FACOLTA' DI MEDICINA E CHIRURGIA

Corso di Dottorato in Salute dell'Uomo

Ciclo XXXIV

Involvement of autophagy in cholestatic liver diseases

Ph.D. dissertation of:

Elisabetta Ninfole

Advisor: Prof. Marco Marzioni

Co-advisor: Dott. Luca Maroni

ABSTRACT

Autophagy is a physiological lysosomal degradation process, essential for cellular homeostasis and ubiquitous in all eukaryotic cells. Dysregulation of hepatic autophagy has been described in several conditions, from obesity to diabetes and cholestatic disease, while stimulation of autophagy seems to ameliorate the liver damage.

The project was focused on the identification of molecular pathways which are activated during autophagic process in response to damage of the bile duct and whether autophagy plays a role in regulating cellular aging processes at the level of the biliary epithelium.

Normal rat cultured cholangiocytes (NRC), a murine intrahepatic bile duct cell line, were used to investigate the autophagic process. We analyzed the role of autophagy in cholangiocytes, the link between autophagy and senescence and the use of autophagic inhibitors and activators.

In vitro, autophagy is activated due to the action of the inductors with a concomitant decline of senescence marker. As further confirmation, we perform experiments to modulate the activity of autophagy itself, by using inhibitor. Our research group has recently shown that the Twinfilin-1 protein (TWF1) modulates the response to damage of cholangiocytes to aging. Subsequently, we investigated if Twf1 may play a role in the early stages of cell fate between autophagy and senescence.

The use of autophagy modulators (inductors / inhibitors) combined with pharmacological agents appears to be a promising strategy to treat a variety of cholestatic conditions. In this settings, Twf1 modulation of cholangiocyte biology may play a relevant role when deciding cell fate between autophagy and senescence.

Further studies will provide the use of experimental models in laboratory animals to strengthen the results obtained *in vitro.*

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1. INTRODUCTION

1.1Autophagy

Autophagy is a physiological lysosomal degradation process essential for cellular homeostasis and energy balance, which is ubiquitous in all eukaryotic cells, being highly conserved from yeast to human. The term 'autophagy', derived from the Greek meaning 'eating of self', was first coined by Christian de Duve over 40 years ago, and was largely based on the observed degradation of mitochondria and other intra-cellular structures within lysosomes of rat liver perfused with the pancreatic hormone, glucagon [1]. Yoshinori Ohsumi, 2016 Nobel Prize in Medicine, has contributed to extraordinary growth in this field with his studies in the yeast *Saccharomyces cerevisiae*. Ohsumi et al. were indeed able to identify the "autophagic bodies" [2], and subsequently of several genes involved in autophagy in yeast [3]. One of the characteristic features of autophagy is its dynamic regulation; through the degradation of cytoplasmic organelles, proteins, and macromolecules, and the recycling of the breakdown products, autophagy plays important roles in the maintenance of cellular homeostasis [4]. Cellular autophagic activity is usually low under basal conditions, but there are several stimuli that can lead to its upregulation. Under physiological conditions, autophagy has several vital roles such as maintenance of the amino acid pool, preimplantation development, clearance of intracellular microbes, and regulation of innate and adaptive immunity [5]. Nutrient starvation is the most well-known inducer of autophagy [6]; further stimuli

include stress, hypoxia or energy depletion, hormonal stimulation and pharmacological agents [7–10]. Although it is commonly seen as a cytoplasmic event, recent studies have unveiled a transcriptional and epigenetic network that regulates autophagy [11]. The identification of transcription factors, microRNAs (miRNAs) and histone modifications connected to autophagy allowed the understanding of both short-term and long-term responses to autophagy [11]. The discovery of evolutionarily conserved genes has enabled scientists to uncover a vast array of homeostatic, developmental, and other physiological functions of autophagy [12]. Accordingly, dysfunction of this process contributes to the pathologies of many human diseases [13]. Autophagy has been widely implicated in many pathophysiological processes such as cancer, metabolic and neurodegenerative disorders as well as cardiovascular and pulmonary diseases. It also has an important role in aging and exercise [14]. Autophagy is distinct from endocytosis-mediated lysosomal degradation of extracellular and plasma membrane proteins, since it is mediated by a unique organelle called the autophagosome [15]. As autophagosomes engulf a portion of cytoplasm, autophagy is generally thought to be a nonselective degradation system. However, autophagy can be selective or nonselective, depending on the targets to which cytoplasmic substrates are delivered [16,17]. Selective autophagy involves damaged or superfluous organelles, whose degradation process is named after its target: mitophagy for mitochondria, pexophagy for peroxisomes, xenophagy for microbes [18], lipophagy involves the degradation of lipid droplets [19]. According to recent findings, autophagy is involved in remodelling of the endoplasmic reticulum (ER) a dynamic organelle that undergoes alterations in morphology, molecular composition and functional specification in response to a variety of stimuli [19,20].

Pathological or physiological challenges may induce ER perturbations that upregulate autophagy to restore homeostasis, a process that has been defined as recovER-phagy (ER-phagy-mediated recovery from ER stress) [20,21]. Cytosolic accumulation and aggregation of misfolded proteins upon defective clearance are involved in conditions such as spongiform neurodegeneration and severe ataxia. Misfolded proteins in the ER are translocated to the cytosol for proteasomal degradation via ER-associated degradation (ERAD) [22]. Those proteins which do not engage ERAD factors are segregated in ER subdomains and delivered to endolysosomes for ER-to-lysosome-associated degradation (ERLAD) under control of ER-phagy receptors [21,22]. The mode of cargo delivery into lysosomes distinguishes three types of autophagy: microautophagy, macroautophagy and chaperone-mediated autophagy (CMA), the latter only found in mammalian cells [23].

1.1.1 Macroautophagy

Macroautophagy (hereafter referred to as autophagy) is the most frequent and well-studied mechanism and occurs at basal rate under normal cellular homeostasis. The understanding of autophagy has taken place by a series of genetic studies in the yeast *Saccharomyces cerevisiae* [2,3]. More than 30 autophagy related genes (ATG) required for autophagy and their mammalian counterparts have been identified, including genes that were not identified in the relatively specific yeast screens [3,24,25]. Membrane dynamics during autophagy are highly conserved from yeast to plants and animals [15,24] -Table1.

