathies pathogenesis.* Following an insult, cholangiocytes become activated and start to release inflammatory mediators (cholangiocytes-dependent events). The resolution of the disease process or alternatively the perpetuation of inflammatory state depends on either cholangiocytes-dependent events or genetic factors, epigenetic modifications and post-transcriptional phenomena. The persistence of the insult determine chronic inflammation establishment which could result in fibrosis, ductopenia, cholestasis and malignant transformation of the biliary epithelium (From Pinto *et al.* [52]).

1.3.1 Primary sclerosing cholangitis and Primary biliary cholangitis: an overview

Primary sclerosing cholangitis (PSC) and Primary biliary cholangitis (PBC) are the two most common cholangiopathies, characterized by progressive vanishing of bile ducts. Despite they possess commonalities (i.e. portal inflammation with lymphocytes infiltration, bile duct injury, fibrosis development and progression to cirrhosis which leads to end-stage liver failure), PSC targets medium to large extrahepatic and intrahepatic bile ducts, while PBC occurs in small intrahepatic bile ducts [65], [66]. Therefore, recognition of biliary epithelium heterogeneity is necessary to unveil the molecular mechanisms underlying cholangiopathies development and progression, and possibly to devise novel therapeutic targets.

PSC is an idiopathic disease whit a mean age of diagnosis of 40 years which predominantly affect men. However, as the population get old, the patient age at diagnosis becomes older [67]. Despite the presence in the 80% of patients of autoantibodies anti p-ANCA, PSC differs from the others autoimmune disease because of the absence of female prevalence and the low responsiveness to immunosuppressive therapy [68]. The characteristic histologic features includes portal inflammation, onion-skin fibrotic lesions and obliterative cholangitis [69]. Bile ducts inflammation and fibrosis lead to bile synthesis and flow impairment and a progressive liver failure. Approximately, 75% of PSC patients are affected by inflammatory bowel disease (IBD), mainly ulcerative colitis (UC), whose pathogenesis have been supposed to be related to gut dysbiosis [70], [71]. Clinical data have shown that PSC patients display portal bacteremia and bacteria-bilia [72] with increasing of 16 S ribosomal RNA (rRNA) in the bile [73].

More interesting, bowel resection after or during liver transplantation, reduced PSC recurrence rate (which hints the 37% of PSC-transplanted patients), suggesting the potential role of gut microbiota in disease relapse [74], [75]. Further evidences supporting the involvement of gut microbiota in PSC development derive from the beneficial effects of oral antibiotics documented in PSC patient treatment [76].

Another hypothesis proposed to explain PSC development is the aberrant mucosal T-cells homing in the liver. Briefly, T-cell memory originally activated in the gut, are aberrantly recruited in the liver and recognize self-antigens of the liver or gut antigens that reach the hepatic compartment via portal blood circulation, triggering an exacerbated inflammatory response [68].

PBC is considered as an autoimmune disease due to the presence of anti-mitochondrial antibody (AMA) in sera of 95% of patients. PBC is a middle-age and older females disease (the average of diagnosis is 55 years), even if some cases of paediatric onset have been reported [77].

The characteristic histological findings includes bile duct lesion with segmental degeneration of ducts and formation of epithelioid granulomas [64]. Different triggers have been linked to the immunological breakdown evidenced in PBC; in particular self/non-self mimicry has been identified as a driving mechanism [78]. Among environmental triggers xenobiotics, microorganisms (*N. aromaticivorans* or *E. coli* which share the amino acidic sequence with the immunodominant epitope of pyruvate dehydrogenase complex-E2 or PDC-E2, the key autoantigen of PBC [79] or hormones, have been identified. Several features evidenced in an aged immune system has been reported in patients affected by PBC.

For example, the modification of T-cell regulatory response [80], the telomere length attrition [81], the DNA damage, the apoptosis (e.g. increasing of the apoptotic markers Bcl-1 and Fas in cholangiocytes), senescence (e.g. increased expression of p16 and p21) [81], downregulation of Ki67 [82] (in human cholangiocytes) and autophagy (e.g. increased expression of LC3) in liver samples of PBC patients (90.5% inflamed bile duct and 25% of non-inflamed bile ducts) with respect to controls [83].

A relevant role in shaping cholangiocyte response to injury is played by genetic variants, which among others, determine whether reactive cholangiocytes regress to a normal phenotype or lead to chronic inflammation of the bile duct, with progression of cholangiopathy. Data collected by 4 different genome wide-association studies (GWASs) highlight 30 susceptibility loci associated with PBC involved in immune cells functions and processes. As for PBC, a number of 31 risk loci related to immune cells responses, have been identified in PSC [84].

1.3.1.1 Therapy

The lack of knowledge on the pathophysiological mechanisms of PSC and PBC development and progression has a severe impact on the availability of therapeutic strategies to treat affected patients. Awareness of the exact molecular pathways characterizing cholangiocytes biology in physiological and pathological conditions may help the identification of specific molecular targets for novel drugs discovery. Up to the present, ursodeoxycholic acid (UDCA) treatment represents the first therapeutic option for PBC patients.

Its effects rely on the promotion of choleresis by upregulation of both cholangiocytes and hepatocellular transporters, thus protecting biliary epithelial cell by toxic bile acids-mediated injury [85]. Intolerant or non-responding PBC patients to UDCA (about 40%) are subjected to obeticholic acid (OCA) administration. Therefore, the combination of the two strategies does not cover all PBC patients [86]. By contrast, no approved therapies is nowadays available for PSC treatment. The administration of high doses of UDCA (28–30 mg/kg) have been associated to serious side effects, such as the increasing risk of liver transplantation and esophageal varices development [87]. The only effective therapies for PSC patients is liver transplantation. A number of clinical trials based on drugs targeting specific molecular pathways (i.e. agonists of nuclear receptors and membrane transporters involved in bile acids homeostasis and metabolisms) related to cholangiocyte biology are currently being investigated for PSC and PBC treatment [86].

1.4 Aging

Aging is the process of functional decline of multiple cells and tissue which ultimately lead to body deterioration and increased susceptibility to death [88], [89]. Several disorders such as cardiovascular diseases [90], pathologies of nervous system [91] or cancer [92] have been related to aging. Few decades ago, it has been possible to identify cellular characteristics and molecular pathways associated to aging process [89].

1.4.1 The hallmarks of aging

The hallmarks of ageing have been identified based on specific criteria, that are: a) the hallmark have to manifest during normal gaining; b) the exacerbation of the hallmark accelerate the aging process; c) the blockage of the hallmark results in retarded aging and, as a consequence, the increase of lifespan.

The aging hallmarks could be divided into three main categories:

- Primary, which are the cause of age-related damage;
- Antagonistic, identified as the response to age-related damage;
- Integrative, that are the consequence of responses and responsible of ageing phenotype;

1.4.1.1 Primary hallmarks

The causes of damage related to ageing are multiple. The accumulation of DNA damage (e.g. mutations, telomeres shortening or gene disruption) is one of the features characterising the aging process [93] which (both genomic and mitochondrial DNA) depends on both exogenous stressors (physical, chemical or biological triggers) and/or endogenous events (DNA replication errors or ROS production) [94]. Therefore, the organisms possess DNA repair machineries which repair damaged nuclear DNA and with lower efficiency mitochondrial DNA [95]. As an example, telomerases are specialized DNA polymerases which preserve telomeres length and function by replicating the terminal sequences of chromosomes. The link between telomeres shortening and aging has been demonstrated in different animal models [96].

Epigenetic alterations such as histone modification, DNA methylation, chromatin remodelling or non-coding RNA, are known to be involved in tissue aging. For instance, experimental mice model lacking sirtuine-6 (SIRT6), a gene belonging to the NAD-dependent protein deacetylases family involved in chromatin function modulation, show an accelerated aging [97]. Accordingly, the overexpression of SIRT6 transgene in mice results in a prolonged life-span [98].

Also the impairment of proteostasis, term which refers to protein homeostasis, has been correlated with aging process [99]. This cellular process plays a critical role in the stabilization of protein exhibiting a correct folding and in mechanisms of protein degradation by proteasome and lysosomes. Thus, the deregulation of proteostasis (due to toxins or free radicals) could lead to the chronic expression of unfolded or misfolded protein or to the accumulation of protein aggregates. This process has been linked to different age-related pathologies of nervous system [100].

1.4.1.2 Antagonistic hallmarks

As a result of primary hallmarks, cell activates compensatory processes known as antagonistic hallmarks. Cellular senescence represents the main response to age-related damage. *In vivo* data have shown the accumulation of senescent cells in aged tissues. Senescence is defined as the irreversible process of cell growth arrest associated to complex cellular changes such as chromatin organization, metabolic reprogramming, increasing of autophagy and the release of a number of proinflammatory mediators and growth factors,which are included in the senescence associated secretory phenotype (SASP) [88], [101]. Senescence process was firstly described by Hayflick and

his colleague [102], [103], around 50 years ago. They observed in cultured normal fibroblast the exhaustion of cells proliferative capacity after serial replicative cycles. Further, it has been shown that the phenomenon observed *in vitro* was due to telomere shortening [104]. Apart from telomere damage, different others stimuli are known to triggers senescence process [105].

The stable replicative arrest of cells is a physiologic process which impedes damaged or cancer cells to divide and expand, thus suppressing tumorigenesis [106]. This compensatory process requires the homeostasis between clearance of senescent cell and mobilization of progenitor cells. The lack of balance, determines the accumulation of senescent cells which contributes to aging.

The growth arrest, which is the intrinsic characteristic of senescent cell, results in the absence of proliferation markers and the acquisition of peculiar morphological changes (e.g. doubling of cell volume and flattened morphology). Cellular senescence is driven by the upregulation of two tumour suppressive pathways, the $p16^{INK4A}$/pRB and $p21^{CIP/WAF1}$/p53 [105], [107]. Along with replicative arrest, senescent cells are characterized by the "secretome" enrichment in proinflammatory cytokines and metalloproteases, known as component of senescence-associated secretory phenotype (SASP) which act in autocrine/paracrine manner mediating angiogenesis (e.g. vascular endothelial growth factors or VEGF), cell growth (e.g. growth-related oncogenes or GROs), chemo resistance, stimulation of epithelial to mesenchymal transition (e.g. IL-6, IL-8 and MMPs), chronic inflammation (e.g. IL-1α , IL-6 , IL-8, MCP or MIP),alteration of stem cells renewal [108] and differentiation, and tissue remodelling [101].

Finally, the role of SASP mediators is to recruit immune cells to eliminate senescent cells [109]. An important feature which allows the identification of senescent cells both in culture conditions and *in vivo*, is the lysosomial β-galactosidase enzyme. The activity of this enzyme results increased in course of replicative senescence, and confers histochemical positivity for the senescent associated β-galactosidase (SA-β- GAL) staining [110].

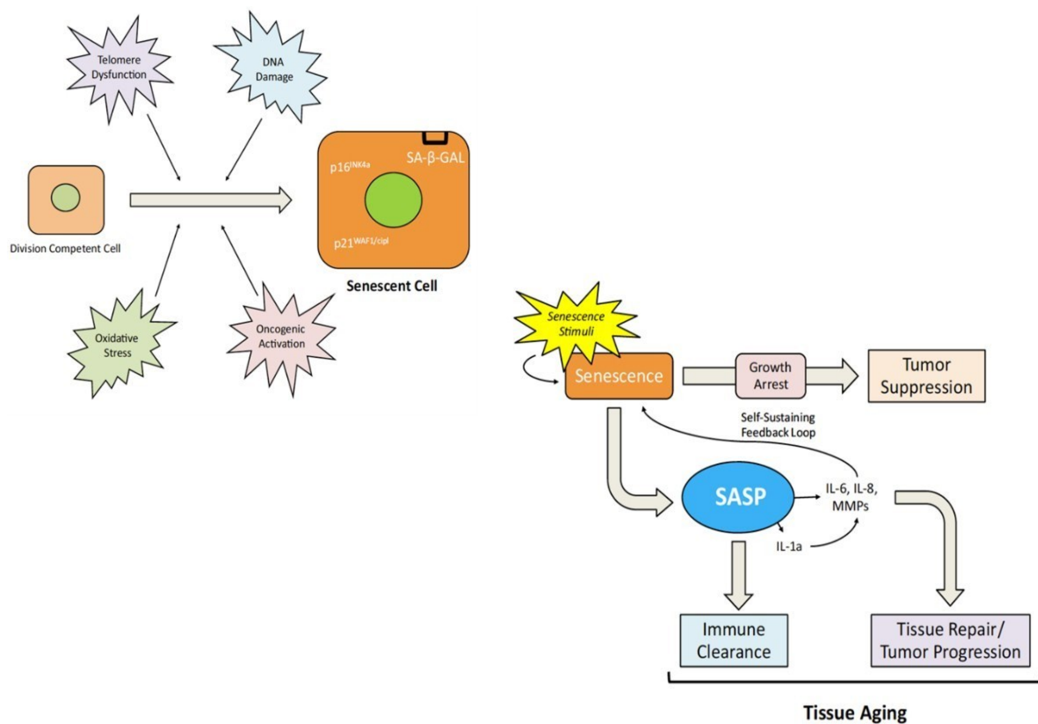


Figure 6. *Cellular Senescence: an overview.* Different triggers could induce cell growth arrest such as telomeres dysfunction, DNA damage and so on. Once cells become senescent, modify their phenotype. The nucleus becomes wider and increases the expression of two nuclear proteins which suppress cell proliferation (p16 and p21) and of the senescence marker, SA-β-GAL). On one hand, senescence establishment prevents cell transformation and thus tumour development. On the other hand, senescent cells secrete a variety of proinflammatory cytokines and other soluble factors which allow tissue repair and the immune clearance of senescent cells. These events lead to tissue aging (From Meng *et al.* 2015 [111]).

Either nutrients sensing deregulation or mitochondrial dysfunction are comprises in the antagonistic hallmarks of ageing. The main physiologic pathway which regulates metabolism is the growth hormone (GH)/insulin-like growth factor (IGF-1) axis. In particular IGF-1 induces insulin releasing in response to glucose sensing. The insulin/IGF-1 signalling pathway targets the transcription factors family FOXO and the mTOR complexes. In both humans and model organisms it has been shown that genetic polymorphisms or mutation which reduces GH, IGF-1 or insulin receptor or downstream effectors, determine an increased lifespan. According with these findings, dietary restrictions increase lifespan of both unicellular and multicellular organisms [112], [113], [114]. Mitochondrial dysfunction has been linked to aging. Different causes have been associated to a reduced efficiency of mitochondria which determines a reduced mitochondria biogenesis such as: deletion of mtDNA, oxidation of mitochondrial proteins, destabilization of the macromolecular organization of respiratory chain complexes, alteration of lipid composition of mitochondrial membranes, loss of mitochondrial homeostasis (as a result of imbalance between fission and fusion events) and defective mitophagy (which physiologically allows the proteolytic degradation of deficient mitochondria) [115], [116].

1.4.1.3 Integrative hallmarks

The decline of regenerative capacity of a tissue is an important characteristic of ageing. Although loss of stem cells proliferation could be detrimental for organisms maintenance, the exacerbation of stem cell growth could led to exhaustion of stem cells niches. A compelling evidence derives

from *Drosophila* intestinal cells. The excessive stem cells proliferation was linked to premature aging.

Changes in cell—cell communication (i.e. endocrine, neuroendocrine or neuronal) have been linked to aging process. Inflammaging, decline of immune system efficiency, accumulation of senescent cells and defective autophagy underlie several age-associated diseases such as diabetes, arthritis, metabolic syndrome and cardiovascular disease [117].

1.5 Aging, senescence and biliary tree disorders

Up to the present, it is not fully understood the influence of ageing in the pathophysiology of liver and biliary diseases. Despite cholangiopathies are not specific of elderly, several data suggest the role of ageing in the clinical course of disease and in the modulation of cholangiocyte pathophysiology.

Previous studies documented age-related alteration of the liver (e.g. hepatic volume reduction, increasing of lipofuscin body or reduction of hepatobiliary functions) as well as molecular changes (e.g. altered response to oxidative stress, a reduced efficiency of DNA repair mechanisms or telomere shortening) which could contribute to decline of liver regenerative capability and to shortening survival rate after liver transplantation [118].

From a clinical point of view, the progression of cholangiopathies seems to be influenced by advanced age. Accordingly, the clinical course of PSC is influenced by patient age at diagnosis with an increasing occurrence of cholangiocarcinoma (CCA) and poor outcome [119].

In addition, donor age has been implicated in the development of biliary complication after liver transplantation [120].

From a molecular point of view, important clues support the role of aging in shaping cholangiocyte biology in course of biliary injury. As an example, analysis of liver samples collected by PSC and PBC patients have shown the increased expression of senescent markers and SASP components in diseased cholangiocytes [42]. Another study of Sasaki, *et al.* demonstrates that small bile ducts of liver tissue collected by early stage PBC-patients, possess elevated positivity to SA-β-GAL staining and increased expression of senescence markers p16 and p21 [82]. As well as in PBC, isolated cholangiocytes from PSC patients show a strong positivity to SA-β-GAL staining and, in addition, a decrease of cholangiocytes proliferation, and the increase of IL-6 and IL-8 levels as compared to control normal human [121]. These intriguingly findings achieved in PSC and PBC patients are corroborated by data collected in animal models of cholestatic liver injury. As an example, isolated cholangiocytes from multi- drug resistance 2 knockout (mdr2 $^{-/-}$) mice, a model of PSC, undergo senescence [122]. The mdr2 $^{-/-}$ mice lack the expression of the canalicular phospholipid flippase mdr2, which serves for bile acids assembly in mixed micelles thus protecting biliary epithelium by toxic bile acids-mediated injury [44]. As a direct consequence of mdr2 lack of expression, toxic bile acids accumulates in the bile triggering bile duct damage.

Cholangiocyte senescence has been recently related to deregulation of cholangiocyte immune response in course of cholangiopathies.

Isolated cholangiocytes from PSC patients subjected to transient stimulation with LPS, known as an important trigger of cholangiocyte injury,

show the increased expression of IL-6 to face cellular damage and cholangiocyte loss. In turns, persistent LPS stimulation induces cholangiocytes senescence as evidenced by either upregulation of protein involved in cell cycle arrest (p16 and p21) and SASP components [42].