Protein function	Yeast	High Eukaryotes
Protein kinase involved in the induction of autophagy and possibly in PAS/phagophore biogenesis	Atg1	ULKs
E2-like enzyme for the ubiquitin-like conjugation system that catalyzes Atg8/LC3's lipidation involved in phagophore expansion	Atg3	ATG3
Cysteine protease processing and delipidating Atg8/LC3, thus involved in phagophore expansion	Atg4	ATG4
Covalently linked to Atg12, generating the Atg12–Atg5 conjugate involved in phagophore expansion	Atg5	ATG5
Component of various PI3K complexes, one of which is involved in induction of autophagy and PAS/phagophore biogenesis	Atg6	Beclin1
E1-like enzyme for the two ubiquitin-like conjugation systems, thus involved in phagophore expansion	Atg7	ATG7
Ubiquitin-like protein involved in phagophore expansion	Atg8	LC3s
E2-like enzyme for the ubiquitin-like conjugation system that mediates the formation of the Atg12–Atg5 conjugate involved in phagophore expansion	Atg10	ATG10
Ubiquitin-like protein involved in phagophore expansion	Atg12	ATG12
Binding partner and regulator of Atg1/ULKs, thus involved in the induction of autophagy and possibly PAS/phagophore biogenesis	Atg13	ATG13
Component of the PI3K complex I involved in induction of autophagy and possibly PAS/phagophore biogenesis	Atg14	ATG14
Associates with Atg12–Atg5 to form a large complex, which acts as an E3 ligase to direct LC3 lipidation on autophagosome membranes, and thus involved in phagophore expansion	Atg16	ATG16
Binding partner and regulator of Atg1/ULKs, thus involved in the induction of autophagy and possibly PAS/phagophore biogenesis	Atg17	FIP200
PtsIns3P-binding proteins possibly involved in PAS/phagophore biogenesis	Atg18	WIPIs
Kinase regulating Vps34 activity; component of various PI3K complexes, one of which is involved in the induction of autophagy and PAS/phagophore biogenesis	Vps15	p150
Component of various PI3K complex, one of which is involved in the induction of autophagy and PAS/phagophore biogenesis	Vps34	PI3K complex

Table 1. **Genes involved in autophagy.** Summary of the most important genes involved in the autophagy process, conserved from yeast to eukaryotes.

The process of autophagy begins with an expanding membrane structure termed the phagophore which enwraps portions of the cytoplasm. This leads to the formation of a double-membrane sequestering vesicle, termed the autophagosome. Autophagosomes subsequently fuse with lysosomes, releasing their inner compartment to the lysosomal lumen. The inner membrane part of the

autophagosome, together with the enclosed cargo, is degraded, and the resulting macromolecules are released into the cytosol through lysosomal membrane permeases for recycling [15].

The autophagy pathway consists of autophagosome initiation, membrane elongation, autophagosome maturation and autophagosome fusion with the lysosome.

The process of autophagy starts with phagophore assembly sites (PAS), that begin to create the membrane source, which enwraps portions of the cytoplasm to be degraded. This assembly was first described in yeast, as a perivacuolar structure visible in fluorescence microscopy where various proteins of the autophagy machinery co-localized. Subsequently, more than 40 autophagy related gene (Atg) have been identified [26]. Among them, one subset including 18 genes is shared by both nonselective and selective autophagy and is required for autophagosome formation, and thus the corresponding gene products are termed the core machinery of autophagosome formation [27]. Although not all membrane sources have been identified, recent data seem to indicate that the endoplasmic reticulum (ER) contributes to form structures called omegasomes. Omegasomes are rich in phosphatidylinositol-3-phosphate (PI3P), a key lipid messenger for autophagy initiation [28]. Other potential membrane sources are the plasma membrane, mitochondria and the Golgi complex [29–31]. Two protein complexes are involved in this initiation step: one complex contains the class III phosphatidylinositol 3-kinase (PI3K) complex [32]. VPS34 is a part of the PI3K complex, which also involves Beclin-1 (BECN1), ATG14 and VPS15 [32]. Inhibition of VPS34 activity by 3-methyladenine (3-MA) or wortmannin leads to inhibition of autophagosome

formation. The ULK1 complex is serine/threonine kinase that phosphorylates the components of PI3K complex, also involved in autophagy initiation; its phosphorylation of BECN1 is important to activate ATG14-bound VPS34 [33]. ULK1 complex includes the focal adhesion kinase-family interacting protein of 200kDa (FIP200), the Unc-51 like autophagy activating kinase (ULK1), ATG101 and ATG13. When nutrients are abundant, binding of the ULK1 complex by the mammalian target of rapamycin (mTOR) complex 1 (mTORC1) inhibits autophagy. The mTORC1 is an important regulator of cell growth and metabolism. It is composed of five subunits that include Raptor, which binds ULK1, and mTOR, a serine-threonine kinase. By phosphorylating ULK1, mTOR inhibits autophagy initiation. In starvation, mTORC1 dissociates from the ULK1 complex, freeing it to trigger autophagosome nucleation and elongation [34–36].

The second step, elongation of the phagophore, results in the formation of autophagosome, which is typically a double-membraned organelle. This step is a simple sequestration, and no degradation occurs. In both yeast and mammals, it relies on two ubiquitin-like reactions. In the first, ATG12 is conjugated to ATG5 in a reaction that requires ATG7 and ATG10 [37]. ATG7 – acting like an E1 ubiquitin-activating enzyme – activates ATG12 in an ATP-dependent manner. ATG12 is then transferred to ATG10, an E2-like ubiquitin carrier protein that potentiates the covalent linkage of ATG12 to ATG5. Conjugated ATG5–ATG12 complexes pair with ATG16L dimers to form a multimeric ATG5–ATG12–ATG16L, resulting in an 800 kDa complex that associates with the expanding phagophore [38,39]. The association of Atg5–Atg12–Atg16L complexes is thought to induce curvature into the growing phagophore through asymmetric recruitment of processed LC3B-II.

Atg5–Atg12 conjugation is not dependent on activation of autophagy and once the autophagosome is formed, Atg5–Atg12–Atg16L dissociates from the membrane, making conjugated Atg5–Atg12 a relatively insufficient marker of autophagy [40].

The second ubiquitin-like reaction involves microtubule-associated protein 1 light chain 3 (LC3), which is encoded by the mammalian homologue of Atg8 [39]. LC3 is expressed in most cell types as a full-length cytosolic protein that, upon autophagy induction, is proteolytically cleaved by ATG4, a cysteine protease, to generate LC3-I [38]. The carboxyterminal glycine exposed by ATG4-dependent cleavage is then activated in an ATP-dependent manner by the E1-like ATG7, similarly to the action of ATG7 on ATG12. Activated LC3-I is then transferred to ATG3, a different E2-like carrier protein, before phosphatidylethanolamine (PE) is conjugated to the carboxyl glycine to generate processed LC3-II [23,38]. LC3B-II has been proposed to act as a receptor for selective substrate, p62/ SQSTM1 [41]. During autophagy the synthesis and processing of LC3 is increased. Recent research indicates that the deacetylation and cytosolic translocation of a nuclear pool of LC3 is required for its lipidation with PE during starvation-induced autophagy [42]. After the closure of the autophagosome ATG16-ATG5-ATG12 complex dissociates from the autophagosome whereas a proportion of LC3B-II remains covalently bound to the membrane and therefore LC3B-II could be used as a marker to monitor levels of autophagy in cells.

The closed autophagosome is transported to the endolysosomal system, where its maturation – through fusion with endocytic vesicles and lysosomes – gives rise to the autophagolysosome, where the sequestered material is degraded. Microtubules and actin filaments, the two main components of the cytoskeleton, have both been

implicated in autophagosome trafficking. To avoid non-specific fusion and ensure proper cargo degradation, the process is tightly regulated, although the exact mechanisms involved in the relevant signalling is not completely understood [43]. Notably, autophagosomes on the way to fusion with lysosomes can fuse with endosomes to form amphisomes [44] -Figure 1. The maturation of early endosomes gives rise to multivesicular bodies (MVBs), late endocytic compartments containing many intraluminal vesicles (ILVs). Fusion of MVBs with the plasma membrane results in the release of ILVs into the extracellular space as exosomes. Alternatively, autophagosomes can fuse with MVBs to form hybrid organelles termed amphisomes [45].