Despite these findings demonstrate a link between senescence establishment and disease presentation, it is unknown whether senescence is the trigger of disease or if it is a consequence of chronic damage [81], [123]. Certainly, non-replicative cholangiocytes play a role in the modulation of biliary microenvironment and organ homeostasis [43]. The loss of proliferative capabilities increases cholangiocytes susceptibility to subsequent injuries, leading to a worsening of inflammation process.

2. AIM OF THE THESIS

My thesis project was focused on the identification of molecular pathways which are activated both during the ageing process and in response to damage of the bile ducts. Cholangiocytes, the cell lining the bile ducts, represent the unique target of cholangiopathies, a group of chronic cholestatic diseases which evolve to end-stage liver disease with liver transplantation as the only curative strategy. Due to the progressive nature of the disease and the lack of effective therapies, a deeper understanding of cholangiocytes pathobiology is needed in order to device novel effective therapeutic strategies. Bile duct injury during the course of cholangiopathies is influenced by a number of factors such as genetic susceptibility, epigenetic events or protein expression modulation by miRs. Several clinical and experimental data have recently identified cellular senescence, the age-related damage response, as an important player in the disease development and progression. The aim of this work is to discover new molecular targets involved in the modulation of cholangiocyte biology in pathophysiological conditions. To reach this goal, bioinformatics tools have been exploited to select intracellular pathways and putative molecular targets to be investigated both in *in vitro* and *in vivo* model as well as in human samples of cholangiopathies.

3. MATERIALS AND METHODS

3.1 Materials

Reagents and antibodies, where not specifically indicated, were purchased respectively by Sigma-Aldrich (St. Louise, MO) and by Santa Cruz Biotechnologies Inc. (Santa Cruz, CA). 3,5-Diethoxycarbonyl-1,4-dihydrocollidine (DDC) was purchased by Sniff GmbH (Soest, Germany). Anti-Ck-19 antibody was purchased by Abcam (Cambridge, United Kingdom). Anti-βactin antibody HRP conjugated was provided by Cell signaling (Danvers, MA). Normal rat cultured cholangiocytes (NRC), a murine intrahepatic bile duct cell line, were a generous gift of Prof. Gianfranco Alpini, Texas A&M University, Temple, TX, USA. siRNA oligonucleotides were purchased from Ambion® (Carlsbad, CA, USA). The transfection reagent INTERFERin® was from Polyplus-transfection (Illkirch, France). The senescence accelerated mouse prone 8 (SAMP8) mice, and the control strain SAM resistant 1 (SAMR1) were bred in house at the Experimental Animal Models for Aging Unit, Scientific Technological Area, IRCCS-INRCA, Ancona, Italy. *Twf1* knockout (C57BL/6NTac *Twf1*$^{-/-}$) mice, and their respective controls were purchased from the European Mouse Mutant Archive (EMMA), National Centre for Biotechnology (Campus de Cantoblanco, Madrid, Spain).

3.2 Methods

3.2.1 MicroRNAs selection

Biomedical literature search on Medline has been carried out in order to find relevant studies about miRs involvement in aging process, irrespective of organ or system. It was possible to identify a total of 12 miRs which have been subsequently investigated in our project in line with the aim of the study.

3.2.2 MicroRNAs testing in cholangiocyte: isolation, reverse transcription and real-time PCR

Total RNA was extracted from cholangiocytes isolated by 8-week DDC-fed mice and control animals, and by young and old mice subjected or not to DDC administration. Cholangiocyte isolation was performed by immune-beads purification as previously reported Following RNA purification (miRNeasy mini Kit, QIAGEN, Hilden, Germany). MiRs expression levels were assessed through the SYBR® Green PCR Kit miR-specific miScript Primer Assay (forward primer) and the miScript SYBR® Green PCR Kit (QIAGEN, Hilden, Germany), containing the miScript Universal Primer (reverse primer) were used for specific miRs expression evaluation. Relative abundance of miRs expression was normalized to U6 expression as internal control.

3.2.3 In silico analysis of microRNAs putative targets and intracellular pathways

To predict putative targets and molecular pathways shared among miRs resulted upregulated in cholangiocytes isolated from DDC-fed mice, the software simple String IDentifier (SID1.0) was used [124]. This program is based on an exhaustive search strategy and is specifically designed to screen shared data (target genes, microRNAs and pathways) available from the TargetScan Human 6.2 and DIANA-MicroT 3.0 target prediction databases (http://diana.cslab.ece.ntua.gr/microT/). The actin- binding protein, Twinfilin-1 (TWF1) was selected for the downstream evaluations.

3.2.4 Expression of Twf1 in cholangiocyte

TWF1 expression was assessed in liver tissues of patients affected by PSC (n=10) and PBC (n=10) by real-time PCR. Normal liver tissue surrounding colorectal hepatic metastases (n=17) was used as internal controls. The isolation of cholangiocyte was carried out as previously [125]. In addition, *TWF1* expression was evaluated by immunohistochemistry staining in liver sections of PSC patients (n=5) compared to control livers (n=3 alcoholic liver disease and n=2 cryptogenic liver disease). Ethical approval was obtained from the research ethics committees at each participating medical centre in accordance with the declaration of Helsinki (S-08872b). After acquisition of informed consent, samples were collected at the time of liver biopsy, surgical liver resection or liver transplantation. Expression levels of *Twf1* were evaluated in cholangiocytes isolated from

both control mice (young and old) and mice (young and old) fed with 0.1% DDC, by real-time PCR.

3.2.5 Cell lines and *in vitro* experiments

NRC were cultured on rat tail collagen type I coated flasks and maintained in Dulbecco's modified Eagle medium: nutrient mixture-F12 (DMEM/F12) supplemented with 5% of fetal bovine serum (FBS), 0.01 ml/ml minimum essential media non-essential amino acids, 0.01 ml/ml of chemically defined lipid concentrate, 0.01 ml/ml insulin transferring selenium, 0.01 ml/ml minimum essential media vitamin solution, 200 mM L-glutamine, 12.7 mg/ml bovine pituitary extract, 393 µg/ml desaxamethasone, 3.4 mg/ml 3, 3', 5- triiodo-L-tyronine, 25 µg/ml epidermal growth factor, 4.11 mg/ml forskolin, 1% penicillin-streptomycin, 10 mg/ml gentamicin. All products have been purchased from Invitrogen (Carlsbad CA, USA). Cell silencing was carried out the day of seeding through the INTERFERin® reagent according to manufacturer's recommendation. The final siRNA concentration was 30 nM in medium without FBS.

3.2.5.1 Evaluation of Twf1 role in cholangiocytes proliferation

To estimate if the absence of *Twf1* could affect cholangiocyte proliferation, NRC were seeded in a 6-well collagen coated plate in complete medium without FBS and incubated with either small-interfering (si) RNA against *Twf1*, which turns off gene expression, or with non- targeting (nt) RNA as control, for 48 h. The day after seeding cells were stimulated with complete medium supplemented with increasing concentration of FBS (0-5-20%) to induce cell growth.

Changes in cells proliferation were assessed by measuring mRNA expression level of *Ki67* and protein expression of proliferating Cell Nuclear Antigen (*Pcna*). Changes in cell growth were further evaluated by sulforhodamine B (SRB) colorimetric assay [126]. This technique has been developed by Skehan and colleagues and it is extensively employed in drug-induced cytotoxicity and cells growth studies. The assay relies on the capability of sulforhodamine dye to bind to protein basic amino acids residues of fixed-cells. Thus, the absorbance measured is proportional to cell number and cellular protein content [127]. Briefly, cells were seeded in a 96-well plates in complete medium without FBS and transfected with siRNA against *Twf1* or with ntRNA. The day after seeding, cells were cultured in medium added with 5% FBS and grown for up to 72 hours. After fixing and washing steps, cells were air dried and stained with 0.4% sulforhodamine B solution for 30 min at room temperature (RT). The incorporated dye was then solubilized by adding the SRB solubilization solution in an equal volume of original culture medium. Growth rates were derived by absorbance values obtained spectrophotometrically using a microplate reader (Tecan, Mannedorf, Switzerland).

3.2.5.2 Evaluation of Twf1 role in cholangiocyte senescence

To address the possible role of *Twf1* in cholangiocyte senescence establishment, NRC were seeded in a 6-well collagen-coated plate in complete medium without FBS and exposed for 48 hours to siRNA against *Twf1* or to corresponding ntRNA. The day after seeding, cells were stimulated or not with 5% FBS.

Total RNA was then isolated and transcribed to cDNA following

manufacturer's instruction (Invitrogen, Carlsbad CA, USA). Cellular senescence was assessed by quantification of $p16^{ink4a}$ and $p21^{waf/cip1}$ expression. SASP component levels were measured by real-time PCR as well. Senescence was further evaluated in NRC knocked-down for *Twf1* expression and in control cells, by quantification of Sudan Black-B (SBB) staining as previously reported [128]. A well- established *in vitro* model of cholangiocyte-induced senescence [42], was used to investigate the role of *Twf1* in cholangiocyte senescence modulation. NRCs were seeded in complete media without FBS and silenced or not for *Twf1* expression. Senescence model was induced by persistent stimulation (10 days) with LPS (200 ng/ml). Media, agonists and siRNA were replaced every 48 hours for up to 10 days. The levels of senescence markers and SASP components were evaluated by real-time PCR. SASP components levels were also assessed by enzyme-linked immunosorbent assay (ELISA) in the same setting of experiments.

3.2.6 Animal models

Seven-week old male senescence accelerated mouse prone 8 (SAMP8) and their respective control SAM resistant 1 (SAMR1) [129], [130] (n = 5 per each experimental group), were raised in house at the Experimental Animal Models for Aging Unit (Scientific Technological Area, IRCCS-INRCA, Ancona, Italy) and fed with either 0.1% 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet or related control diet for 4 weeks. The experimental DDC diet and the standard diet were also administered to seven-week old male *Twf1* knockout (C57BL/6NTac $Twf1^{-/-}$) and to wildtype counterpart ($Twf1^{+/+}$ mice) for 8 weeks. As widely described in literature, DDC

-fed mice develop onion skin-like periductal and portal-portal fibrosis, display ductular reaction and a marked increase of serum bile acid levels. All together these features resemble the hallmarks of chronic cholangiopathies [131]. Changes in cholangiocyte proliferation were determined by measuring differences in intrahepatic bile duct mass (by quantitative immunohistochemistry for CK-19 in liver sections), as previously reported [132]. Changes in collagen deposition were assessed by quantitative histochemical staining for Sirius Red. Hepatic content of collagen was evaluated by hydroxyproline quantification [133]. Animal study protocols were performed in compliance with local Institution guidelines and in accordance with the ARRIVE guidelines.

3.2.6.1 SAMR1 and SAMP8

SAMP8 is an accelerated aging model developed by Takeda and his colleagues [129]. SAMP8 strain displays a reduced lifespan of 50% with respect to SAMR1 controls and signs of aging such as ruffled and dull coat, loss of hair, abnormal curvature of the spine and inflammation of the periorbital areas. The phenotypic characteristic are shown in the figure below (Figure 7).

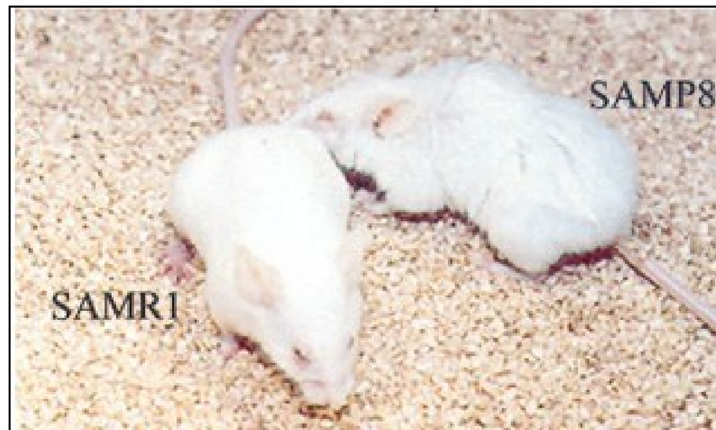


Figure 7. *SAMR1 and SAMP8 mice: phenotypic characteristics*. The signs of aging typical of SAMP8 mice includes loss of skin glossiness, increased coarseness or hair loss, curvature of the spine and inflammation of periorbital areas. (From Ye *et al* 2004 [130]).

SAMP8 and SAMR1 mice belong to the SAM family that derives from continuous sister-brother mating of five AKR mice with severe deterioration, and three AKR mice with normal aging [134]. The grade of aging has been assigned phenotypically by evaluating characteristics such as loss of skin glossiness, increased coarseness or hair loss (Figure 7). SAM family includes 9 SAMP (P1-P9) and 3 SAMR (R1-R3) substrains. Among these, SAMP8 mice exhibit signs of liver degeneration. In particular, biochemical markers of hepatic function such as alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (AP) are increased in SAMP8 mice as compared to the relative controls. Notably, AP increase is associated to bile canaliculi damage or cholestasis. SAMP8 mice display hepatic steatosis (macrovesicular fat) and hepatocellular degeneration (e.g. enlarge cell nuclei, swelling cell bodies and necrosis with deposition of cell debris) in the centrilobular zone of hepatic acinus. At this level, immune cell infiltration, fibrosis and cell degeneration has been found. It is not fully elucidated the mechanism driving the liver degeneration in this animal model.

One of the proposed mechanism relies on the increasing titers of MuLV in liver of SAMP8 (50 fold) with respect to controls. MuLV is a retrovirus that following viral RNA retrotranscription in DNA, integrates his genome in the host genome [135], [136]. Once integrated, viral genome is replicated together with the host chromosomal DNA. However, there are no data that link MuLV to liver disease development. Another mechanisms proposed to explain the liver degeneration occurring in SAMP8 is the hypoxia establishment. With this regard, increased levels of lipid peroxide have been evidenced in liver of SAMP8 compared to SAMR1, since early stage of life [137].

3.2.6.2 *Twf1*$^{+/+}$ *and Twf1*$^{-/-}$

Twf1 knockout (C57BL/6NTac *Twf1*$^{-/-}$) are transgenic mice generated through the "knockout-first" strategy (Figure 8). Null allele is generated through allele splicing to LacZ trapping element included in the targeting cassette. This knockout could be easily modified due to the presence of particular sequences in the trapping cassettes. In particular, the Flippase (Flp) recombinase recognizes the Flp recombinase recognition target (FRT) and it is able to revert the mutation to wild-type upon sequence recognition leaving the *LoxP* sites in either sides of the gene exon of interest. The crossing with *Cre* mice results in *Cre* recombinase-mediated exon excision upon *LoxP* sequences recognition. The cleavage induces a frameshift mutation resulting in mutant transcript decay.

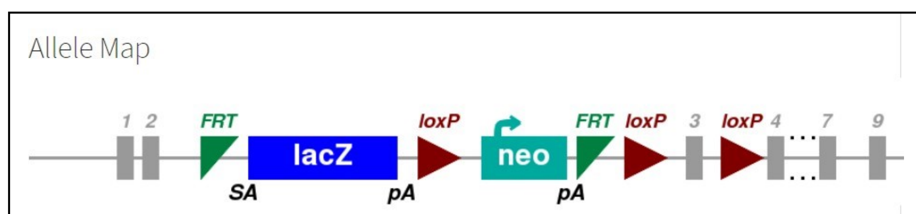


Figure 8. *Illustration of Twf1$^{-/-}$ allele map*

The animals lacking for the expression of *Twf1* have been genotyping before starting the treatment. Genomic DNA was extracted from mouse tail with the DirectPCR lysis Reagent Tail (Viagen Biotech Inc, Los Angeles, USA) following manufacturer's instructions. The protocol for mice genotyping includes separate PCR reactions that detect LacZ, the gene-specific wild type allele, and the mutant specific allele. The set of primers used for the allele discrimination have been listed in the table below (Table 1).

	sense (5´→3´)	**antisense (5´→3´)**
Wild-type allele	TCGTGGAGCATCTAC AGTTGG	CAGACATGCACTTCAGCACG
Mutant allele	TCGTGGAGCATCTAC AGTTGG	TCGTGGTATCGTTATGCGCC
LacZ	TATCACGACGCGCTGTATC	ACATCGGGCAAATAATATCG

Table 1. *Primers set used for mouse genotyping.*

The thermal profile for both PCRs are listed below:

Wild type and mutant PCRs Cycle LacZ PCR

94° C 5 min **94 ° C 30 sec** ┐ **58 ° C 30 sec** ├ **x 35** **72 ° C 45 sec** ┘ **72 ° C 5 min** **12 ° C forever**	**94° C 5 min** **94 ° C 30 sec** ┐ **60 ° C 30 sec** ├ **x 35** **72 ° C 30 sec** ┘ **72 ° C 5 min** **12 ° C forever**

3.2.7 RNA extraction, reverse transcription and real-time PCR

Total RNA was isolated from NRC or liver tissues using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) or alternatively following a manual protocol. A quantity of 1 μg was reverse-transcribed to complementary cDNA with random primers using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City CA, USA) according to the kit's instruction. Primers for real-time PCR were designed with Oligo software version 6.71 (Molecular Biology Insight, Cascade, CO) using reference mRNA sequences accessed through GenBank. The specificity of primers was confirmed by BLAST analysis. Real-time PCR was performed using a Rotor-Gene 6000 instrument (Corbett Life Science Pty. Ltd., Mortlake, NSW, Australia) through the SYBR Green fluorophore. Relative abundance of target genes was normalized to *Peptidylprolyl isomerase B* (*Cyclophilin B*) as internal control. The Gene Expression Macro Genex developed by Bio-Rad (Milan, Italy) was used to calculate relative expression values from real-time PCR data. Oligonucleotide sequences of primers used for real-time PCR are listed in the table above:

Gene name	**sense 5’-> 3’**	**antisense 5’-> 3’**
Mouse *Ppia*	CAGTGCTCAGAGCTCGAAAGT	GTGTTCTTCGACATCACGGC
Mouse *Twf1*	CATGTCCCACCAGACAGGCA	TCTGTATTTCCCGTTTCTGGCTC
Rat *Ppia*	CTGGAAGGCATGGATGTGGTAC	TCTCTCTACTCCTTGGCAATGGC
Rat *Ki67*	CCTTGCAGCATTACAAAGCA	GCTTCTCACCTGTTGCTTCC
Rat *Twf1*	GTCAGTTCATCCCCATTGTCT	TATTCAATGCGCGGATACACA
Rat *p16*^{INK4A}	ATGGAGTCCTCTGCAGATAGA	ATCGGGGGTACGACCGAAAGTGTT
Rat *p21*$^{CIP/WAF1}$	CTGGTGATGTCCGACCTGTTC	CTGCTCAGTGGCGAAGTCAAA
Rat *Il-1α*	ACTACTTCACATCCGCAGCT	TGCGAGTGACTTAGGACGAG
Rat *Il-1β*	CAGGAAGGCAGTGTCACTCA	AAAGAAGGTGCTTGGGTCCT
Rat *Igf-1*	GCTATGGCTCCAGCATTCG	TCCGGAAGCAACACTCATCC
Rat *PAI-1*	GAGGATGAAAGAAACAGCCAGCT	CCCGCTATGAAATTAGATTCACGT

Table 2 .*Primers set used for real-time PCR*

3.2.8 ELISA

Cell Culture media were collected and centrifuged to remove cell debris, and used for Il-1α, Il-1β and Igf-1 levels measurement according to manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The absorbance was read at 450 nm against 620 nm, as reference wavelength, using a microplate reader (Tecan, Mannedorf, Switzerland).

3.2.9 Western Blotting

Total protein lysates were obtained by NRC and liver tissues using a RIPA lysis buffer (Santa Cruz Biotechnology, Texas, USA) supplemented with protease inhibitor cocktails, PMSF and sodium orthovanadate (Santa Cruz Biotechnology, Texas, USA). Liver samples have been mechanically homogenized in 1 ml of RIPA solution by using the Tissue Lyser (QIAGEN, Hilden, Germany). The proteic fractions recovered from cell lysis or tissue homogenization were quantified with the Bradford assay. The absorbance was spectrophotometrically read at 595 nm by using the Sunrise instrument (Tecan, Mannedorf, Switzerland). Thereafter, a total of 60 μg of protein lysates was analysed by Western blot (WB). This technique uses three elements to accomplish the task: separation by size, transfer to a solid support and marking target proteins through an appropriated primary and secondary antibody. The separation by size was carried out by sodium dodecyl sulfate-polyacrlyamide gel electrophoresis (SDS-PAGE) on 12% acrylamide gel. Once the gel has been polymerized, the running buffer (25 mM Tris-HCl, 192 mM Glicine, 0,1% SDS [w/v] pH 8.3) was introduced into the electrophorator. The gel was placed inside the electrophorator and completely covered with the buffer. Each protein fraction collected was then

added with loading buffer 5X (0,25 M Tris-HCl pH 6.8,10% SDS, 50% glycerol, 0,5 M DTT added before loading sample). Once quantified, protein lysates were denatured at 100° C for 5 minutes and then added in each well. A protein ladder marker has been loaded beside the protein fractions to evaluate the molecular weight of each proteic sample. The gel electrophoresis has been carried out at 100 mV for 2 hours. After proteins separation, the blotting in a polyvinylidene fluoride membrane (PVDF) of the same dimension of the gel has been performed by using the instrument iBlot (Invitrogen, Carlsbad CA, USA) for 5 or 7 minutes depends on protein of interest molecular weight. At the end of the transfer, the nitrocellulose membrane has been placed on the blocking solution TBS-T (20 mM Tris- HCl pH 7.2-7.6, 300 mM NaCl, 0,3% Tween 20) added with 5 % non-fat dry milk [w/v] for 1 hours at room temperature. At the end of this step, the membrane was incubated in agitation at 4°C overnight, with the specific primary antibody properly diluted in TBS-T 5% non-fat dry milk. The binding of primary antibody with the protein of interest, have been displayed using a secondary antibody conjugated with peroxidase (HRP) diluted 1:2000 in blocking solution, incubated for 1 hours at room temperature. Every step of incubation has been followed by 3 washing with TBS-T for 5 minutes each in agitation. The HRP in the presence of *ECL mix* constituted by two detection reagents at a ratio 40:1, catalysed the oxidation of the chemiluminescent substrate (Pierce, Waltham, MA). The intensity of the bands was determined by scanning video densitometry using the Chemi Doc imaging system (UVP, LCC, Upland, CA).

Anti β-actin antibody conjugated with HRP (Cell signalling, Danvers, MA) has been used as *equal loading* control and for normalization.

Densitometry has been carried out with Image J software.

3.2.10 Immunohistochemistry and histochemistry analysis

Five µm-tick histologic slides were analysed for TWF1 and CK-19 expression by immunohistochemistry. Collagen deposition was assessed by quantitative histochemistry for Sirius Red (saturated picric acid containing 0,1% Direct red 80 and 0,1% di Fast Green FCF) as previously described [133]. For immunohistochemistry evaluation, glass slides were incubated with non-immune serum to fill non-desirable antigenic sites. Serum was obtained by animal belonging to the same species of those used for secondary antibody production. Hepatic sections have been pre-treated with 3% H_20_2 for 10 minutes to inhibit endogenous peroxidases. Samples were incubated with primary antibody overnight at 4° C and subsequently washed in Phosphate saline buffer 1X (PSB). The binding between the specific primary antibody and the relative antigen has been evidenced with the secondary antibody HRP-conjugated, incubated for 30 minutes at room temperature. After this step, slides were washed to remove the unbound secondary antibody and then incubated with diaminobenzidine (DAB) to detect the protein of interest. Counterstaining has been carried out with 5 minutes Mayer haematoxylin. Histological preparations were observed under an optical microscope (20X).

3.2.11 Liver hydroxyproline content quantification

In order to quantify the hepatic content of collagen, hydroxyproline assay was performed as previously described [133]. Liver samples (100 mg) were homogenized in ddH_2O, vortexed and precipitated in Trichloracetic acid (TCA) for 20 minutes. Following this step, samples were centrifuged at 6000 rpm for 10 minutes at 4°C. Supernatants were discarded and pellets were resuspended in 100% of cold ethanol. The last two steps were then repeated. Recovered pellets were dry precipitated for 5- 10 minutes. Samples were resupended in 800 µl of 6 M HCl and placed in heat block for 18 hours at 110°C. After hydrolysis, samples were centrifuged (14000 rpm for 10 minutes), neutralized with 10 M NaOH, oxidized for 25-30 minutes with Chloramine T solution (1,27 g Chloramine T, 20 mL of 50 % N- Propanol in dd H_2O, and Acetate Citrate Buffer up to 100 ml) and incubated in Ehrlich's perchloric acid solution (1,5 g 4- dimethylaminobenzaldehyde in 10 ml of N-Propanol/Perchloric acid mix in 1: 2 ratio). Absorbance was read at 560 nm wavelength in a microplate reader (Tecan Group Ltd., Männedorf, Switzerland).

3.2.12 Statistical analysis

Data are expressed as mean ± SD. 95% confidence interval (CI) was calculated. Differences between groups were analyzed by Student's t-test or analysis of variance (ANOVA), as appropriate. Differences between groups were considered significant when the *p* value was lower than 0.05.

4. RESULTS

4.1 Age-related miRs are upregulated in diseased cholangiocytes

A set of 12 miRs known to be involved in the regulation of ageing processes, were selected from the literature search on Medline. Each miR has a functional role which is summarized in the table below (Table 3).

miR	Role	Ref.
miR-1a	Upregulated in Zmpste24-/- progeroid mice , modulates Igf-1 transcription	PMC3034170
miR-29b	Upregulated in response to DNA damage in a p53-dep manner, upregulated in Zmpste24-/- progeroid mice	PMC3117645
miR-30e	Represses B-Myb expression (oncogene)	PMC3021067
miR-669c	Increased in aged livers, targets glutathione S-transferases	PMID: 18561983
miR-709	Increased in aged livers	PMID: 18561983
miR-214	Increased in aged livers	PMID: 18561983
miR-93	Increased in aged livers, targets glutathione S-transferases; involved in oxidative stress (mitochondrial damage)	PMID: 18561983
miR-34a	Tumour suppressor gene, inhibits SIRT1 expression (cell cycle and apoptosis)	PMID: 19221490
miR-146a	Upregulated during senescence; targets IRAK1 and limits IL-6 and IL-8 expression. Also downregulated (increases ROS production)	PMID: 20148189
miR-146b	Upregulated during senescence, targets IRAK1 and limits IL-6 and IL-8 expression. Also downregulated (increases ROS production)	PMID: 20148189
miR-20a	Repress LRF thus activating p19AFR (inhibition of cell proliferation and induction of senescence)	PMID: 21114763
miR-24	Decreased in replicative senescence associated with p16 increased expression	PMID: 18365017

Table 3. *Age-related miRs selected from literature search.*

The mRNA expression levels of age-related miRs were screened in cholangiocytes isolated from mice subjected to 8-week DDC feeding, as a model of sclerosing cholangitis. As shown in Figure 9, the levels of *miR-1a*, *miR-30e*, *miR-93*, *miR-34a*, *miR-146b* and *miR-20a* resulted increased in diseased cholangiocytes with respect to controls. No changes could be detected in the expression of the remaining miRs (data not shown).

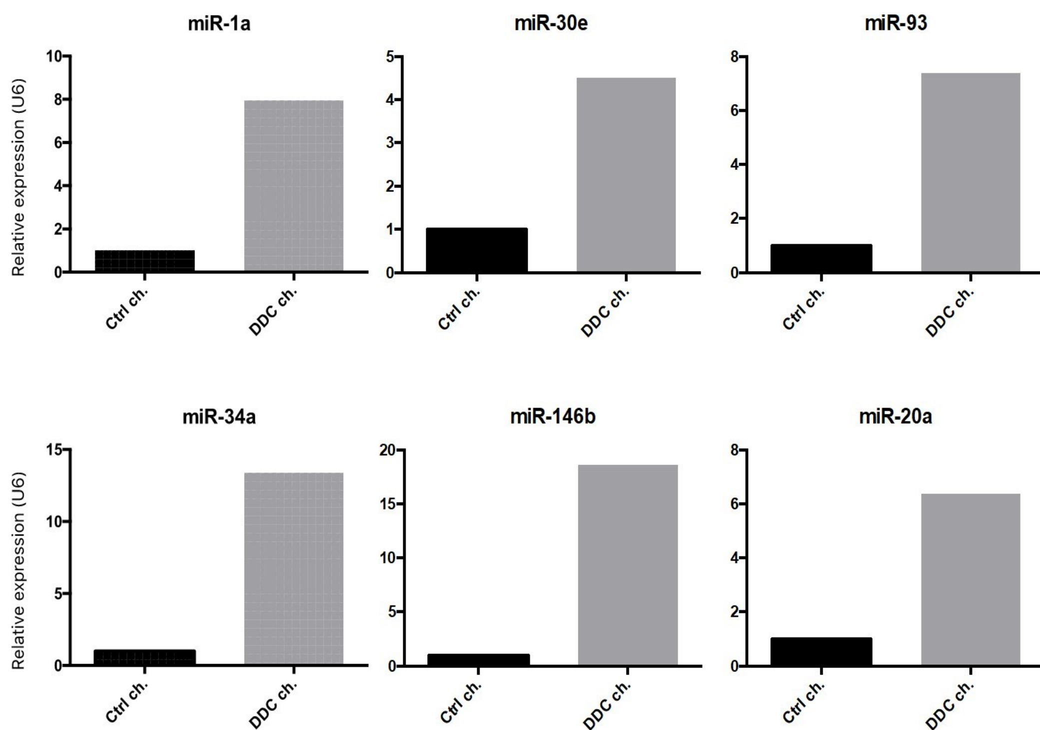


Figure 9. *Age-related miRs are increased in diseased cholangiocytes*. Cholangiocytes were isolated from mice subjected to either 8-week DDC feeding or control feeding. Gene expression of miR-1a, miR-30e, miR-93, miR-34a, miR- 146b and miR-20a is increased in cholangiocytes of injured animals as compared to controls. Data are expressed as mean of a pool sample of at least 3 animals for each experimental group.

4.2 Twf1 is the molecular target of upregulated miRs

In silico analysis was carried out so as to identify putative intracellular pathways and molecular targets commonly regulated by the 6 miRs whose levels are increased in DDC-isolated cholangiocytes. We found 30 signaling pathways regulated by all the 6 upregulated miRs (*miR-1a*, *miR-30e*, *miR-93*, *miR-34a*, *miR-146b* and *miR-20a*) (Table 4).

KEGG pathway ID	KEGG pathway name
mmu00564	Glycerophospholipid metabolism
mmu04130	SNARE interactions in vesicular transport
mmu04020	Calcium signaling pathway
mmu04620	Toll-like receptor signaling pathway
mmu04916	Melanogenesis
mmu04330	Notch signaling pathway
mmu05020	Parkinson's disease
mmu04320	Dorso-ventral axis formation
mmu04514	Cell adhesion molecules (CAMs)
mmu04530	Tight junction
mmu04060	Cytokine-cytokine receptor interaction
mmu04070	Phosphatidylinositol signaling system
mmu04120	Ubiquitin mediated proteolysis
mmu04520	Adherens junction
mmu04080	Neuroactive ligand-receptor interaction
mmu04310	Wnt signaling pathway
mmu05213	Endometrial cancer
mmu05221	Acute myeloid leukemia
mmu04810	Regulation of actin cytoskeleton
mmu04630	Jak-STAT signaling pathway
mmu04110	Cell cycle
mmu05210	Colorectal cancer
mmu04010	MAPK signaling pathway
mmu04012	ErbB signaling pathway
mmu05222	Small cell lung cancer
mmu05223	Non-small cell lung cancer
mmu04350	TGF-beta signaling pathway
mmu05212	Pancreatic cancer
mmu04360	Axon guidance
mmu05220	Chronic myeloid leukemia

Table 4. *Signaling pathways regulated by the upregulated miR.*

Moreover, based on the analysis of the TargetScanHuman6.2 database, it was possible to identify 6 molecular targets shared among more than one miRs screened (Table 5).

Annotation	**Gene Name**	**Common miRs**
ZC3H7B	**Zinc finger CCCH-type containing 7B**	***miR-93; miR-34a; miR-1a***
SLC7A6	**Solute carrier family 7 (amino acid transporter light chain, y+L system), member 6**	***miR-30e; miR-34a; miR-1a***
CTNND2	**Catenin (cadherin-associated protein), delta 2 (neural plakophilin-related arm-repeat protein)**	***miR-30e; miR-34a; miR-1a***
GLCE	**Glucuronic acid epimerase**	***miR-30e; miR-34a; miR-1a***
TWF1	**Twinfilin, actin-binding protein, homolog 1 (Drosophila)**	***miR-30e; miR-20a; miR-1a***
LCOR	**Ligand dependent nuclear receptor corepressor**	***miR-146b; miR-30e; miR-1a***

Table 5. *Molecular targets common to more than one upregulated miRs*

Of note, the acting-binding protein Twinfilin 1 (TWF1), is the common putative target of *miR-1a*, *miR-20a* and *miR30e.* TWF1 is an actin-binding protein conserved from yeast to mammals. It belongs to the central cytoskeleton regulators which sequesters actin monomer and caps filament barber ends [138]. In particular it inhibits the actin- polymerization and regulates cell motility by capping F-actin barbed ends.

4.3 Twf1 and related miRs are upregulated in old and diseased cholangiocytes

In order to investigate the possible role of TWF1 in the modulation of ageing process and cholangiocytes biology in course of biliary injury, mRNA expression levels of *Twf1* and its related miRs were evaluated in isolated cholangiocytes from young (2-month old) and old (22-month old) mice subjected to DDC diet. As shown in Figure 10A, the expression of *miR-1a*, *miR-20a* and *miR-30e* were modulated in aged and injured cholangiocytes. Similarly, *Twf1* mRNA levels were increased in old- diseased cholangiocytes as compared to young controls. Intriguingly, *Twf1* levels tended to increase progressively from young-diseased cholangiocytes, to old cholangiocytes and in aged cholangiocytes isolated by standard diet- fed mice (Figure 10B).

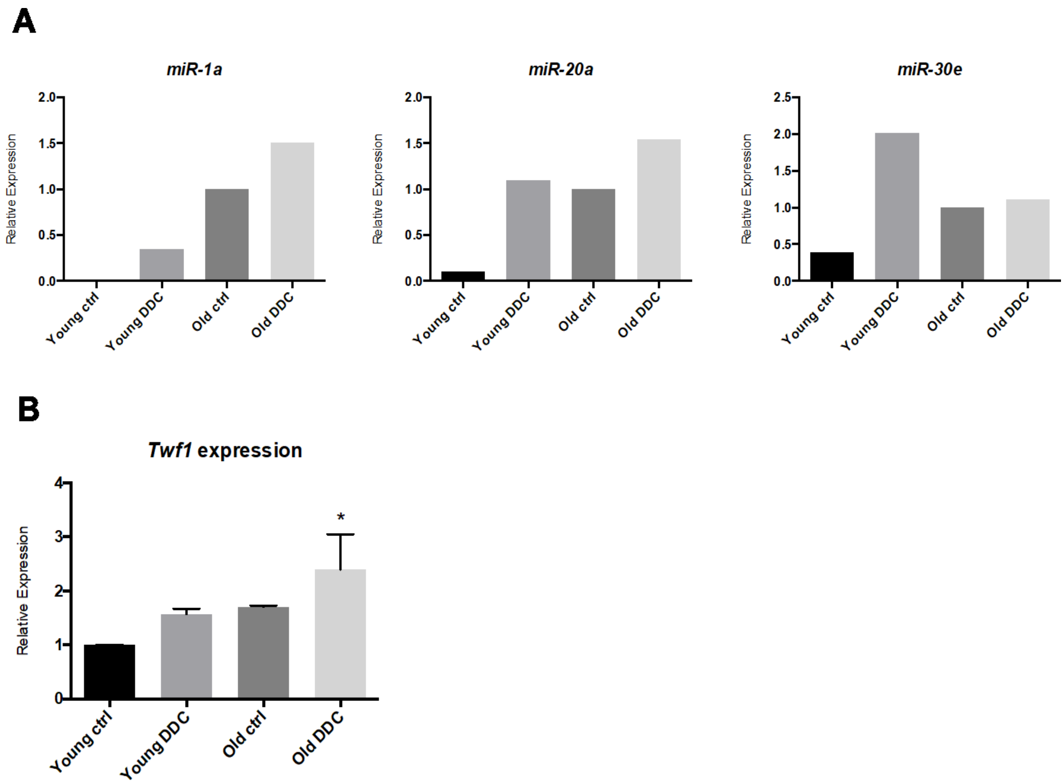


Figure 10. *Twf1 and related miRs are upregulated in both aged and injured of cholangiocytes. (A)* Expression levels of miRs (*miR-1a, miR-20a and miR-30e*) was measured in cholangiocytes isolated from young and aged mice, either subjected or not to DDC-feeding for 8 weeks. The mRNA levels resulted upregulated in both old and diseased cholangiocytes. (B) Expression levels of *Twf1* were upregulated in old-DDC cholangiocytes with respect to young control animals and tended to be higher in young cholangiocytes subjected to DDC and in old cholangiocytes of control mice with respect to young control animals. Data are expressed as mean of a pool sample of at least 3 animals per condition; *: $p < 0.05$ vs. young control (ANOVA).