When autophagosome formation is completed, LC3B-II attached to the outer membrane is cleaved from PE by Atg4 and released back to the cytosol. However, the retrieval and uncoating mechanisms of other Atg proteins remain to be studied. In mammalian cells, the fusion event is thought to require the lysosomal membrane protein LAMP-2 and the small GTPase Rab7. After fusion, degradation of the inner vesicle is dependent on a series of lysosomal/vacuolar acid hydrolases, including proteinases A and B (encoded by PEP4 and PRB1, respectively) and the lipase Atg15 in yeast and cathepsin B, D (a homolog of proteinase A), and Lin mammalian cells. The resulting small molecules from the degradation, particularly amino acids, are transported back to the cytosol for protein synthesis and maintenance of cellular functions under starvation conditions. The identification of Atg22, together with other vacuolar permeases (such as Avt3 and Avt4) as vacuolar amino acid effluxes during yeast autophagy, has helped in the understanding of the mechanisms of

nutrient recycling; these permeases represent the last step in the degradation and recycling process [46].

A detailed review of the autophagy apparatus has been provided elsewhere [47,48].

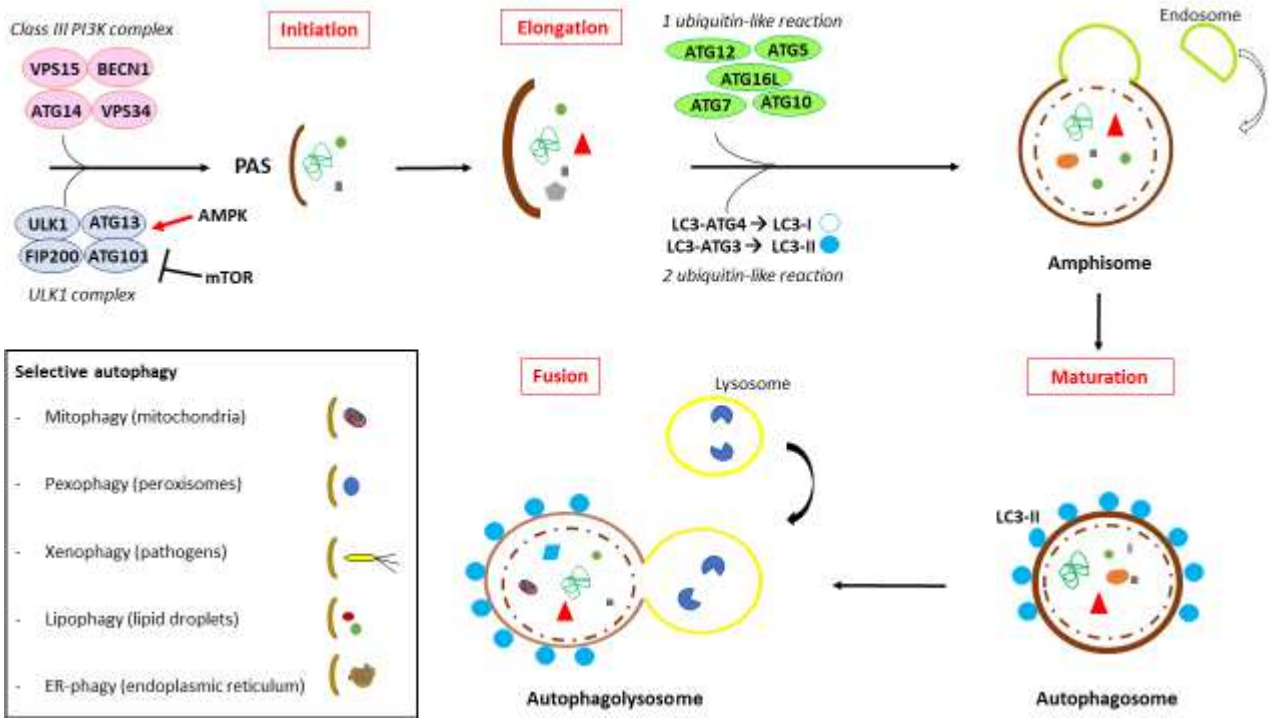

Figure 1. **Molecular and signalling pathways regulating autophagy** (From Pinto *et al* 2021 [49] Autophagy is a degradation process involving four key steps: autophagosome initiation from PASs, which begin to create the membrane source through the action of ULK1 and class III PI3K complex; membrane elongation, which allows capturing the autophagic substrates and relies on two ubiquitin-like reactions; autophagosome maturation; and autophagosome fusion with the lysosome to form the autophagolysosome, where the sequestered material is degraded. The autophagy process may be selective (e.g., mitophagy, pexophagy, xenophagy and lipophagy) or non-selective depending on its target.

1.1.2 Microautophagy

Microautophagy involves the direct engulfment of cytoplasmic cargo into the lysosome through invagination of the lysosomal membrane. Microautophagy is important in the maintenance of organellar size, membrane homeostasis and cell survival under nitrogen restriction [23,50]. Macro- and microautophagy can both engulf large structures by selective and non-selective mechanisms.

1.1.3 Chaperone-mediated autophagy

Chaperone-mediated autophagy (CMA) involves the direct translocation of cytoplasmic proteins across the lysosomal membrane in a complex with chaperone proteins that are recognized by the lysosomal membrane receptor LAMP-2A (lysosomal-associated membrane protein 2A), resulting in their unfolding and degradation [51]. CMA is highly selective, resulting in the degradation of a population of cytosolic proteins that contain a KFERQ peptide [51]. CMA is also involved in regulating lipolysis in the liver through lipid droplet degradation. Perilipins (PLINs) are proteins that coat lipid droplets; PLIN removal is required for cytosolic lipases and autophagy to gain access to the lipids in the droplet [52]. Upregulation of the ubiquitous PLIN2 has been reported to suppress autophagy, whereas its downregulation stimulates triglyceride catabolism via autophagy [52].

1.2 Autophagy in cholestatic liver disease

Autophagy plays a critical role in the regulation of liver physiology and the balancing of liver metabolism. Numerous recent studies have indicated that autophagy may participate in the pathogenesis of liver diseases, such as liver hepatitis, steatosis, fibrosis, cirrhosis, and hepatocellular carcinoma [53]. Although autophagy regulates several cell functions, it was first found to maintain energy balance in liver cells. In conditions of food deprivation (starvation), hepatic autophagy activation provides nutrients via degradation of intracellular materials [29]. Degradation and recycling of essential components thus contribute to the energy stores and, at least in the liver, closely depend on the duration of nutrient deficiency. Liver autophagy provides starved cells with amino acids, glucose and free fatty acids for use in energy production and synthesis of new macromolecules and controls the quality and quantity of organelles such as mitochondria [54]. Hormones (insulin and glucagon) and amino acids are important stimuli in adaptation to starvation [54,55]. A growing body of evidence has shown that liver autophagy contributes to basic hepatic functions, including glycogenolysis, gluconeogenesis and β-oxidation, through selective turnover of specific cargos controlled by a series of transcription factors. In mice, starvation-induced autophagy is important in the conversion of amino acids to glucose via gluconeogenesis, to maintain blood glucose concentrations [56]. However, if nutrient shortage persists, glycophagy and lipophagy are also activated, providing glucose and free fatty acids (FFAs) as preferential cargo [57]. The mammalian target of rapamycin (mTOR) is the key cellular nutrient sensor that regulates cell growth and metabolism. This protein