4.4 Evaluation of TWF1 expression in human samples

TWF1 expression was evaluated in liver tissues of patients affected by primary cholangiopathies. Intriguingly, *TWF1* mRNA levels were upregulated in PBC and PSC cholangiocytes with regard to controls (Figure 11A). *TWF1* expression was further assessed by immunohistochemistry in liver sections of PSC patients and in liver samples collected by patients affected by other liver disease for comparison. *TWF1* staining positivity was evidenced at the portal triad level (Figure 11B). In particular, *TWF1* expression tends to be increased in cholangiocytes of proliferating bile ducts of PSC patients as compared to the relative controls. A score ranging from 0 to 5 based on *TWF1* staining intensity has been assigned for each patient for semi-quantitative evaluation. These data suggest that expression of *TWF1* is not restricted to PSC cholangiocytes but is linked to cholangiocyte proliferation, which occurs in lower level also in liver diseases other than cholangiopathies.

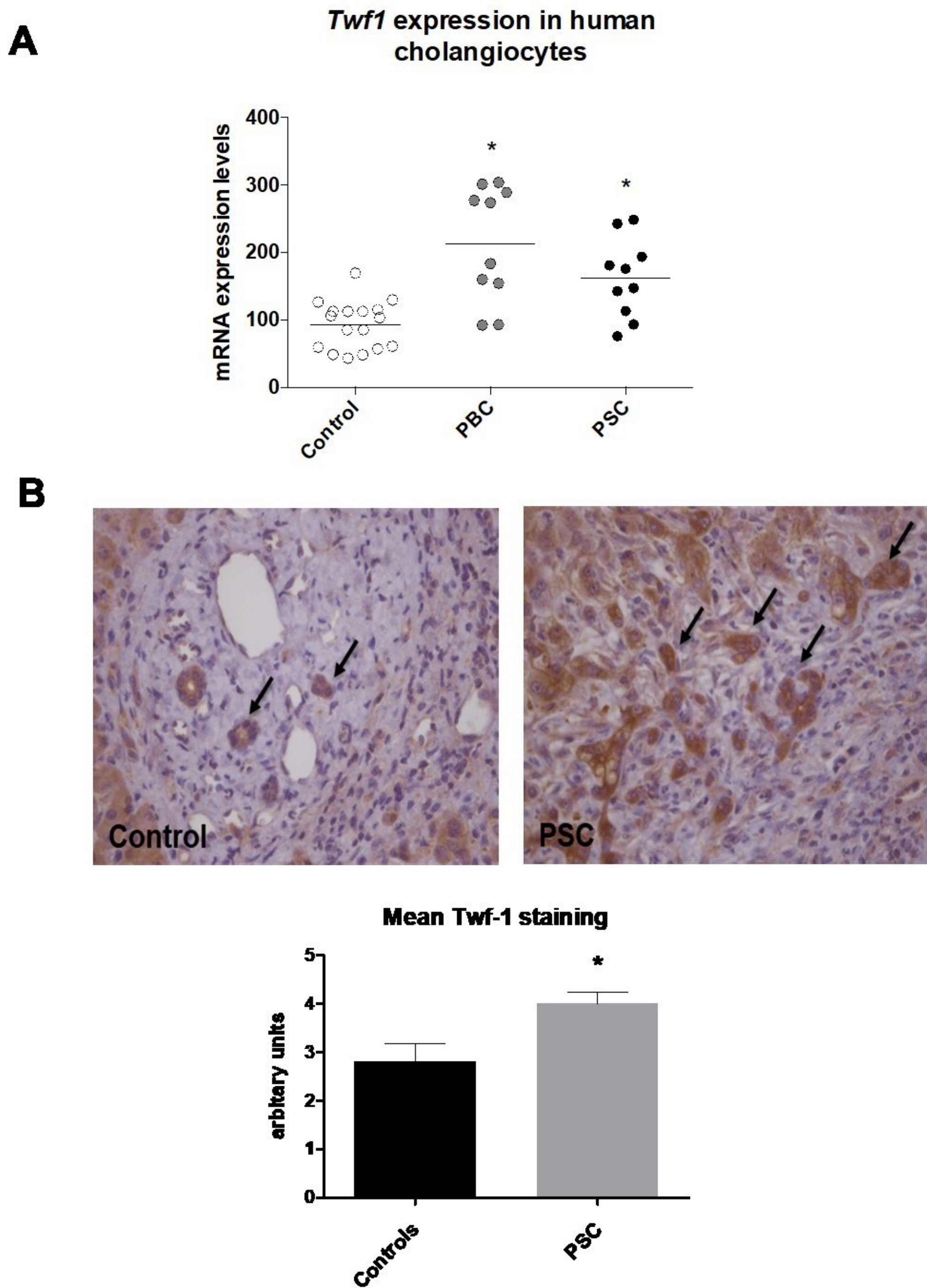


Figure 11. *TWF1 expression in human reactive cholangiocytes.* (A) mRNA expression levels of TWF1 were higher in liver tissue from PBC and PSC patients compared to control subjects; *: $p < 0.05$ vs. control (ANOVA). (B) The expression of TWF1 was evaluated by immunohistochemistry in biopsy samples of PSC patients and compared to patients affected by other liver diseases. The staining showed an increased protein expression in reactive cholangiocytes of the portal tracts of PSC patients compared to those belonging to patients affected by other liver diseases. *: $p < 0.05$ vs. control (t-Test). Original magnification 20 X.

4.5 Twf1 is involved in cholangiocytes proliferation

In order to verify the involvement of TWF1 in biliary proliferation, *Twf1* knocked-down cholangiocytes and control cells were stimulated with 5% and 20% FBS. As shown in Figure 12A, the absence of *Twf1* gene expression following siRNA transfection, decreased the expression of the proliferation marker *Ki67* in serum stimulated cells. Accordingly, *Pcna* protein expression t was reduced in *Twf1* Knocked-down cholangiocytes as compared to the relative controls (Figure 12C). On a functional level, cholangiocyte proliferation evaluation was carried out by SRB assay. Cell growth rate was significantly reduced in *Twf1* knocked-down cells with respect to control cells, at both 48 hours and 72 hours after cell seeding (Figure 12B). These data suggest a possible involvement of TWF1 in cholangiocyte proliferation, which represents one of the compensatory process activated by reactive cholangiocytes to face bile ducts injury.

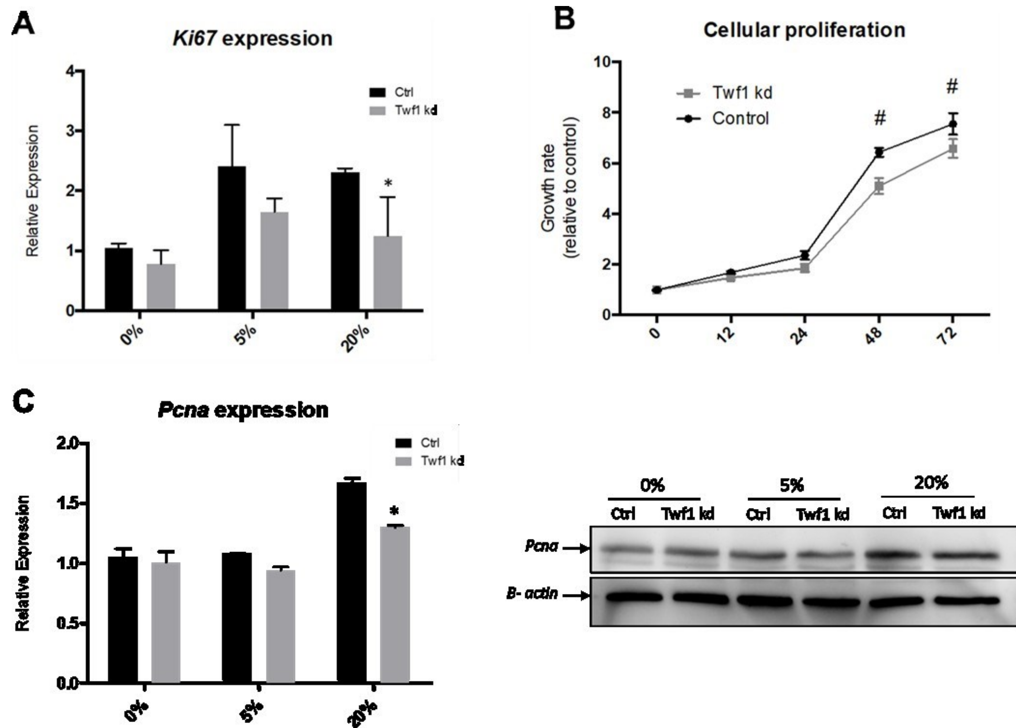


Figure 12. *Twf1 supports biliary proliferation in vitro.* (A) Gene expression of the proliferation marker *Ki67* was significantly reduced in *Twf1* knocked-down cholangiocytes stimulated with 20% serum as compared to control cells. (B) In normal culture medium, growth curves of *Twf1* knocked-down cholangiocyte was significantly lower at 48 and 72 hours after seeding compared to control cells. (C) Protein expression levels of *Pcna* are significantly decreased in *Twf1* knocked- down cholangiocytes stimulated with 20% serum with respect to relative controls. Data are expressed as mean ± SD of at least 3 experiments; *: $p < 0.05$ vs. 20% control; #: $p < 0.05$ vs. respective control (ANOVA).

4.6 Twf1 is involved in cholangiocytes senescence

One of the aim of our study was to examine the role of TWF1 in ageing process modulation. As widely described in the previous paragraphs (section 1.4.1), the upregulation of senescence markers and the secretome enrichment of SASP components, are pathological mechanisms activated in response to age-related damage. Senescence was evaluated in cholangiocytes silenced for *Twf1* expression by measuring the levels of senescence and SASP markers and by quantification of lipofuscin deposition assessed by SBB staining. As depicted in Figure 13A, the lack of *Twf1* expression determines the induction of senescence as evidenced by the significant increasing of mRNA levels of $p16^{ink4a}$ in *Twf1* knocked-down cells exposed to a pro-proliferative stimulus. However, any changes in the alternative senescence marker $p21^{cip/Waf1}$ have been detected on mRNA level. Accordingly, the ratio of positive cells for SBB staining was markedly increased in *Twf1* knocked-down cholangiocytes compared to control cells, thus indicating the establishment of cellular senescence. The expression levels of *Twf1* were measured in a well-established *in vitro* model capable to induce cellular senescence. On a mRNA levels, the expression of *Twf1* is increased in LPS-induced senescence cholangiocytes compared to control cells. The induction of *Twf1* expression triggered by persistent LPS stimulus (10 days) is abolished in cells subjected to *Twf1* knockdown. These data are in line with our previous results, suggesting that *Twf1* expression is induced in response to cellular senescence possibly as a compensatory mechanisms stimulating cholangiocyte proliferation.

.

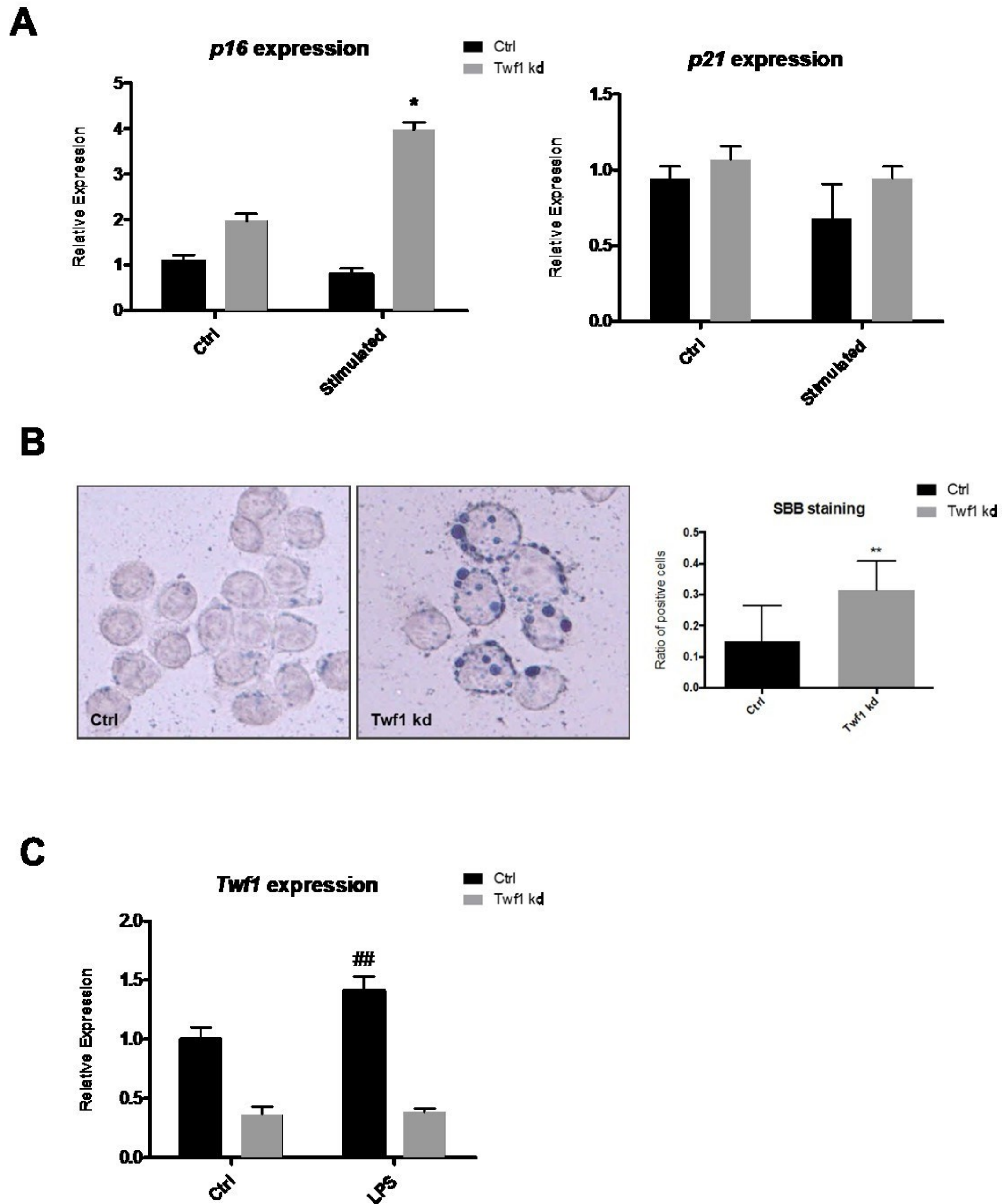


Figure 13. *Senescence is induced in Twf1 knocked-down cholangiocytes in vitro.* (A) The expression levels of the senescence markers *p16* and *p21* were evaluated in *Twf1* knocked-down cholangiocytes and in control cells, either or not exposed to a pro-proliferative medium. A significant increase in *p16* expression was evidenced in proliferating *Twf1*-deficient cholangiocytes. No differences could be detected in *p21* expression. (B) The ration of positive cell to SBB staining was significantly increased in *Twf1* knocked-down cholangiocytes, suggesting an enhanced cellular senescence. (C) *Twf1* expression was induced in LPS-treated cholangiocytes. Data are expressed as mean ± SD of at least 3 experiments; *: p < 0.05 vs. control; **: p < 0.01 vs. control; ##: p< 0.01 vs. control.

On the same line, senescence associated secretome enrichment (*Il- 1α*, *Il-1β* and *Igf-1)* could be demonstrated by real-time PCR in cholangiocytes silenced for *Twf1* gene expression with respect to relative controls. *Pai-1* expression was increased as well, however the increase does not reach statistical significant (Figure 14). Levels of SASP component were measured also in cell culture media as secretor factors by ELISA. *Il-1α* protein levels were increased in the cell supernatants collected by *Twf1* knocked-down cholangiocytes with regards to controls. Conversely, the levels of *Il-1β* and *Igf-1* were under the detection limit of the test (data not shown). Taken together these data suggest that *Twf1* induction could dampen the activation of senescence.

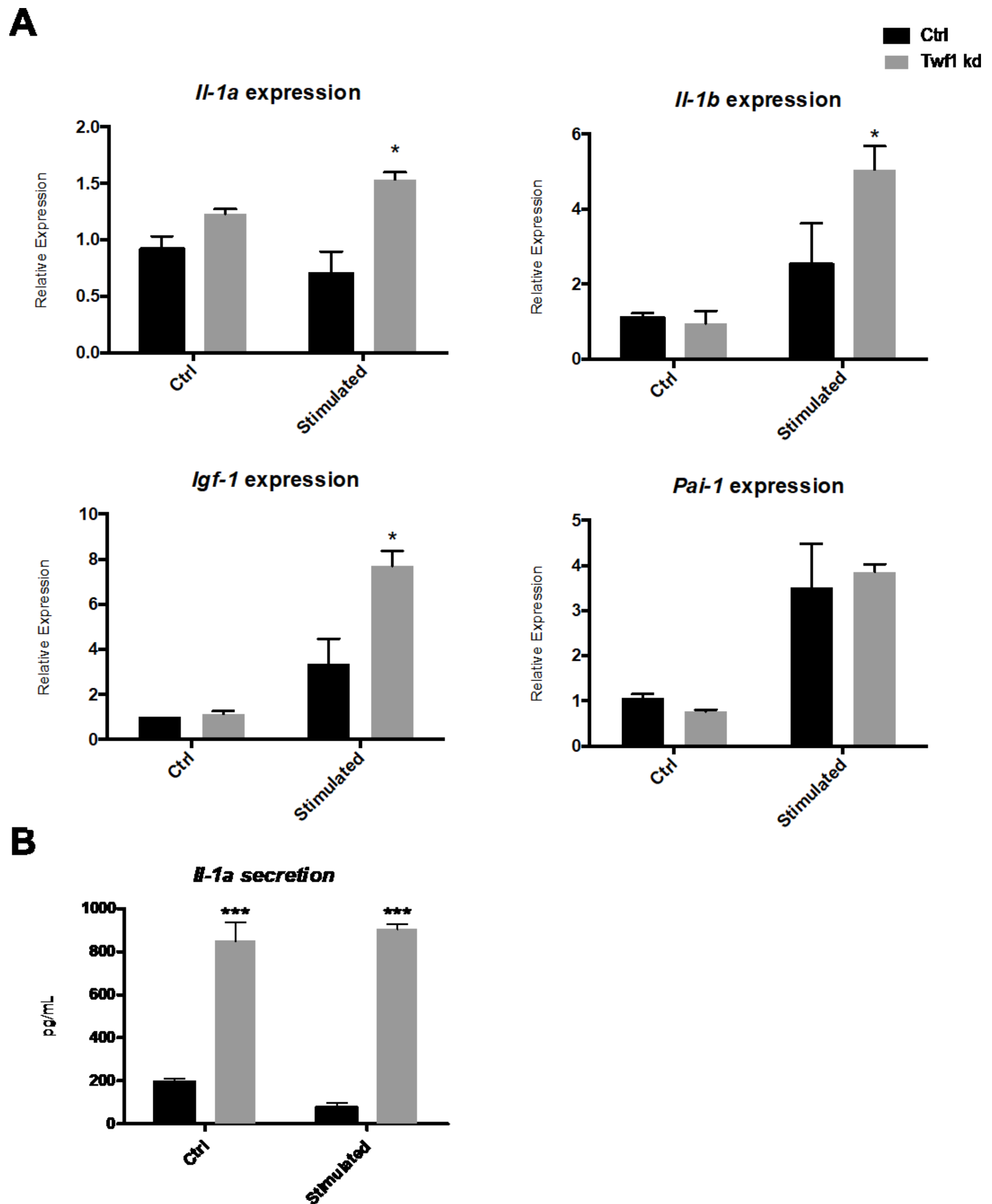


Figure 14. *Senescence associated secretory phenotype is induced in Twf1 knocked-down cholangiocytes in vitro.* The expression of SASP markers was evaluated in *Twf1* knocked-down cholangiocytes subjected to a pro-proliferative stimulus, with respect to relative controls. Levels of *Il-1α*, *Il-1β* and *Igf-1* were increased in *Twf1* knocked-down cholangiocytes, whereas no difference in *Pai-1* expression could be evidenced. (C) Levels of *Il-1α* released in cell culture media were significantly increased in *Twf1* knocked-down cholangiocytes either subjected or not to pro-proliferative stimulus. Data are expressed as mean ± SD of at least 3 experiments; *: $p < 0.05$ vs. respective control; ***: $p < 0.05$ vs. respective control (ANOVA).

The levels of senescence markers *p16* and *p21* and of SASP components, were evaluated in LPS-induced cholangiocyte senescence. Expression of either senescence markers or SASP components was upregulated in LPS-treated cholangiocytes exposed to siRNA against *Twf1* (Figure 15A-B). No differences could be found in *Il-1*α and *Pai-1* expression. On the whole, these data show that *Twf1* induction observed in response to endotoxic stimulus, may slow down or reduce senescent phenotype establishment in response to noxious stimuli.

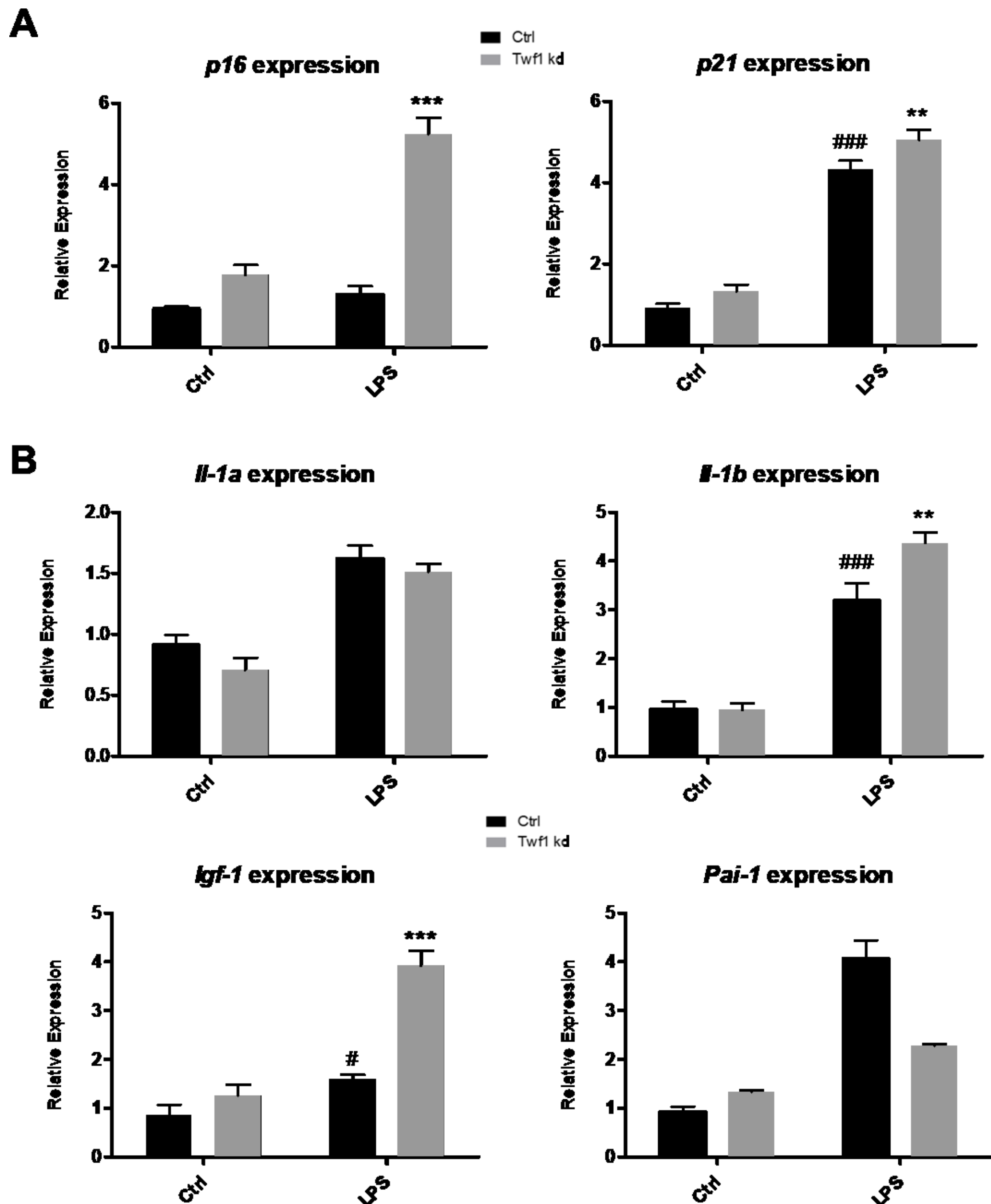
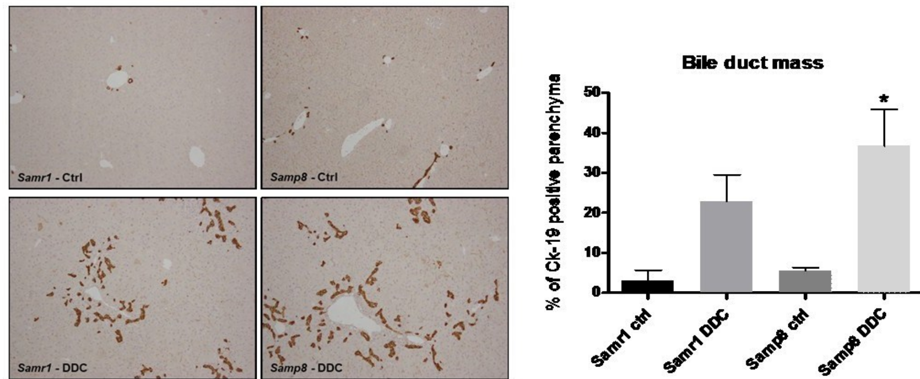


Figure 15. *Twf1 may dampen cholangiocyte senescence in vitro*. (A) The expression of senescence markers p16 and p21 was evaluated in Twf1 knocked-down cholangiocytes subjected to a pro-senescence stimulus. Levels of p16 and p21 were increased in Twf1 knocked-down treated cholangiocytes, (B). SASP component expression was investigated in the same experimental setting. Levels of Il-1β and Igf-1 resulted increased in LPS-treated cholangiocytes lacking Twf1 expression. Any variation in Il-1α and Pai-1 expression could be evidenced. Data are expressed as mean ± SD of at least 3 experiments; **: $p < 0.01$ vs. LPS-treated control;***: $p <0.001$ vs. LPS-treated control; #: $p < 0.05$ vs. respective control; ###: $p < 0.001$ vs. respective control (ANOVA)

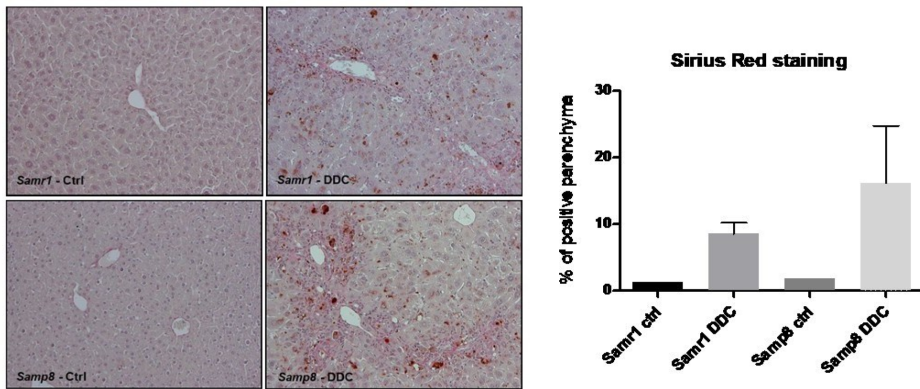
4.7 Accelerated aging exacerbates biliary injury *in vivo*

To investigate the possible role of aging in the modulation of cholangiocytes biology, bile ducts damage was inflicted to an *in vivo* model of accelerated senescence, the SAMP8 mice, and to relative controls SAMR1. The two experimental groups were fed with DDC experimental diet for 4 weeks, as a model of sclerosing cholangitis, or alternatively with standard diet. As shown in Figure 16A, the intrahepatic bile mass assessed by Ck-19 immunohistochemistry and quantification, tended to be higher in SAMP8 mice subjected to DDC diet compared to SAMR1, subjected to the same experimental diet. Fibrosis occurrence was evaluated by quantification of Sirius Red staining and hydroxyproline liver content. The percentage of Sirius Red positive liver parenchyma was slightly increased in SAMP8 DDC-fed mice compared to the relative controls. Unfortunately, the increase is not statistical significant (Figure 16B). As depicted in Figure 16C the hepatic content of hydroxyproline showed a similar trend. Albeit hydroxyproline content was increased in SAMP8 mice subjected to DDC with respect to injured SAMR1, the differences were not significant (Figure 15C). The data provided, suggest that aging process probably aggravates biliary response to injury.

A



B



C

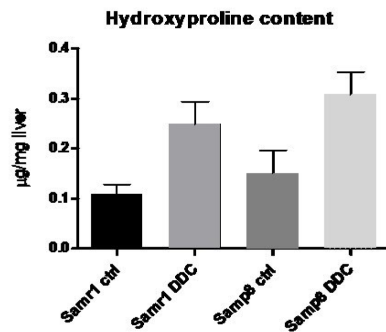


Figure 16. *Cholangiocyte response to injury is worsened in a mouse model of accelerated aging*. SAMP8 and SAMR1 mice were subjected to 4-week DDC diet as a model of sclerosing cholangitis or alternatively to standard diet. (A) As shown in the representative pictures (magnification 20X) of *Ck-19* staining (positive cholangiocytes in brown), the intrahepatic bile duct mass was increased in SAMP8 DDC-fed mice with respect to SAMR1 injured mice. (B) The percentage of positive liver parenchyma for Sirius Red staining was increased in SAMP8 mice in response to DDC treatment as compared to the relative controls. (C) Hydroxyproline content was increased in SAMP8 mice, either or not subjected to DDC feeding, with respect to SAMR1 controls. Data are mean ± SE. *: $p < 0.05$ vs. SAMR1 on DDC diet.

In order to verify changes of *Twf1* protein expression in course of biliary injury and in aging process, *Twf1* protein levels were evaluated in liver samples of SAMP8 and SAMR1 mice, subjected or not to DDC feeding. As widely reported, SAMP8 display a reduced life span. In particular 2-month SAMP8 mice phenotypically resemble old mice (about 14 months of age). As expected Twf1 expression resulted upregulated by aging process. Interestingly, the increase was exacerbated when aged mice are subjected to bile duct injury (Figure 17).

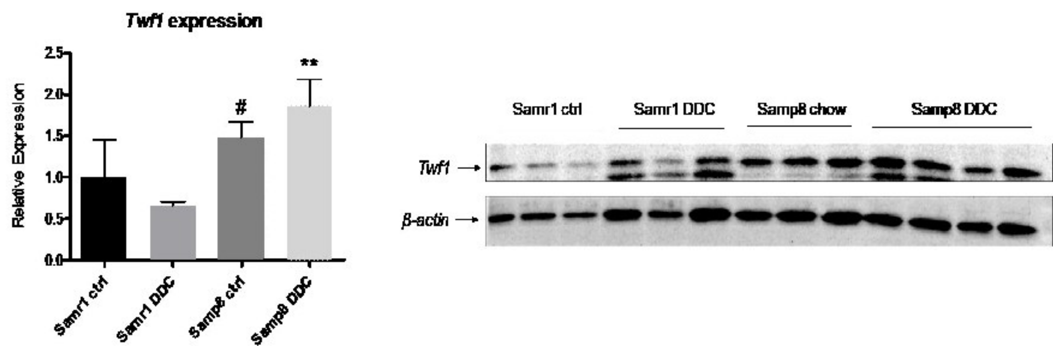
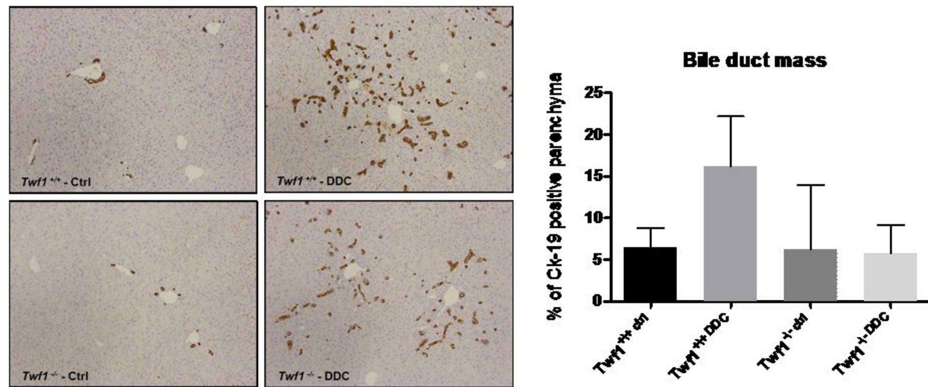


Figure 17. *Twf1 expression is modulated by cholangiocyte senescence and injury in vivo. Twf1* expression was evaluated at protein level in SAMP8 and SAMR1 mice subjected or not to DDC-feeding. *Twf1* protein levels were upregulated in aged mice. The increasing was more pronounced when the injurious stimulus is inflicted on aged mice. Data are mean ± SE. **: $p < 0.01$ vs. SAMR1 on DDC diet; #: $p < 0.05$ vs. SAMR1 on DDC diet.

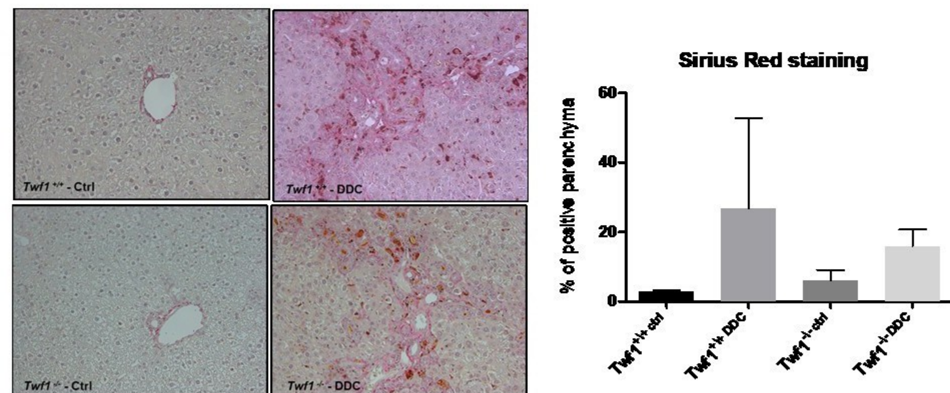
4.8 The absence of Twf1 reduces biliary response to injury *in vivo*

To investigate the influence of *Twf1* in cholangiocytes response to injury *Twf1* $^{-/-}$ mice and the relative wildtype counterparts, were subjected to standard diet or DDC diet administration for 8 weeks, as a model of sclerosing cholangitis. As previously, biliary proliferation and fibrosis were evaluated respectively by Ck-19 immunohistochemistry and quantification, and by quantification of Sirius Red staining as well as by determination of liver hydroxyproline content. Biliary mass assessed by Ck-19 immunohistochemistry and quantification, tended to be decreased in *Twf1*$^{-/-}$ mice compared to diseased wildtype mice (Figure 18A). As shown in Figure 18B, collagen deposition was lower in *Twf1*$^{-/-}$ as compared to the relative controls. However, the existing differences do not reach statistical significant. In addition to histochemical staining, fibrosis has been evaluated by quantification of hydroxyproline in the liver. Hepatic content of hydroxyproline was significantly decreased in *Twf1*$^{-/-}$ mice with respect to *Twf1*$^{+/+}$ fed with DDC diet (Figure 18C). These data reinforce the hypothesis whereby the absence of *Twf1* expression improves cholangiocyte response in course of biliary injury.