kinase is composed of two complexes: mTORC1, which is involved in nutrient homeostasis, and mTORC2 [58]. In the presence of nutrients, mTORC1 directly phosphorylates and inhibits the autophagy-initiating kinase ULK1. Pacer - a recently discovered regulator of hepatic autophagy and liver homeostasis, and a key player in connecting metabolic signals to late steps of autophagy regulation - is directly phosphorylated by mTORC1 in nutrient-rich conditions; its absence impairs autophagosome maturation and lipid catabolism both in vitro and in vivo. Balanced mTOR activity is critical for physiological liver function [59]. In young Atg5 knockout (L-Atg5 KO) mice, mTOR ablation attenuated hepatomegaly, liver injury and inflammation, but not fibrosis [60]. mTOR inhibitors such as rapamycin and its derivatives have considerably improved autophagy regulation. They include everolimus (RAD001), decolorises (AP23573) and temsirolimus (CCI-779), whereas second-generation mTOR inhibitors include MLN0128 (sapanisertib), CC-233 or NVP-BEZ235 (dactolisib) and AZD-8055 [34,61].

Autophagy also regulates energy supply by controlling the number, quality, and dynamics of organelles such as mitochondria or endoplasmic reticulum (ER). Mitochondria are the key energy-producing organelles and cellular source of reactive species. Mitochondrial dysfunction is associated with both acute and chronic liver diseases with emerging evidence indicating that mitophagy plays a key role in the liver's physiology and pathophysiology [62]. Blocking liver autophagy accelerates time to mortality in the murine sepsis model, suggesting that liver autophagy plays a protective role for organ failure through degradation of damaged mitochondria, as well as prevention of apoptosis [63]. Autophagy plays a protective role in alcohol-related liver disease (ALD) and drug-induced liver injury (DILI) by

selectively removing damaged mitochondria (mitophagy), lipid droplets (lipophagy), protein aggregates and adducts in hepatocytes [64]. Autophagy also protects against ALD by degrading interferon regulatory factor 1 (IRF1) and damaged mitochondria in hepatic macrophages [64]. The involvement of autophagy and endoplasmic reticulum (ER) stress during early-stage liver injury remains to be fully elucidated. In a recent work, a murine model of liver injury was induced by intraperitoneally injecting lipopolysaccharide (LPS) and D-galactosamine (GalN); autophagy and ER stress occurred in early-stage liver injury induced by LPS-GalN administration in mice [65]. Sirtuin-1 (SIRT1), a NAD^+-dependent deacetylase, can be activated by caloric restriction or by pharmacological activators, particularly resveratrol. SIRT1-autophagy pathway and decreased ER stress are universally required for the protective effects of moderate caloric restriction (30%) and resveratrol on high-fat diet-induced hepatic steatosis [66].

Dysregulation of liver autophagy has been described in severe metabolic disorders such as obesity, hepatic steatosis and diabetes [57]. Yet, it is still unclear whether autophagy favours or prevents the progression of liver injury. Fibrosis is the result of the wound-healing response of the liver to repeated injury. The main causes of liver fibrosis include chronic viral infection such as hepatitis B and C, alcohol abuse (alcoholic steatohepatitis) and non-alcoholic fatty liver disease (NAFLD) [67–69]. NAFLD and non-alcoholic steatohepatitis (NASH), a progressive form of NAFLD, can evolve to advanced liver disease, cirrhosis and hepatocellular carcinoma [69,70]. Liver sinusoidal endothelial cells (LSECs), which line the sinusoidal lumen, play a key role in liver injury due to their unique position and provide the first line of defence. NASH is associated with a defect in liver endothelial autophagy due to

inhibition of adenosine monophosphate-activated protein kinase (AMPK)α activity, the master regulator of autophagy [71]. The deficiency induces endothelial inflammation, endothelial-to-mesenchymal transition and endothelial cell death. Moreover, upon exposure to a high-fat diet, LSECs deficient in autophagy rapidly and strongly modulate some genes involved in inflammation [71]. In vivo, autophagy has been investigated in a transgenic mouse line bearing a deletion of Atg7 expression in endothelial cells (Atg7endo mice). Following mild acute liver injury, LSECs isolated from such mice displayed worse endothelial dysfunction compared with their control littermates (Atg7control). LSEC autophagy also regulates the antioxidant response, as demonstrated by elevated intracellular O^{-2} production in Atg7endo mice subjected to mild acute liver injury. Since autophagy exerts a protective role in early liver injury, its potentiation may prove an attractive approach to prevent disease progression [72]. Following acute injury, the inflammatory milieu activates resident macrophages (Kupffer cells) or injured hepatocytes to replace necrotic or apoptotic cells. Persistence of hepatic injury and failed liver regeneration induce activation of hepatic stellate cells (HSCs) through α-SMA and collagen-I expression and deposition of large amounts of extracellular matrix [67]. Dimethyl α-ketoglutarate has been demonstrated to inhibit collagen deposition in a carbon tetrachloride (CCl4)-induced liver fibrosis model in vivo. LC3B and α-SMA (a marker of HSC activation) signalling were both reduced in fibrotic livers treated with DMKG, suggesting that DMKG may inhibit HSC activation by inhibiting autophagy. These effects have been confirmed in vitro using the HSC-T6 cell line [73]. In the liver, extracellular vesicles (EVs) from injured hepatocytes and LSECs have been reported to induce HSC activation. Binding of

platelet-derived growth factor (PDGF) – a key molecule in liver fibrosis progression – to PDGF receptor (PDGFR) induces tyrosine autophosphorylation, which recruits important downstream signalling molecules, such as Src homology 2 domain protein phosphatase 2 (SHP2). PDGF and SHP2 induce EVs release from HSCs through activation of mTOR signalling, which inhibits autophagy, and Rho-associated protein kinase 1 signalling. HSC autophagy has been found to mitigate liver fibrosis by reducing fibrogenic HSC-derived EVs release [74]. Recently, a non-canonical form of autophagy, LC3-associated phagocytosis (LAP), has been seen to exert beneficial antifibrogenic effects. Autophagy and LAP are distinct both functionally and mechanistically; notably, the latter is independent of the autophagy activating kinase ULK1 but requires components such as the P13K complex and Atg5 and Atg7. LAP, which is enhanced in blood monocytes from the liver of cirrhosis patients and in animal models, exerts an anti-inflammatory action. Sustaining LAP would mitigate both systemic and hepatic inflammation and may open therapeutic prospects for chronic liver disease [75].

Recently, bile acids (BAs) and their receptor Farnesoid X Receptor (FXR) have been implicated in the regulation of hepatic autophagy, hence in cholestatic diseases. BAs have been reported to inhibit autophagy degradation in vitro and may also play a role in impaired hepatic autophagy in FXR KO mice in vivo; moreover, by reducing Rab7 expression, they induced decreased autophagosomal-lysosomal fusion in primary cultured mouse hepatocytes [76]. These findings suggest a possible link between BAs and impaired autophagy in BA-induced hepatotoxicity and liver tumorigenesis [76].

The potential role of autophagy in cancer has been analysed in several studies. To date, it remains unclear whether the activation of the autophagic process favours or prevents the progression of tumour alterations. In CCA, several pieces of evidence strongly suggest a correlation with autophagy. The use of autophagy modulators (inductors or inhibitors) combined with pharmacological agents seems a very promising strategy to treat different cancers, thus enhancing apoptosis by ER stress or due to mitochondrial dynamics [77].

1.2.1 Biliary tree

The biliary tree is a network of ducts arising from the canals of Hering up to the choledochus, 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 [78,79].

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 [80].

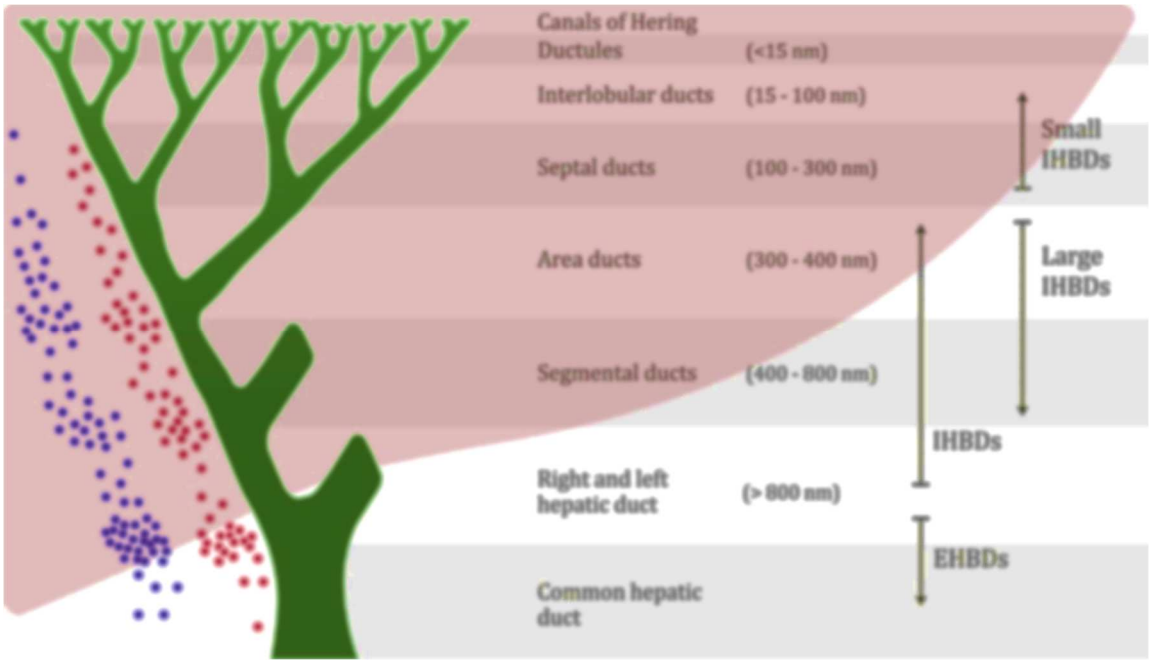

Figure 2. **Biliary tree architecture** (Image modified from de Jong *et al.* 2018 [81])

Small and large cholangiocytes are two different populations that have been characterized in the intrahepatic bile ducts. When large cholangiocytes are damaged, the small cholangiocytes proliferate and acquire phenotypic and functional features of large cholangiocytes. In this regard, small cholangiocytes are thought to be committed biliary progenitors, evidence corroborated by integrated differential gene expression studies [82].

Blood supply of biliary tree is ensured by the peribiliary biliary plexus (PBP) which stems from the hepatic artery branches and flows into the hepatic sinusoids. The PBP supports the transport of bile substances reabsorbed from cholangiocytes to hepatocytes [83]. The liver is innervated by two autonomic nervous fibers, the

sympathetic and the parasympathetic fibers [84]. These fibers release several neuropetides and neurotransmitters that regulate cholangiocytes absorptive/secretory activities and proliferation through the expression of relative specific receptors on cholangiocytes membrane [85].

1.2.2 Cholangiocytes

Cholangiocytes are the epithelial cells which line the bile ducts and are the central target of a group of diseases with different etiology, 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) [86]. 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 [87] and maintain cell polarity [88]. Cell-cell communication between adjacent cells is also ensured by the presence of gap junctions [89]. Along the apical membrane, cholangiocytes possess several microvilli protruding in the bile duct lumen, which increase of 5-fold cell surface area [90]. Moreover, biliary cells are provided with a primary cilium which exerts sensory functions, transmitting extracellular-derived information inside the cells thus modulating different intracellular pathways involved in cellular biological functions (e.g., differentiation, proliferation or secretion) [91]. The primary cilium is a nonmotile structure composed by a microtubule-based core surrounded by axoneme, which extends from the basal body up to cell membrane [80,91]. At the primary cilium level, it is possible to find several proteins and receptors whose

activation results in the upregulation or downregulation of different signaling pathways [91].

As well as other cell types, cholangiocytes cytoplasm, possesses 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 [92]. Cholangiocyte exocytic capabilities lie in multivesicular bodies (MVBs) and exosomes releasing, processes that take place in the apical domain of plasma membrane [93]. 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 [94]. 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 [95].

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 [90]. Unlike small cholangiocytes, large cholangiocytes have a columnar morphology and a small nucleus to cytoplasm ratio [90]. 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 [78]. On the other hand, small cholangiocytes can 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 [96].

1.2.3 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 led to secretion into the bile of water, Cl^- and $HCO3^-$, and extraction of glucose, bile acids and amino acids [97]. The releasing of $HCO3^-$ 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 [80,97]. 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 - Figure 3 [98].

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 [98]. In the ileum most bile acids (95%) are absorbed and then recycled in the liver via enterohepatic circulation [97]. Differently, through the cholehepatic shunt, a portion of bile acids (unconjugated) secreted by hepatocytes, after being passively absorbed by cholangiocytes, return to hepatocytes [80]. Subsequently, these unconjugated bile acids are secreted again by hepatocytes stimulating once again $HCO3^-$ secretion. One of the most important molecules which regulates biliary secretion is the secretin (STC) hormone, whose specific receptor is expressed at the basolateral domain of large cholangiocytes membrane [99]. Briefly, in response to peptides and acidic pH produced after a meal, duodenal and jejunal S cells release the STC into the portal blood [80]. 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 [86]. The existing Cl- gradient across the two sides of plasma membrane activates the anion exchanger 2 (AE2) and, therefore, the net releasing of HCO3 - associated to the osmotic influx of H2O through the aquaporin 1 (AQP1) [86,100].

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 [101]. 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^- [102]. 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^- exchanger, K^+ channel (SK2) [80]. 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) [80]. As mentioned above, bile contains organic cations excreted by hepatocytes which could be noxious for cholangiocytes [98]. 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 can excrete again into the bile, exogenous or endogenous lipophilic compounds [102,103]. 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 or and MRP4) [104].