A



B



C

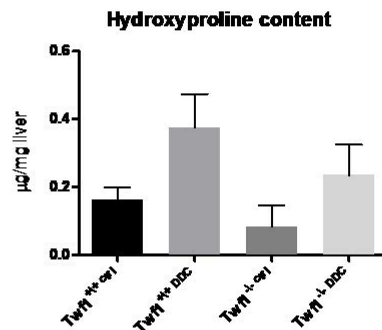


Figure 18. *Twf1 lack of expression reduces reactive cholangiocytes response to injury. Twf1* $^{+/+}$ *and Twf1* $^{-/-}$ mice were subjected to 8-week DDC feeding as amodel of sclerosing cholangitis or alternatively to standard diet. (A) As depicted in the representative pictures (magnification 20X) of *Ck-19* staining (positive cholangiocytes in brown), the intrahepatic bile duct mass tended to decrease in DDC-fed mice lacking for *Twf1* expression, as compared to *Twf1* $^{+/+}$ DDC-fed mice. (B) The percentage of positive liver parenchyma to Sirius Red staining, showed a reduction of collagen deposition in *Twf1*$^{-/-}$ mice, with regard to DDC-fed *Twf1*$^{+/+}$mice. (C) Quantification of hydroxyproline content was significant lower in *Twf1*$^{-/-}$ DDC-mice compared to diseased wildtype animals. Data are mean ± SE. **: $p < 0.01$ vs. *Twf1* $^{+/+}$ on DDC diet.

5. CONCLUSIONS

In the current study it was possible to demonstrate that:

1. Twf1 upregulation occurs in response to aging and in course of bile ducts injury;
2. Twf1 modulates cholangiocyte pathobiology by inducing cholangiocytes proliferation and by preventing senescence establishment;
3. Bile ducts injury is worsened in a mouse model of accelerated senescence;
4. Intrahepatic bile duct mass and fibrosis are reduced in *Twf1* $^{-/-}$ mice.

The overall findings suggest that the pathophysiological mechanisms underlying the aging process and cholangiocytes response to biliary injury, may be similar and that these processes may progress through the activation of common pathways.

Up to now, it is still unexplored the way by which aging influences the onset or the progression of cholangiopathies. A number of studies unveil the appearance of anatomic alteration and molecular changes in the liver linked to aging. Clinical data showed that the clinical course and evolution of PSC, seems to be related to the patient age at diagnosis with older patients at increased risk of developing cholangiocarcinoma [119]. Moreover, the donor age is among the most important risk factors predisposing to the development of biliary complications after liver transplantation [120].

Further data derive from human liver samples from PSC and PBC patients. In particular, it has been shown that diseased cholangiocytes upregulate the expression of genes involved in cell cycle arrest, show a strong positivity to SA-B-GAL staining [82], [121] and increase the levels of proinflammatory mediators belonging to the SASP secretome [42]. On the same line, cholangiocytes of multi-drug resistance 2 knockout (mdr2 $^{-/-}$) mice, an *in vivo* model of bile damage which resembles the features of PSC in human, undergo senescence [122].

Other clues support the role of senescence in cholangiocyte immune capabilities deregulation, in the setting of biliary tree disorders. The persistent endogenous insult with LPS, a component of bacterial cell wall, induces the upregulation of IL-6, as compensatory mechanisms to insult, and the establishment of cholangiocyte senescence and senescence- associated secretory phenotype [42]. Non-replicative cholangiocytes are more susceptible to noxious stimuli thus promoting the inflammatory process [43]. Despite these findings shed light on the possible link between cellular senescence establishment and disease presentation, it is not fully understood if senescence represents the trigger of non-replicative state of cholangiocytes or if it is merely the effect of persistent biliary damage.

Certainly, non-replicative cholangiocytes play a role in the modulation of biliary microenvironment and organ homeostasis. A literature search on Medline has been carried out in order to select miRs known to be involved in aging process in different organs (Table 3). This involves short (about 21- 24 nucleotides) non-coding regulatory RNAs which modulate genes activity by directly bind to complementary sequences localized in
different mRNA targets. To preliminary verify the possible role of the age-

related miRs in cholangiocyte biology modulation in course of biliary damage, we tested the expression of a panel of 12 miRs in cholangiocytes isolated from mice subjected to DDC diet, as a model of sclerosing cholangitis. Of the 12 miRs taken into account, 6 were increased (*miR-1a*, *miR-30e*, *miR-93*, *miR-34a*, *miR-146b* and *miR-20a*) in pooled samples of diseased cholangiocytes isolated by DDC-fed mice with respect to controls (Figure 9). These results prove that a relation between biliary tree disease and aging may exist. In order to confirm these preliminary data, the expression of the upregulated miRs was tested in young (2-month) and old (22-month) mice subjected to either standard diet or DDC diet. In this experimental setting, we could demonstrate that the modulation of *miR-1a*, *miR-20a* and *miR30e* expression occurs in response to age and in response to injury, with a possible synergic effect when the damage is inflicted to aged cholangiocytes (Figure 10A). By *in silico* analysis, intracellular pathways and molecular targets shared among the age-related miRs upregulated, have been identified. As shown in Table 4, these miRs modulate several molecular pathways and many of these have been widely studied for their involvement in pathological mechanisms linked to cholangiocytes response to damage [139]. Through a bioinformatics tool it was possible to assess putative targets of selected miRs (Table 5). The TargetScanHuman 6.2 database allowed the identification, among others, of TWF1 as a putative target shared among *miR-1a*, *miR-20a* and *miR30e*. TWF1 is a monomer-actin binding protein highly conserved form yeast to mammals which is involved in motile function and cytoskeleton remodeling [140]. It is localized to cortical G-actin rich-structures and it is probably

regulated by RAC1, a small GTPase. TWF1 is able to bind and sever actin

filaments, thus promoting actin structures remodeling and turnover [141], [142]. The expression levels of *Twf1* were significantly increased in aged mice as compared to young controls. Intriguingly, mRNA levels of *Twf1* were upregulated in young diseased cholangiocytes with respect to controls whereas intermediate values have been found in old cholangiocytes (Figure 10B). The expression of TWF1 have been evaluated also in human samples of cholangiopathies. Expression levels of *TWF1* were increased in cholangiocytes isolated by both PBC and PSC patients as compared to controls patient (Figure11A). Of note, positive for TWF1 immunohistochemistry tended to be higher in reactive cholangiocytes of PSC patients compared to the relative control, as shown by the staining intensity in the Figure 11B. The compensatory response of biliary epithelial cells is characterized by modification of their phenotype and biology. Cholangiocyte proliferation is effective to maintain bile duct mass and to preserve the secretory/absorptive function of biliary epithelia. When the balance between cell death phenomena and cell proliferation come less, ductopenia occurs.

Cultured cholangiocytes lacking for *Twf1* expression and exposed to a pro-proliferative stimulus, showed a decreased expression of *Ki-67* on a mRNA level (Figure 12A) and of *Pcna* on a protein level (Figure 12C). Accordingly, on a functional level biliary proliferation was reduced in Twf1 knocked-down cholangiocytes as assessed by SRB assay (Figure 12B). The upregulation of *Twf1*, which is observed in response to injury and in aged cholangiocytes, could be a compensatory mechanism functional to sustain biliary mass and preserve cholangiocyte physiologic functions. The effect of *Twf1* on cholangiocyte proliferation is in part mediated by the modulation of

cholangiocyte senescence. With this regard, the expression levels of the senescence marker *p16* is increased in cholangiocytes silenced for *Twf1* expression exposed to a pro-proliferative stimulus (Figure 13A). However, no differences have been observed in *p21* expression (Figure 13A).

On a functional level, the expression of specific lipofuscin assessed by SBB staining was significantly increased in cholangiocytes exposed to siRNA against *Twf1* (Figure 13B) thus indicating the establishment of cellular senescence [128]. Along with the upregulation of genes involved in cell cycle arrest, senescent cell acquire the so-called senescence-associated secretory phenotype (SASP). SASP components include soluble mediators which exert complex effects (e.g. inflammation, growth, chemotaxis, extracellular matrix remodeling) [143]. In our *in vitro* experiments, the absence of *Twf1* expression results in the upregulation of *Il-1α*, *Il-1β* and *Igf-1,* soluble mediators belonging to SASP markers (Figure 14A). According to mRNA data, *Il-1a* secretion is increased in cholangiocytes lacking for *Twf1* expression (Figure 14B). No differences could be evidenced for the other markers, *Il-1β* and *Igf-1* (data not shown). Similar results were obtained in a well-established in vitro model which relies on the persistent stimulation (10 days) with lipopolysaccharide (LPS) to induce cell senescence. The expression levels of *Twf1* were increased in LPS- induced senescent cholangiocytes as compared to controls (Figure 14C). Based on our data we could speculate that *Twf1* expression induced in response to cellular senescence, represents a possible compensatory mechanism thus stimulating cholangiocyte proliferation. The levels of senescence markers and senescence associated secretome are significantly

increased in cholangiocytes exposed to LPS for up to 10 days (Figure 15A-

B). As discussed above, cholangiocyte senescence has been recently demonstrated to play an important pathogenetic role in cholangiopathies development [42], [121]. The overall findings provide evidence that common molecular pathways may be activated in course of cholangiocyte response to injury and aging. The upregulation of *Twf1* observed in diseased and aged cholangiocytes may be functional to sustain cholangiocyte proliferation probably by preventing cellular senescence establishment and SASP releasing in course of bile duct damage. Further studies are needed to elucidate if the dysregulation of such mechanism could promote disease development or predispose to premature aging. In order to evaluate whether or not aging process could enhance cholangiocyte susceptibility to bile duct damage, we performed *in vivo* study on a mouse model of accelerated aging subjected to DDC feeding, to induce biliary alteration resembling PSC [129], [144]. As shown in Figure 16 panel A, intrahepatic bile duct mass assessed by Ck-19 immunohistochemistry and quantification, is significantly increased in SAMP8 mice exposed to a model of biliary insult as compared to SAMR1 as control group. On the same line, collagen deposition tended to be higher in DDC-fed SAMP8 mice as assessed by Sirius Red and hepatic hydroxyproline quantification (Figure 16 B-C). By contrast, in *Twf1* $^{-/-}$ mice is observed an improvement of cholangiocyte reaction to injury. Mice lacking the expression of *Twf1* subjected to DDC model, show a reduced biliary proliferation, assessed by Ck-19 quantification, and fibrosis occurrence, as evidenced by Sirius Red evaluation and hepatic hydroxyproline content quantification that was significantly lower in *Twf1* $^{-/-}$ mice than in controls (Figure 18).

These data suggest that bile ducts injury induces the activation of compensatory response including the increasing expression of *Twf1* which, in

turn, contributes to cholangiocyte proliferation and to dampen biliary senescence. When the noxious stimulus hits an aged biliary tree, such mechanisms are exacerbated. The final long-term effects of such modulations remain open to further research. On one hand, cholangiocyte activation (e.g. proliferation, proinflammatory cytokines releasing) may exert beneficial effects in response to an acute insult, on the other the persistent activation of reactive phenotype of cholangiocytes may lead to perpetuation of inflammatory process due to the ability of biliary epithelial cells to release a great amount of soluble mediators [145]. In this setting, *Twf1* modulation of cholangiocyte biology (increase of cholangiocyte proliferation and reducing of senescence phenotype establishment) may play a relevant role in altering cancer immune surveillance in these cells, predisposing to cholangiocarcinoma development [146]. In conclusion, Twf1 is a key mediator of both biliary disease and aging. Either processes may evolve by activation of similar pathways and share a common molecular signature. Dissecting of such mechanisms could be useful to identify novel target for cholangiopathies treatment.

6. BIBLIOGRAPHY

[1] M. Strazzabosco and L. Fabris, "Functional anatomy of normal bile ducts," *Anat. Rec.*, vol. 291, no. 6, pp. 653–660, 2008.

[2] K. S. Yoo, W. T. Lim, and H. S. Choi, "Biology of cholangiocytes: From bench to bedside," *Gut Liver*, vol. 10, no. 5, pp. 687–698, 2016.

[3] J. H. Tabibian, A. I. Masyuk, T. V. Masyuk, S. P. O'Hara, and N. F. LaRusso, "Physiology of cholangiocytes," *Compr. Physiol.*, vol. 3, no. 1, pp. 541–565, 2013.

[4] A. G. Kanno N, LeSage G, Glaser S, Alvaro D, "Functional Heterogeneity of the Intrahepatic Biliary Epithelium," pp. 555–561, 2016.

[5] M. M. Marzioni, S. S. Glaser, H. Francis, J. L. Phinizy, G. LeSage, and G. Alpini, "Functional heterogeneity of cholangiocytes," *Seminars in Liver Disease*, vol. 22, no. 3. pp. 227–240, 2002.

[6] A. Benedetti, C. Bassotti, K. Rapino, L. Marucci, and A. M. Jezequel, "A morphometric study of the epithelium lining the rat intrahepatic biliary tree," *J. Hepatol.*, vol. 24, no. 3, pp. 335–342, 1996.

[7] S. S. Glaser, E. Gaudio, A. Rao, L. M. Pierce, P. Onori, A. Franchitto, H. L. Francis, D. E. Dostal, J. K. Venter, S. Demorrow, R. Mancinelli, G. Carpino, D. Alvaro, S. E. Kopriva, J. M. Savage, and G. D. Alpini, "Morphological and functional heterogeneity of the mouse intrahepatic biliary epithelium," *Lab. Investig.*, 2009.

[8] I. E. M. de Jong, O. B. van Leeuwen, T. Lisman, A. S. H. Gouw, and R. J.

Porte, "Repopulating the biliary tree from the peribiliary glands," *Biochimica et Biophysica Acta - Molecular Basis of Disease*, vol. 1864, no. 4. pp. 1524–1531, 2018.

[9] K. Yamamoto and M. J. Phillips, "A hitherto unrecognized bile ductular plexus in normal rat liver," *Hepatology*, 1984.

[10] L. Landau, "Electron Microscope Study of Vertebrate Liver Innervation," *Arch. histol. jap.*, 1937.

[11] M. Marzioni, G. Fava, D. Alvaro, G. Alpini, and A. Benedetti, "Control of cholangiocyte adaptive responses by visceral hormones and neuropeptides," *Clinical Reviews in Allergy and Immunology*. 2009.

[12] L. Maroni, B. Haibo, D. Ray, T. Zhou, Y. Wan, F. Meng, M. Marzioni, and G. Alpini, "Functional and Structural Features of Cholangiocytes in Health and Disease," *C. Cell. Mol. Gastroenterol. Hepatol.*, vol. 1, no. 4, pp. 368–380, 2015.

[13] M. Ishii, B. Vroman, and N. F. LaRusso, "Isolation and morphologic characterization of bile duct epithelial cells from normal rat liver," *Gastroenterology*, 1989.

[14] K. Shin, V. C. Fogg, and B. Margolis, "Tight Junctions and Cell Polarity," *Annu. Rev. Cell Dev. Biol.*, 2006.

[15] H. P. Bode, L. F. Wang, D. Cassio, M. F. Leite, M. V. St-Pierre, K. Hirata, K. Okazaki, M. L. Sears, P. Meda, M. H. Nathanson, and J. F. Dufour, "Expression and regulation of gap junctions in rat cholangiocytes," *Hepatology*, 2002.

[16] A. P. Mansini, E. Peixoto, K. M. Thelen, C. Gaspari, S. Jin, and S. A. Gradilone, "The cholangiocyte primary cilium in health and disease," *Biochimica et Biophysica Acta - Molecular Basis of Disease*. 2018.

[17] R. B. Doctor and L. Fouassier, “Emerging roles of the actin cytoskeleton in cholangiocyte function and disease,” *Seminars in Liver Disease*. 2002.

[18] A. I. Masyuk, B. Q. Huang, C. J. Ward, S. A. Gradilone, J. M. Banales, T. V. Masyuk, B. Radtke, P. L. Splinter, and N. F. LaRusso, “Biliary exosomes influence cholangiocyte regulatory mechanisms and proliferation through interaction with primary cilia,” *AJP Gastrointest. Liver Physiol.*, vol. 299, no. 4, pp. G990–G999, 2010.

[19] S. Kanemoto, R. Nitani, T. Murakami, M. Kaneko, R. Asada, K. Matsuhisa, A. Saito, and K. Imaizumi, “Multivesicular body formation enhancement and exosome release during endoplasmic reticulum stress,” *Biochem. Biophys. Res. Commun.*, 2016.

[20] J. Zhang, S. Li, L. Li, M. Li, C. Guo, J. Yao, and S. Mi, “Exosome and exosomal microRNA: Trafficking, sorting, and function,” *Genomics, Proteomics and Bioinformatics*. 2015.

[21] S. Sell, “Heterogeneity and plasticity of hepatocyte lineage cells,” *Hepatology*, vol. 33, no. 3, pp. 738–750, 2001.

[22] H. Jones, G. Alpini, and H. Francis, “Bile acid signaling and biliary functions,” *Acta Pharmaceutica Sinica B*. 2015.