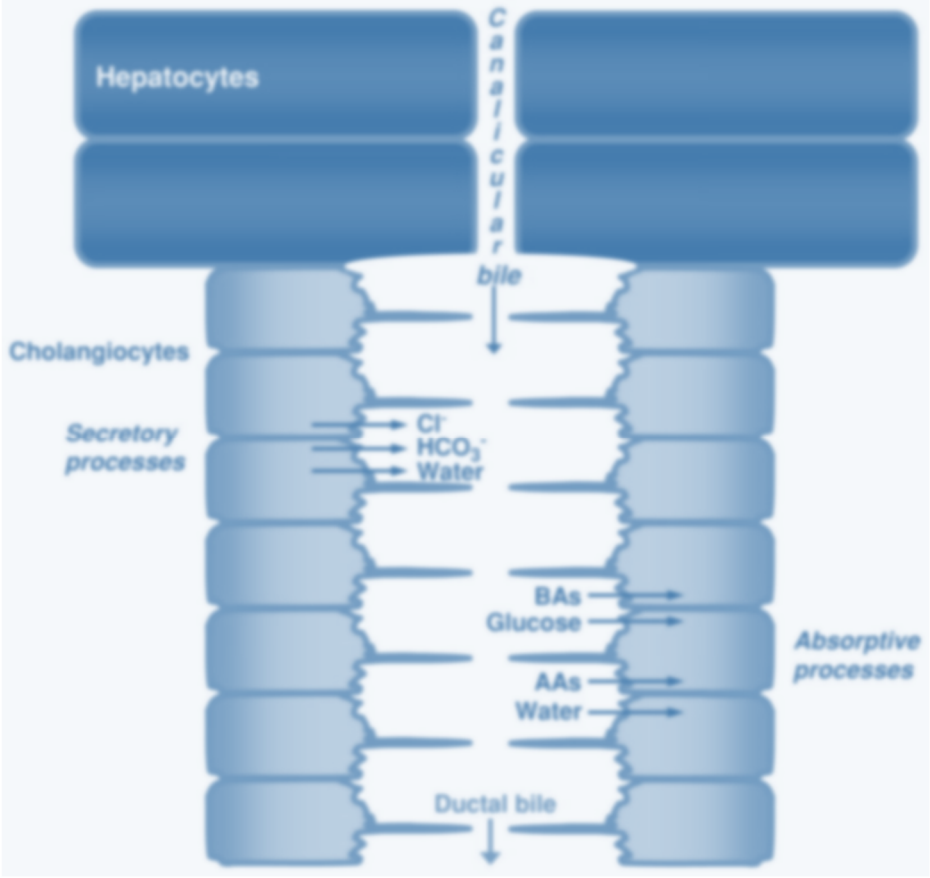

Figure 3. **Hepatic bile production and modification, an overview.** Hepatocytes synthetize the canalicular primary bile which, as it flows into the bile duct lumen, is modified by cholangiocytes through either secretory (Cl-, HCO3- and water extrusion) or absorptive processes (glucose, bile acids, water, and amino acids absorption). BAs: bile acids; AAs: amino acids (Image modified from Tabibian *et al.* [80])

1.2.4 Functional role in disease state and therapeutic strategies

In physiological conditions cholangiocytes are mitotically dormant, until biliary tree damage occurs. In response to exogenous/endogenous stimuli, cholangiocytes actively participates in inflammatory and reparative processes within the liver - Figure 4. Furthermore, cholangiocytes interact with the immune system and microorganism and are involved in drug metabolism [105]. Cholangiocytes represent

the primary cell target of a diverse group of genetic and acquired biliary disorders, collectively called "cholangiopathies". Many of the cholangiopathies are, at their early stages, site restricted along the biliary tree. For instance, primary biliary cirrhosis (PBC), drug-induced cholangiopathies, and graft vs. host disease (GVHD) involving the liver affect primarily the small bile ducts. In contrast, primary sclerosing cholangitis (PSC) and cholangiocarcinoma mainly involve the large intra- and extrahepatic bile ducts [105].

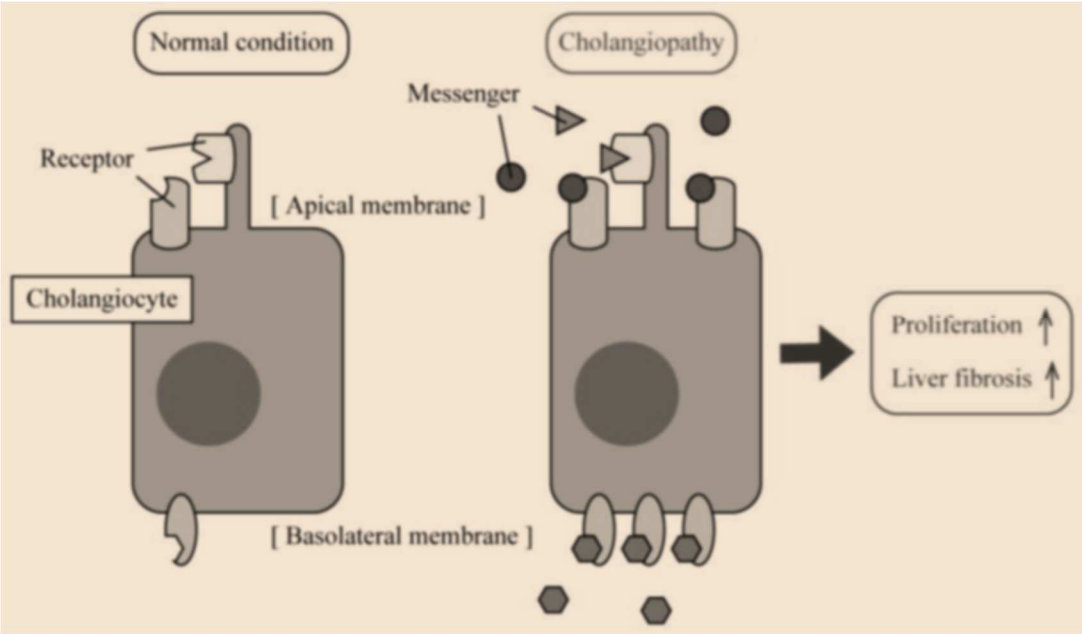

Figure 4. **Working model of cholangiocyte responses during cholangiopathies** (Image modified from Sato *et al*. 2018, [106])

Chronic cholestatic liver diseases are a universal manifestation of cholangiopathies. Cholangiopathy progression is often accompanied by an imbalance between cholangiocyte proliferation and death; this leads to the gradual disappearance of bile ducts, which also characterizes conditions such as PBC, PSC as well as drug-induced ductopenia and cystic fibrosis-related liver disease [107]. Accumulation of

toxic bile acids (BAs) is the main cause of cell damage in cholestasis patients [108]. The mechanisms by which cholestasis induces liver damage require further investigation, but they include at least mitochondrial dysfunction (hence oxidative stress) [109], unbalanced apoptosis, and necrosis [110], which can lead to liver fibrosis [111], and organelle (mainly ER) stress [112]. As noted above, such cellular damage induces an adaptive response that includes autophagy activation. There is mounting evidence that autophagy is altered in cholestatic conditions. BAs cause the build-up of insoluble p62 and ubiquitinated proteins and increase the rate of apoptosis [113]; such events are accompanied by autophagosome accumulation and suppression of the autophagic flux, as also seen in hepatocytes treated with BAs, which show BECN1 inhibition. Moreover, pharmacological or genetic inhibition of autophagy increases BA-induced cell death in hepatocytes [113]. Notably, animal bile duct ligation (BDL) experiments have documented the accumulation of p62 and ubiquitinated proteins as seen in human liver. Autophagy stimulation seemed to ameliorate the liver damage [114]. In BDL mice fed cholic acids, a BA that is typically increased in human cholestasis, Mallory bodies and p62-positive aggregates increased [114]. Autophagy activation by rapamycin-induced inhibition of mTOR signalling led to the disappearance of these hepatic inclusion bodies [115]. In PIZZ mice, a model of induced liver injury, the A1AT mutant Z protein accumulated in the ER and polymerized into a complex quaternary structure, the typical lesion of the condition [116]. Such polymers have been detected in autophagosomes, suggesting that autophagy is a possible mechanism for their degradation [117]; indeed, autophagy induction has proved a useful therapeutic strategy to reduce liver injury in PIZZ mice [118,119]. In general, mice with defective autophagy – such as Atg7 and Atg5 KO

mice and mice treated with autophagy inhibitors – have more severe cholestatic liver injury [120,121]. Although there are few human studies, due to the technical difficulties attendant to testing the true autophagic flux, p62-positive hepatocellular inclusion bodies are commonly found in patients with cholestatic liver diseases such as PBC or cystic fibrosis [122–124]. Impaired autophagy, reflected by increased levels of LC3 and p62, has also been described in other cholestatic liver diseases including PSC and genetic cholestasis [125]. Increased LC3 and p62 protein expression and decreased expression of Rab7 (involved in vesicular traffic) have been seen in tissue from patients with hepatolithiasis com-pared with normal tissue [126].

Despite the evidence for a possible protective role of autophagy stimulation in cholestasis, no strategies aimed at its induction have yet been tested in cholangiopathy patients. The majority of cholangiopathy research focuses on PSC because of the well-established research models with limited preclinical and therapeutic studies for PBC and biliary atresia [127]. Current therapeutic strategies directed at replenishing the bile ducts of ductopenic patients are limited to protecting cholangiocytes from death induced by the immunological response [128,129]. The first-line treatments to counteract cholangiocyte death are hydrophilic ursodesoxycholic acid (UDCA) as well as immunosuppressive and anti-inflammatory agents. UDCA is the drug of first choice for cholestasis, particularly PBC but less so for PSC [129,130]. UDCA increases the autophagic flux in human patients, while in vitro studies have lent further support to its therapeutic potential in cholestasis [125]. UDCA is also believed to rebalance the autophagic responses in cholestasis patients and to act as an FXR antagonist [131]. Furthermore, in a rat model of NASH, UDCA exerted favorable effects by reducing apoptosis and stimulating

autophagy through AMPK phosphorylation [132]. These data suggest that its ability to enhance autophagy could also be harnessed to treat in other diseases that would benefit from autophagy induction. 24-norursodeoxycholic acid (norUDCA) is a side chain shortened homologue of UDCA that has shown high potential in preclinical mouse models of cholestatic and fibrotic liver disease [133–137]. Notably, it is also an autophagy inducer; in the PIZZ mouse model, norUDCA significantly reduced ATZ globules by inducing autophagy [119,138] while exerting favorable effects on various parameters such as serum liver enzymes and casp-3 and -12 (markers of ER stress-induced apoptosis), it reduced compensatory liver proliferation and increased in the expression of various genes involved in autophagy [119]. Similar to UDCA, norUDCA appears to induce autophagy via AMPK activation through the mTOR/ULK1 pathway [138]. OCA is a second-line treatment strategy for PBC patients who do not respond to or tolerate UDCA [139]. However, OCA has been shown to impair the autophagic flux both in vitro and in vivo [125]. In clinical settings, its anti-cholestatic properties seem to outweigh the potential negative effects of reduced autophagy [140]. In summary, the pro-autophagic effect of current cholangiopathy medications may help reduce the damage induced by cholestatic disease.

1.3 Autophagy and ageing

1.3.1 Ageing

Aging is the process of functional decline of multiple cells and tissue which ultimately lead to body deterioration and increased susceptibility to death [141,142]. Several disorders such as cardiovascular diseases [143], pathologies of nervous system [144] or cancer [145] have been related to aging. 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.

The causes of primary hallmarks are multiple. The accumulation of DNA damage (e.g., mutations, telomeres shortening or gene disruption) is one of the features characterising the aging process [146] which depends on both exogenous stressors (physical, chemical or biological triggers) and/or endogenous events (DNA replication errors or ROS production) [147]. 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 [148]. Accordingly, the overexpression of SIRT6 transgene in mice

results in a prolonged lifespan [149]. Also, the impairment of proteostasis, term which refers to protein homeostasis, has been correlated with aging process [150].

As a result of primary hallmarks, cell activates compensatory processes known as antagonistic hallmarks. Cellular senescence represents the main response to age-related damage. Senescence is defined as the irreversible process of cell growth arrest associated to complex cellular changes such as chromatin organization, metabolic reprogramming including autophagy and the release of several proinflammatory mediators and growth factors, which are included in the senescence associated secretory phenotype (SASP) [141,151]. The stable replicative arrest of cells is a physiologic process which impedes damaged or cancer cells to divide and expand, thus suppressing tumorigenesis [152]. 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 [153,154]. 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 [155] and

differentiation, and tissue remodelling [151]. Senescence may play a role in biliary atresia, primary sclerosing cholangitis, cellular rejection, and primary biliary cirrhosis, four liver diseases affecting cholangiocytes and the biliary system [156].

Finally, the role of SASP mediators is to recruit immune cells to eliminate senescent cells [157]. 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 [158].

Either nutrient sensing deregulation and 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. 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 [159–161]. 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) [162,163]. 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 [164].

1.3.2 How autophagy is correlated with ageing

Autophagy is a highly regulated cellular program responsible for recycling intracellular proteins and damaged/non-functional organelles. Autophagy seems to be involved in various longevity pathways. Investigations into the correlation between autophagy and ageing and how it may affect lifespan or healthspan have found that autophagic activity decreases with age in numerous species [68,165–167]. The work carried out in *Caenorhabditis elegans,* and *Drosophila melanogaster* has highlighted several conserved pathways with a key role in longevity, including insulin/IGF-1 signalling, calorie restriction, mitochondrial respiration and TOR signalling. Notably, autophagy activation has been documented to extend animal lifespan, suggesting that autophagy is one of the convergent mechanisms of several longevity pathways [168–172]. Beclin-1 (Bec-1) is required for lifespan extension in nematodes, as it impairs the insulin signalling pathway [168]. Delayed manifestation of age-related changes after tissue-specific deletion of critical autophagy genes has been described in mouse tissues such as kidney and heart [173,174]. An increase in lifespan has been reported in mice overexpressing Atg5 [175] and as a result of pharmacological and physiological autophagy induction (SPD and caloric restriction) [176–178]. In contrast, autophagy inhibition is related to premature ageing, as seen in numerous loss-of-function studies of key factors of the autophagic