[23] J. L. Boyer, “Bile Formation and Secretion,” *Compr Physiol.*, 2013.

[24] A. Franchitto, P. Onori, A. Renzi, G. Carpino, R. Mancinelli, D. Alvaro, and E. Gaudio, “Recent advances on the mechanisms regulating cholangiocyte proliferation and the significance of the neuroendocrine regulation of cholangiocyte pathophysiology,” *Ann. Transl. Med.*, 2013.

[25] A. I. Masyuk and N. F. LaRusso, “Aquaporins in the hepatobiliary system,” *Hepatology*. 2006.

[26] D. Alvaro, G. Alpini, A. M. Jezequel, C. Bassotti, C. Francia, F. Fraioli, R.

Romeo, L. Marucci, G. Le Sage, S. S. Glaser, and A. Benedetti, “Role and mechanisms of action of acetylcholine in the regulation of rat cholangiocyte secretory functions,” *J. Clin. Invest.*, 1997.

[27] N. Minagawa, J. Nagata, K. Shibao, A. I. Masyuk, D. A. Gomes, M. A. Rodrigues, G. Lesage, Y. Akiba, J. D. Kaunitz, B. E. Ehrlich, N. F. Larusso, and M. H. Nathanson, “Cyclic AMP Regulates Bicarbonate Secretion in Cholangiocytes Through Release of ATP Into Bile,” *Gastroenterology*, vol. 133, no. 5, pp. 1592–1602, 2007.

[28] A. Gigliozzi, F. Fraioli, P. Sundaram, J. Lee, A. Mennone, D. Alvaro, and J. L. Boyer, “Molecular identification and functional characterization of mdr1a in rat cholangiocytes,” *Gastroenterology*, 2000.

[29] A. Benedetti, A. Di Sario, L. Marucci, G. Svegliati-Baroni, C. D. Schteingart, H. T. Ton-Nu, and A. F. Hofmann, “Carrier-mediated transport of conjugated bile acids across the basolateral membrane of biliary epithelial cells,” *Am J Physiol*, 1997.

[30] K. Sato, M. Marzioni, F. Meng, H. Francis, S. Glaser, and G. Alpini, “Ductular reaction in liver diseases: pathological mechanisms and translational significances,” *Hepatology*, 2018.

[31] M. M. Giordano DM, Pinto C, Maroni L, Benedetti A, “Inflammation and the Gut-Liver Axis in the Pathophysiology of Cholangiopathies,” *Int. J. Mol. Sci.*, vol. 19, no. 10, p. E3003, 2018.

[32] D. Alvaro, M. G. Mancino, S. Glaser, E. Gaudio, M. Marzioni, H. Francis, and G. Alpini, “Proliferating Cholangiocytes: A Neuroendocrine Compartment in the Diseased Liver,” *Gastroenterology*, vol. 132, no. 1, pp. 415–431, 2007.

[33] D. Alvaro, A. Gigliozzi, and A. F. Attili, “Regulation and deregulation of

cholangiocyte proliferation," *Journal of Hepatology*. 2000.

[34] S. R. Popper H, Kent G, "Ductular cell reaction in the liver in hepatic injury," *J Mt Sinai Hosp N Y.*, vol. 24, no. 5, pp. 551–6, 1957.

[35] G. Svegliati-Baroni, S. De Minicis, and M. Marzioni, "Hepatic fibrogenesis in response to chronic liver injury: Novel insights on the role of cell-to-cell interaction and transition," *Liver International*. 2008.

[36] N. Y. Yasoshima M, Kono N, Sugawara H, Katayanagi K, Harada K, "Increased expression of interleukin-6 and tumor necrosis factor-alpha in pathologic biliary epithelial cells: in situ and culture study.," *Lab. Investig.*, vol. 78, no. 1, pp. 89–100, 1998.

[37] T. Ezure, T. Sakamoto, H. Tsuji, J. G. Lunz, N. Murase, J. J. Fung, and A. J. Demetris, "The development and compensation of biliary cirrhosis in interleukin-6-deficient mice," *Am. J. Pathol.*, vol. 156, no. 5, pp. 1627– 1639, 2000.

[38] K. N. Lazaridis, M. Strazzabosco, and N. F. Larusso, "The cholangiopathies: Disorders of biliary epithelia," *Gastroenterology*. 2004.

[39] G. Svegliati-Baroni, F. Ridolfi, Z. Caradonna, D. Alvaro, M. Marzioni, S. Saccomanno, C. Candelaresi, L. Trozzi, G. Macarri, A. Benedetti, and F. Folli, "Regulation of ERK/JNK/p70S6Kin two rat models of liver injury and fibrosis," *J. Hepatol.*, 2003.

[40] G. Lesage, D. Alvaro, A. Benedetti, S. Glaser, L. Marucci, L. Baiocchi, W. Eisel, A. Caligiuri, J. L. Phinizy, R. Rodgers, H. Francis, and G. Alpini, "Cholinergic system modulates growth, apoptosis, and secretion of cholangiocytes from bile duct-ligated rats," *Gastroenterology*, 1999.

[41] F. Yang, E. Gaudio, P. Onori, C. Wise, G. Alpini, and S. S. Glaser, "Mechanisms of Biliary Damage," *Journal of Cell Death*, vol. 2010. pp. 13–

21, 2010.

[42] J. H. Tabibian, S. P. O'Hara, P. L. Splinter, C. E. Trussoni, and N. F. Larusso, "Cholangiocyte senescence by way of N-Ras activation is a characteristic of primary sclerosing cholangitis," *Hepatology*, vol. 59, no. 6, pp. 2263–2275, 2014.

[43] A. D. Aravinthan and G. J. M. Alexander, "Senescence in chronic liver disease: Is the future in aging?," *Journal of Hepatology*, vol. 65, no. 4. pp. 825–834, 2016.

[44] P. Fickert, A. Fuchsbichler, M. Wagner, G. Zollner, A. Kaser, H. Tilg, R. Krause, F. Lammert, C. Langner, K. Zatloukal, H. U. Marschall, H. Denk, and M. Trauner, "Regurgitation of bile acids from leaky bile ducts causes sclerosing cholangitis in Mdr2 (Abcb4) knockout mice," *Gastroenterology*, 2004.

[45] S. Milani, H. Herbst, D. Schuppan, and H. Stein, "Transforming Growth Factors β1 and β2 Are Differentially Expressed in Fibrotic Liver Disease," *Am. J. Pathol.*, 1991.

[46] Z. Li, J. a Dranoff, E. P. Chan, M. Uemura, J. Sévigny, and R. G. Wells, "Transforming growth factor-beta and substrate stiffness regulate portal fibroblast activation in culture.," *Hepatology*, vol. 46, no. 4, pp. 1246–56, 2007.

[47] S. Yokomuro, H. Tsuji, J. G. Lunz, T. Sakamoto, T. Ezure, N. Murase, and A. J. Demetris, "Growth control of human biliary epithelial cells by interleukin 6, hepatocyte growth factor, transforming growth factor β1, and activin A: Comparison of a cholangiocarcinoma cell line with primary cultures of non- neoplastic biliary epithelial cells," *Hepatology*, vol. 32, no. 1, pp. 26–35, 2000.

[48] E. Kruglov, R. Nathanson, T. Nguyen, and J. Dranoff, “Secretion of {MCP-1/CCL2} by bile duct epithelia induces myofibroblastic transdifferentiation of portal fibroblasts,” *Am J Physiol. - Gastrointest Liver Physiol.*, vol. 290, no. 4, pp. G765–G771, 2006.

[49] B. K. Davis, H. Wen, and J. P.-Y. Ting, “The Inflammasome NLRs in Immunity, Inflammation, and Associated Diseases,” *Annu. Rev. Immunol.*, vol. 29, no. 1, pp. 707–735, 2011.

[50] S. Akira and K. Takeda, “Toll-like receptor signalling,” *Nature Reviews Immunology*. 2004.

[51] J. Tschopp and K. Schroder, “NLRP3 inflammasome activation: The convergence of multiple signalling pathways on ROS production?,” *Nature Reviews Immunology*, vol. 10, no. 3. pp. 210–215, 2010.

[52] C. Pinto, D. M. Giordano, L. Maroni, and M. Marzioni, “Role of inflammation and proinflammatory cytokines in cholangiocyte pathophysiology,” *Biochimica et Biophysica Acta - Molecular Basis of Disease*, vol. 1864, no. 4. pp. 1270–1278, 2018.

[53] D. H. Adams and S. C. Afford, “The role of cholangiocytes in the development of chronic inflammatory liver disease,” *Front. Biosci.*, 2002.

[54] K. Harada, S. Shimoda, H. Ikeda, M. Chiba, M. Hsu, Y. Sato, M. Kobayashi, X. S. Ren, H. Ohta, S. Kasashima, A. Kawashima, and Y. Nakanuma, “Significance of periductal Langerhans cells and biliary epithelial cell-derived macrophage inflammatory protein-3?? in the pathogenesis of primary biliary cirrhosis,” *Liver Int.*, vol. 31, no. 2, pp. 245– 253, 2011.

[55] T. Imai, K. Hieshima, C. Haskell, M. Baba, M. Nagira, M. Nishimura, M. Kakizaki, S. Takagi, H. Nomiyama, T. J. Schall, and O. Yoshie,

"Identification and molecular characterization of fractalkine receptor CX3CR1, which mediates both leukocyte migration and adhesion," *Cell*, vol. 91, no. 4, pp. 521–530, 1997.

[56] K. Isse, K. Harada, Y. Zen, T. Kamihira, S. Shimoda, M. Harada, and Y. Nakanuma, "Fractalkine and CX3CR1 are involved in the recruitment of intraepithelial lymphocytes of intrahepatic bile ducts," *Hepatology*, 2005.

[57] C. Te Wu, P. A. Davis, V. A. Luketic, and M. E. Gershwin, "A review of the physiological and immunological functions of biliary epithelial cells: Targets for primary biliary cirrhosis, primary sclerosing cholangitis and drug-induced ductopenias," in *Clinical and Developmental Immunology*, 2004, vol. 11, no. 3–4, pp. 205–213.

[58] R. C. Ayres, J. M. Neuberger, J. Shaw, R. Joplin, and D. H. Adams, "Intercellular adhesion molecule-1 and MHC antigens on human intrahepatic bile duct cells: effect of pro-inflammatory cytokines.," *Gut*, vol. 34, no. 9, pp. 1245–9, 1993.

[59] S. M. Cruickshank, J. Southgate, P. J. Selby, and L. K. Trejdosiewicz, "Expression and cytokine regulation of immune recognition elements by normal human biliary epithelial and established liver cell lines in vitro," *J. Hepatol.*, 1998.

[60] E. Radaelli, F. del Piero, L. Aresu, F. Sciarrone, N. Vicari, S. Mattiello, S. Tagliabue, M. Fabbi, and E. Scanziani, "Expression of major histocompatibility complex class II antigens in porcine Leptospiral nephritis," *Vet. Pathol.*, 2009.

[61] J. Y. Sung, J. W. Costerton, and E. A. Shaffer, "Defense system in the biliary tract against bacterial infection," *Digestive Diseases and Sciences*, vol. 37, no. 5. pp. 689–696, 1992.

[62] K. Harada and Y. Nakanuma, "Biliary innate immunity: Function and modulation," *Mediators of Inflammation*, vol. 2010. 2010.

[63] K. Harada, K. Ohba, S. Ozaki, K. Isse, T. Hirayama, A. Wada, and Y. Nakanuma, "Peptide antibiotic human beta-defensin-1 and -2 contribute to antimicrobial defense of the intrahepatic biliary tree," *Hepatology*, vol. 40, no. 4, pp. 925–932, 2004.

[64] K. N. Lazaridis and N. F. Larusso, "The cholangiopathies," *Mayo Clinic Proceedings*, vol. 90, no. 6. pp. 791–800, 2015.

[65] S. N. Sclair, E. Little, and C. Levy, "Current concepts in primary biliary cirrhosis and primary sclerosing cholangitis," *Clinical and Translational Gastroenterology*. 2015.

[66] R. M. Marchioni Beery, H. Vaziri, and F. Forouhar, "Primary Biliary Cirrhosis and Primary Sclerosing Cholangitis: a Review Featuring a Women's Health Perspective.," *J. Clin. Transl. Hepatol.*, 2014.

[67] J. Frith, D. Jones, and J. L. Newton, "Chronic liver disease in an aging population," *Age and Aging*. 2009.

[68] A. T. Borchers, S. Shimoda, C. Bowlus, C. L. Keen, and M. E. Gershwin, "Lymphocyte recruitment and homing to the liver in primary biliary cirrhosis and primary sclerosing cholangitis," *Seminars in Immunopathology*. 2009.

[69] J. E. Eaton, J. A. Talwalkar, K. N. Lazaridis, G. J. Gores, and K. D. Lindor, "Pathogenesis of primary sclerosing cholangitis and advances in diagnosis and management," *Gastroenterology*, vol. 145, no. 3. pp. 521–536, 2013.

[70] J. H. Ngu, R. B. Gearry, A. J. Wright, and C. A. M. Stedman, "Inflammatory bowel disease is associated with poor outcomes of patients with primary sclerosing cholangitis," *Clin. Gastroenterol. Hepatol.*, vol. 9,

no. 12, pp. 1092–1097, 2011.

[71] J. H. Tabibian, J. A. Talwalkar, and K. D. Lindor, “Role of the microbiota and antibiotics in primary sclerosing cholangitis,” *BioMed Research International*, vol. 2013. 2013.

[72] J. Pohl, A. Ring, W. Stremmel, and A. Stiehl, “The role of dominant stenoses in bacterial infections of bile ducts in primary sclerosing cholangitis,” *Eur. J. Gastroenterol. Hepatol.*, vol. 18, no. 1, pp. 69–74, 2006.

[73] K. Hiramatsu, K. Harada, K. Tsuneyama, M. Sasaki, S. Fujita, T. Hashimoto, S. Kaneko, K. Kobayashi, and Y. Nakanuma, “Amplification and sequence analysis of partial bacterial 16S ribosomal RNA gene in gallbladder bile from patients with primary biliary cirrhosis,” *J. Hepatol.*, 2000.

[74] E. Alabraba, P. Nightingale, B. Gunson, S. Hubscher, S. Olliff, D. Mirza, and J. Neuberger, “A re-evaluation of the risk factors for the recurrence of primary sclerosing cholangitis in liver allografts,” *Liver Transplant.*, 2009.

[75] A. Vera, S. Moledina, B. Gunson, S. Hubscher, D. Mirza, S. Olliff, and J. Neuberger, “Risk factors for recurrence of primary sclerosing cholangitis of liver allograft.,” *Lancet (London, England)*, vol. 360, no. 9349, pp. 1943–4, 2002.

[76] M. Färkkilä, A. L. Karvonen, H. Nurmi, H. Nuutinen, M. Taavitsainen, P. Pikkarainen, and P. Kärkkäinen, “Metronidazole and ursodeoxycholic acid for primary sclerosing cholangitis: A randomized placebo-controlled trial,” *Hepatology*, 2004.

[77] D. S. Smyk, E. I. Rigopoulou, A. Lleo, R. D. Abeles, A. Mavropoulos, C. Billinis, P. Invernizzi, and D. P. Bogdanos, “Immunopathogenesis of

primary biliary cirrhosis: An old wives' tale," *Immunity and Aging*. 2011.

[78] D. P. Bogdanos, K. Choudhuri, and D. Vergani, "Molecular mimicry and autoimmune liver disease: Virtuous intentions, malign consequences," *Liver*. 2001.

[79] J. Van de Water, H. Ishibashi, R. L. Coppel, and M. E. Gershwin, "Molecular mimicry and primary biliary cirrhosis: Premises not promises," *Hepatology*. 2001.

[80] F. Bernuzzi, D. Fenoglio, F. Battaglia, M. Fravega, M. E. Gershwin, F. Indiveri, A. A. Ansari, M. Podda, P. Invernizzi, and G. Filaci, "Phenotypical and functional alterations of CD8 regulatory T cells in primary biliary cirrhosis," *J. Autoimmun.*, 2010.

[81] M. Sasaki, H. Ikeda, J. Yamaguchi, S. Nakada, and Y. Nakanuma, "Telomere shortening in the damaged small bile ducts in primary biliary cirrhosis reflects ongoing cellular senescence," *Hepatology*, 2008.

[82] M. Sasaki, H. Ikeda, H. Haga, T. Manabe, and Y. Nakanuma, "Frequent cellular senescence in small bile ducts in primary biliary cirrhosis: A possible role in bile duct loss," *J. Pathol.*, 2005.

[83] M. Sasaki, M. Miyakoshi, Y. Sato, and Y. Nakanuma, "Autophagy mediates the process of cellular senescence characterizing bile duct damages in primary biliary cirrhosis," *Lab. Investig.*, 2010.

[84] X. Jiang and T. H. Karlsen, "Genetics of primary sclerosing cholangitis and pathophysiological implications," *Nature Reviews Gastroenterology and Hepatology*. 2017.

[85] M. G. Roma, F. D. Toledo, A. C. Boaglio, C. L. Basiglio, F. A. Crocenzi, and E. J. Sánchez Pozzi, "Ursodeoxycholic acid in cholestasis: linking action mechanisms to therapeutic applications," *Clin. Sci.*, 2011.

[86] J. Goldstein and C. Levy, “Novel and emerging therapies for cholestatic liver diseases,” *Liver Int.*, vol. 38, no. 9, pp. 1520–1535, 2018.

[87] K. D. Lindor, K. V. Kowdley, V. A. C. Luketic, M. E. Harrison, T. McCashland, A. S. Befeler, D. Harnois, R. Jorgensen, J. Petz, J. Keach, J. Mooney, C. Sargeant, T. Bernard, D. King, E. Miceli, J. Schmoll, T. Hoskin, P. Thapa, and F. Enders, “High-dose ursodeoxycholic acid for the treatment of primary sclerosing cholangitis,” *Hepatology*, 2009.

[88] D. McHugh and J. Gil, “Senescence and aging: Causes, consequences, and therapeutic avenues,” *Journal of Cell Biology*. 2018.

[89] C. López-otín, M. A. Blasco, L. Partridge, and M. Serrano, “The Hallmarks of Aging (copy without figures, can’t seem to remove),” vol. 153, no. 6, pp. 1194–1217, 2013.

[90] B. J. North and D. A. Sinclair, “The intersection between aging and cardiovascular disease,” *Circulation Research*. 2012.

[91] H. W. Querfurth and F. M. Laferla, “Alzheimer’s Disease,” pp. 329–344, 2018.

[92] J. P. De Magalhães, “How aging processes influence cancer,” *Nature Reviews Cancer*. 2013.

[93] A. A. Moskalev, M. V. Shaposhnikov, E. N. Plyusnina, A. Zhavoronkov, A. Budovsky, H. Yanai, and V. E. Fraifeld, “The role of DNA damage and repair in aging through the prism of Koch-like criteria,” *Aging Research Reviews*. 2013.

[94] J. H. J. Hoeijmakers, “DNA Damage, Aging, and Cancer,” *N. Engl. J. Med.*, 2009.

[95] A. W. Linnane, T. Ozawa, S. Marzuki, and M. Tanaka, “MITOCHONDRIAL DNA MUTATIONS AS AN IMPORTANT

CONTRIBUTOR TO AGING AND DEGENERATIVE DISEASES," *Lancet*, 1989.

[96] M. Armanios, J. K. Alder, E. M. Parry, B. Karim, M. A. Strong, and C. W. Greider, "Short Telomeres are Sufficient to Cause the Degenerative Defects Associated with Aging," *Am. J. Hum. Genet.*, 2009.

[97] R. Mostoslavsky, K. F. Chua, D. B. Lombard, W. W. Pang, M. R. Fischer, L. Gellon, P. Liu, G. Mostoslavsky, S. Franco, M. M. Murphy, K. D. Mills, P. Patel, J. T. Hsu, A. L. Hong, E. Ford, H. L. Cheng, C. Kennedy, N. Nunez, R. Bronson, D. Frendewey, W. Auerbach, D. Valenzuela, M. Karow, M. O. Hottiger, S. Hursting, J. C. Barrett, L. Guarente, R. Mulligan, B. Demple, G. D. Yancopoulos, and F. W. Alt, "Genomic instability and aging-like phenotype in the absence of mammalian SIRT6," *Cell*, 2006.

[98] Y. Kanfi, S. Naiman, G. Amir, V. Peshti, G. Zinman, L. Nahum, Z. Bar-Joseph, and H. Y. Cohen, "The sirtuin SIRT6 regulates lifespan in male mice," *Nature*, 2012.

[99] H. Koga, S. Kaushik, and A. M. Cuervo, "Protein homeostasis and aging: The importance of exquisite quality control," *Aging Research Reviews*. 2011.

[100] E. T. Powers, R. I. Morimoto, A. Dillin, J. W. Kelly, and W. E. Balch, "Biological and Chemical Approaches to Diseases of Proteostasis Deficiency," *Annu. Rev. Biochem.*, 2009.

[101] J. Campisi, "Aging, cellular senescence, and cancer," *Annu. Rev. Physiol.*, 2013.

[102] L. Hayflick, "The limited in vitro lifetime of human diploid cell strains," *Exp. Cell Res.*, 1965.

[103] L. Hayflick and P. S. Moorhead, "The serial cultivation of human diploid

cell strains," *Exp. Cell Res.*, 1961.

[104] A. G. Bodnar, M. Ouellette, M. Frolkis, S. E. Holt, C. P. Chiu, G. B. Morin, C. B. Harley, J. W. Shay, S. Lichtsteiner, and W. E. Wright, "Extension of life-span by introduction of telomerase into normal human cells," *Science (80-.).*, 1998.

[105] J. Campisi and F. D'Adda Di Fagagna, "Cellular senescence: When bad things happen to good cells," *Nature Reviews Molecular Cell Biology*. 2007.

[106] R. Sager, "Senescence as a mode of tumor suppression," in *Environmental Health Perspectives*, 1991.

[107] C. J. Collins and J. M. Sedivy, "Involvement of the INK4a/Arf gene locus in senescence.," *Aging Cell*, vol. 2, no. 3, pp. 145–150, 2003.

[108] J. Doles, M. Storer, L. Cozzuto, G. Roma, and W. M. Keyes, "Age- associated inflammation inhibits epidermal stem cell function," *Genes Dev.*, 2012.

[109] W. Xue, L. Zender, C. Miething, R. A. Dickins, E. Hernando, V. Krizhanovsky, C. Cordon-Cardo, and S. W. Lowe, "Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas," *Nature*, 2007.

[110] G. P. Dimri, X. Lee, G. Basile, M. Acosta, G. Scott, C. Roskelley, E. E. Medrano, M. Linskens, I. Rubelj, and O. Pereira-Smith, "A biomarker that identifies senescent human cells in culture and in aging skin in vivo.," *Proc. Natl. Acad. Sci.*, 1995.

[111] L. Meng, M. Quezada, P. Levine, Y. Han, K. McDaniel, T. Zhou, E. Lin, S. Glaser, F. Meng, H. Francis, and G. Alpini, "Functional role of cellular senescence in biliary injury," *American Journal of Pathology*. 2015.

[112] R. J. Colman, T. M. Beasley, J. W. Kemnitz, S. C. Johnson, R. Weindruch,

and R. M. Anderson, “Caloric restriction reduces age-related and all-cause mortality in rhesus monkeys,” *Nat. Commun.*, 2014.

[113] L. Fontana, L. Partridge, and V. D. Longo, “Extending healthy life span- from yeast to humans,” *Science*. 2010.

[114] J. A. Mattison, G. S. Roth, T. Mark Beasley, E. M. Tilmont, A. M. Handy, R. L. Herbert, D. L. Longo, D. B. Allison, J. E. Young, M. Bryant, D. Barnard, W. F. Ward, W. Qi, D. K. Ingram, and R. De Cabo, “Impact of caloric restriction on health and survival in rhesus monkeys from the NIA study,” *Nature*, 2012.

[115] E. Sahin and R. A. DePinho, “Axis of aging: Telomeres, p53 and mitochondria,” *Nature Reviews Molecular Cell Biology*. 2012.

[116] K. Wang and D. J. Klionsky, “Mitochondria removal by autophagy,” *Autophagy*. 2011.

[117] K. Szarc Vel Szic, K. Declerck, M. Vidaković, and W. Vanden Berghe, “From inflammaging to healthy aging by dietary lifestyle choices: Is epigenetics the key to personalized nutrition?,” *Clinical Epigenetics*. 2015.

[118] D. L. Schmucker, “Age-related changes in liver structure and function: Implications for disease?,” *Experimental Gerontology*. 2005.

[119] T. J. Weismüller, C. P. Strassburg, P. J. Trivedi, G. M. Hirschfield, P. J. Trivedi, A. Bergquist, K. Said, M. Imam, K. N. Lazaridis, B. D. Juran, A. Cheung, K. D. Lindor, T. J. Weismüller, H. Lenzen, M. P. Manns, C. Y. Ponsioen, U. Beuers, K. Holm, S. Naess, T. H. Karlsen, E. Schrumpf, K. M. Boberg, D. Gotthardt, C. Rupp, M. A. Färkkilä, K. Jokelainen, H. U. Marschall, M. Benito de Valle, D. Thorburn, F. Saffioti, R. K. Weersma, J. Fevery, T. Mueller, O. Chazouillères, K. Schulze, C. Schramm, S. Almer, S. P. Pereira, C. Levy, A. Mason, C. L. Bowlus, A. Floreani, E. Halilbasic, M.

Trauner, K. K. Yimam, P. Milkiewicz, P. Milkiewicz, D. K. Huynh, A. Pares, C. N. Manser, G. N. Dalekos, B. Eksteen, P. Invernizzi, C. P. Berg, G. I. Kirchner, C. Sarrazin, V. Zimmer, L. Fabris, F. Braun, M. Marzioni, C. Schramm, R. W. Chapman, R. W. Chapman, K. D. Lindor, M. Imam, K. D. Lindor, S. Naess, T. H. Karlsen, E. Schrumpf, K. M. Boberg, B. E. Hansen, B. E. Hansen, and B. E. Hansen, "Patient Age, Sex, and Inflammatory Bowel Disease Phenotype Associate With Course of Primary Sclerosing Cholangitis," *Gastroenterology*, 2017.

[120] T. H. Welling, D. G. Heidt, M. J. Englesbe, J. C. Magee, R. S. Sung, D. A. Campbell, J. D. Punch, and S. J. Pelletier, "Biliary complications following liver transplantation in the model for end-stage liver disease era: Effect of donor, recipient, and technical factors," *Liver Transplant.*, 2008.

[121] J. H. Tabibian, C. E. Trussoni, S. P. O'Hara, P. L. Splinter, J. K. Heimbach, and N. F. LaRusso, "Characterization of cultured cholangiocytes isolated from livers of patients with primary sclerosing cholangitis," *Lab. Investig.*, 2014.

[122] J. H. Tabibian, S. I. MacUra, S. P. O'Hara, J. L. Fidler, J. F. Glockner, N. Takahashi, V. J. Lowe, B. J. Kemp, P. K. Mishra, P. S. Tietz, P. L. Splinter, C. E. Trussoni, and N. F. Larusso, "Micro-computed tomography and nuclear magnetic resonance imaging for noninvasive, live-mouse cholangiography," *Lab. Investig.*, 2013.

[123] M. Sasaki, M. Miyakoshi, Y. Sato, and Y. Nakanuma, "Modulation of the microenvironment by senescent biliary epithelial cells may be involved in the pathogenesis of primary biliary cirrhosis," *J. Hepatol.*, 2010.

[124] M. C. Albertini, F. Olivieri, R. Lazzarini, F. Pilolli, F. Galli, G. Spada, A. Accorsi, M. R. Rippo, and A. D. Procopio, "Predicting microRNA

modulation in human prostate cancer using a simple String IDentifier (SID1.0)," *J. Biomed. Inform.*, 2011.

[125] J. M. Banales, E. Sáez, M. Úriz, S. Sarvide, A. D. Urribarri, P. Splinter, P. S. Tietz Bogert, L. Bujanda, J. Prieto, J. F. Medina, and N. F. Larusso, "Up-regulation of microRNA 506 leads to decreased Cl -/HCO 3- anion exchanger 2 expression in biliary epithelium of patients with primary biliary cirrhosis," *Hepatology*, vol. 56, no. 2, pp. 687–697, 2012.

[126] E. Orellana and A. Kasinski, "Sulforhodamine B (SRB) Assay in Cell Culture to Investigate Cell Proliferation," *BIO-PROTOCOL*, 2016.

[127] V. Vichai and K. Kirtikara, "Sulforhodamine B colorimetric assay for cytotoxicity screening," *Nat. Protoc.*, 2006.

[128] E. A. Georgakopoulou, K. Tsimaratou, K. Evangelou, P. J. Fernandez-Marcos, V. Zoumpourlis, I. P. Trougakos, D. Kletsas, J. Bartek, M. Serrano, and V. G. Gorgoulis, "Specific lipofuscin staining as a novel biomarker to detect replicative and stress-induced senescence. A method applicable in cryo-preserved and archival tissues," *Aging (Albany. NY).*, 2013.

[129] T. Takeda, M. Hosokawa, and K. Higuchi, "Senescence-Accelerated Mouse (SAM): A novel murine model of senescence," in *Experimental Gerontology*, 1997.

[130] X. Ye, H. C. Meeker, P. B. Kozlowski, J. Wegiel, K. C. Wang, H. Imaki, and R. I. Carp, "Pathological changes in the liver of a senescence accelerated mouse strain (SAMP8): a mouse model for the study of liver diseases.," *Histol. Histopathol.*, vol. 19, no. 4, pp. 1141–51, 2004.

[131] P. Fickert, U. Stöger, A. Fuchsbichler, T. Moustafa, H. U. Marschall, A. H. Weiglein, O. Tsybrovskyy, H. Jaeschke, K. Zatloukal, H. Denk, and M. Trauner, "A new xenobiotic-induced mouse model of sclerosing cholangitis

and biliary fibrosis," *Am. J. Pathol.*, vol. 171, no. 2, pp. 525–536, 2007.

[132] M. Marzioni, S. Saccomanno, L. Agostinelli, C. Rychlicki, S. De Minicis, I. Pierantonelli, M. Trauner, P. Fickert, T. Müller, K. Shanmukhappa, L. Trozzi, C. Candelaresi, G. S. Baroni, and A. Benedetti, "PDX-1/Hes-1 interactions determine cholangiocyte proliferative response to injury in rodents: Possible implications for sclerosing cholangitis," *J. Hepatol.*, vol. 58, no. 4, pp. 750–756, Apr. 2013.

[133] S. De Minicis, C. Rychlicki, L. Agostinelli, S. Saccomanno, L. Trozzi, C. Candelaresi, R. Bataller, C. Millán, D. A. Brenner, M. Vivarelli, F. Mocchegiani, M. Marzioni, A. Benedetti, and G. Svegliati-Baroni, "Semaphorin 7A contributes to TGF-β-mediated liver fibrogenesis," *Am. J. Pathol.*, 2013.

[134] T. Takeda, M. Hosokawa, S. Takeshita, M. Irino, K. Higuchi, T. Matsushita, Y. Tomita, K. Yasuhira, H. Hamamoto, K. Shimizu, M. Ishii, and T. Yamamuro, "A new murine model of accelerated senescence," *Mech. Aging Dev.*, 1981.

[135] W. P. Rowe, W. E. Pugh, and J. W. Hartley, "Plaque assay techniques for murine leukemia viruses," *Virology*, 1970.

[136] W. P. Rowe and J. W. Hartley, "STUDIES OF GENETIC TRANSMISSION OF MURINE LEUKEMIA VIRUS BY AKR MICE," *J. Exp. Med.*, 1972.

[137] R. Sultana, G. Cenini, and D. A. Butterfield, "SAMP8 : A model tounderstand the role of oxidative stress in age-related diseases including Alzheimer ' s disease," pp. 361–370.

[138] V. O. Paavilainen, E. Oksanen, A. Goldman, and P. Lappalainen, "Structure of the actin-depolymerizing factor homology domain in complex with

actin," *J. Cell. Biol.*, 2008.

[139] S. P. O'Hara, J. H. Tabibian, P. L. Splinter, and N. F. LaRusso, "The dynamic biliary epithelia: Molecules, pathways, and disease," *J. Hepatol.*, 2013.

[140] S. Palmgren, M. Vartiainen, and P. Lappalainen, "Twinfilin, a molecular mailman for actin monomers," *J. Cell Sci.* , 2002.

[141] A. B. Johnston, A. Collins, and B. L. Goode, "High-speed depolymerization at actin filament ends jointly catalysed by Twinfilin and Srv2/CAP," *Nat. Cell Biol.*, 2015.

[142] J. B. Moseley, K. Okada, H. I. Balcer, D. R. Kovar, T. D. Pollard, and B. L. Goode, "Twinfilin is an actin-filament-severing protein and promotes rapid turnover of actin structures in vivo," *J. Cell Sci.*, 2006.

[143] T. Tchkonia, Y. Zhu, J. Van Deursen, J. Campisi, and J. L. Kirkland, "Cellular senescence and the senescent secretory phenotype: Therapeutic opportunities," *Journal of Clinical Investigation*. 2013.

[144] T. Takeda, "Senescence-accelerated mouse (SAM): A biogerontological resource in aging research," *Neurobiol. Aging*, 1999.

[145] L. Maroni, L. Agostinelli, S. Saccomanno, C. Pinto, D. M. Giordano, C. Rychlicki, S. De Minicis, L. Trozzi, J. M. Banales, E. Melum, T. H. Karlsen, A. Benedetti, G. S. Baroni, and M. Marzioni, "Nlrp3 Activation Induces Il-18 Synthesis and Affects the Epithelial Barrier Function in Reactive Cholangiocytes," *Am. J. Pathol.*, vol. 187, no. 2, pp. 366–376, 2017.

[146] T. W. Kang, T. Yevsa, N. Woller, L. Hoenicke, T. Wuestefeld, D. Dauch, A. Hohmeyer, M. Gereke, R. Rudalska, A. Potapova, M. Iken, M. Vucur, S. Weiss, M. Heikenwalder, S. Khan, J. Gil, D. Bruder, M. Manns, P.

Schirmacher, F. Tacke, M. Ott, T. Luedde, T. Longerich, S. Kubicka, and L. Zender, "Senescence surveillance of pre-malignant hepatocytes limits liver cancer development," *Nature*, 2011.

7. TABLES INDEX

Table 1. Primers set used for mouse genotyping 41

Table 2. Primers set used for real-time PCR 43

Table 3. Age-related miRs selected from literature search 48

Table 4. Signaling pathways regulated by the upregulated miRs 50

Table 5. *Molecular targets common to more than one upregulated miRs* 51

8. FIGURES INDEX

Figure 1. Biliary tree architecture 2

Figure 2.Hepatic bile production and modification, an overview 9

Figure 3. Overview of cholangiocytes immune biology 10

Figure 4.Crosstalk between activated cholangiocytes and resident/non-resident cells in response to biliary injury 14

Figure 5.Schematic representation of cholangiopathies pathogenesis 18

Figure 6. Cellular Senescence: an overview 26

Figure 7. SAMR1 and SAMP8 mice: phenotypic characteristics 39

Figure 8. Illustration of Twf1-/- allele map 41

Figure 9. Age-related miRs are increased in diseased cholangiocytes. 49

Figure 10. Twf1 and related miRs are upregulated in both aged and injured of cholangiocytes 53

Figure 11. TWF1 expression in human reactive cholangiocytes 55

Figure 12.Twf1 supports biliary proliferation in vitro 57

Figure 13.Senescence is induced in Twf1 knocked-down cholangiocytes in vitro 59

Figure 14.Senescence associated secretory phenotype is induced in Twf1 knocked-down cholangiocytes in vitro. 61

Figure 15.Twf1 may dampen cholangiocyte senescence in vitro. 63

Figure 16.Cholangiocyte response to injury is worsened in a mouse model of accelerated aging 65

Figure 17.Twf1 expression is modulated by cholagniocyte senescence and injury in vivo 66
Figure 18.Twf1 lack of expression reduces reactive cholangiocytes response to injury 68