machinery [171,173]. Loss-of-function mutations in Atg1 (Unc-51), Atg7, Atg18 and Bec-1 reduced *C. elegans* lifespan [171], whereas deficient Atg1, Atg8 and Sestrin1 expression reduced *Drosophila melanogaster* lifespan due to triglyceride accumulation, mitochondrial dysfunction, muscle degeneration and cardiac malfunction, which are typically [167,179]. In contrast to the lifespan extension effect in lower organism, it has been reported that overexpression of ULK3, the mammalian homolog of Atg1, induces premature senescence in human fibroblast [180]. Similarly, the expression of various proteins required for autophagy induction is reduced in ageing and pathological conditions; for instance, Atg5, Atg7 and BECN1 are downregulated in the normal human aged brain [181], SIRT1 is downregulated in subjects with insulin resistance and metabolic syndrome [182], and ULK1, BECN1 and LC3 are downregulated in osteoarthritis patients [183], suggesting that insufficient autophagy may contribute to the ageing phenotype. Importantly, SIRT1 is one of an evolutionarily conserved family of NAD^+ dependent deacetylases that play important role in several processes, including metabolism and ageing. Autophagy mediates downregulation of mammalian SIRT1 protein during senescence and *in vivo* aging. This causes the loss of SIRT1 during aging of several tissues related to the immune and hematopoietic system in mice and in $CD8^+$ $CD28^-$T cells from aged human donors [184]. Furthermore, in several species pharmacological inhibition of autophagy prevents the anti-ageing effects of caloric restriction [185]. Therefore, autophagy may both induce and prevent senescence, which may be dependent on cell type or the type of the experimental model. However, it is still an open question as to whether autophagy is necessary for senescence to occur or inhibits the senescence processes. In IMR90 cells, autophagy

is activated during senescence and its activation is correlated with negative feedback in the PI3K-mammalian target of rapamycin (mTOR) pathway [186]. A subset of autophagy-related genes, including ULK3, induces autophagy and senescence; these data suggest that autophagy, and its consequent protein turnover, mediate the acquisition of the senescence phenotype [186]. Autophagy also promotes senescence through degradation of nuclear lamina and chromatin [187]. This process may be critical for the SASP, and immune clearance of DNA based cells by triggering cyclic GMP-AMP synthase (cGAS) and stimulator of interferon genes (STING) [188–190]. Kang *et al* [191] reported that the transcription factor GATA4 is a senescence and SASP regulator, cleared in normal cells by the autophagy adaptor p62. This process is suppressed when senescence is induced, and these findings suggest that GATA4 may represent a separate branch of DNA damage repair that favours senescence.

1.3.3 Ageing and autophagy in the liver

The aging of the population, the increased prevalence of chronic liver disease in elderly and the need to broaden the list of potential liver donors suggest to us to better understand the molecular mechanism of the ageing liver [192]. Emerging evidence shows that modulating autophagy seems to be effective in improving the age-related alterations of the liver. These alterations not only impair the metabolic capacity of the liver but also represent important factors in the in the pathogenesis of malignant liver disease [193]. Deregulated autophagy may be related to the autoimmune process against mitochondrial antigens in PBC [194]. The inhibition of autophagy suppressed cellular senescence in biliary epithelial cells (BECs) in damaged small bile ducts in primary biliary cirrhosis (PBC). These findings suggest

that autophagy may mediate the process of biliary epithelial senescence and involve in the pathogenesis of bile duct lesions in PBC [195]. Moreover, ER stress may play a role in the pathogenesis of deregulated autophagy and cellular senescence in biliary epithelial lesions in PBC [196]. In the liver, ageing is also associated with lipid accumulation. This further impairs autophagic activity, since it appears to prevent autophagosome acidification and to reduce the expression of proteolytic enzymes, as reported in a mouse model of genetically induced obesity [197]. The age-related reduction of autophagy efficiency also affects lipophagy, which plays an important role in lipid metabolism [198,199]. The decline of lipophagy in the aged or steatotic liver slows down the breakdown of lipids accumulated in the liver and results in a lower FFA intake for lipid metabolism, compounding the cell function impairment [200,57]. The concomitant increase in the number of senescent hepatocytes further weakens cell function by damaging mitochondria, thus leading to lower FA oxidation, reduced ATP synthesis and production of large amounts of ROS [201]. High ROS levels contribute to HSC activation and eventually to the development of liver fibrosis and structural impairment, besides inducing increased hepatocyte apoptosis and hepatic inflammation [202–204]. Normal mitochondrial function is crucial for hepatic metabolism. Ageing also reduces the mitochondrial turnover rate by affecting mitophagy efficiency [205], resulting in a constant increase in the number of dysfunctional mitochondria and in a gradual rise in ROS production that compound the steatosis. The role of autophagy in hepatic fibrosis is more debated. Ageing itself is considered as a major risk factor for fibrosis development [206]. In the past decade, several studies have demonstrated that autophagy activation may have a pro-fibrotic role by providing the energy for HSC activation, mainly through

lipophagy and mitophagy [207–209]. On the other hand, autophagy may play a protective role in alcohol-induced hepatic injury by selectively eliminating dysfunctional mitochondria and lipid droplets [210]. Moreover, the use of a known autophagy inducer such as rapamycin in a rat study improved the panel of hepatic fibrosis markers, possibly through an adverse effect on HSC proliferation [211].

2. AIM OF THE THESIS

The project was focused on the identification of molecular pathways which are activated during autophagic process in response to damage of the bile ducts. Cholangiocytes, the cell lining the bile ducts, represent the unique target of cholangiopathies. Due to the progressive nature of the disease and the lack of effective therapies, a deeper understanding of cholangiocytes pathobiology is needed to device novel effective therapeutic strategies. 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. Moreover, emerging evidence shows that modulating autophagy seems to be effective in improving the age-related alterations. 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, we analyzed the role of autophagy in cholangiocytes, the link between autophagy and senescence and the use of autophagic inhibitors and activators. Bioinformatics tools have been exploited to select intracellular pathways and putative molecular targets to be investigated by *in vitro* studies. Further studies will provide the use of experimental models in laboratory animals to strengthen the results obtained *in vitro.*

3. MATERIALS AND METHODS

3.1 Materials

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. Autophagy Assay Kit, chloroquine diphosphate, Rapamycin and Everolimus were from abcam. Antibody used are listed in the table above-Table 2.

B-actin HRP-linked	Cell signaling
LC3-II	Cell signaling
p53	abcam
p62	Cell signaling
Twf1	Sigma-aldrich

Table 2. **Antibody set used for Western blotting**

3.2 Methods

3.2.1 Cell line 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) (Gibco), 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 Lglutamine, 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.

3.2.2 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 [212]. A well-established in vitro model of cholangiocyte-induced senescence [213], 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.

3.2.3 RNA extraction, reverse transcription, and real-time PCR

Total RNA was isolated from NRC using the RNeasy Mini Kit (QIAGEN, Hilden, Germany). A quantity of 1 μg was reverse transcribed to complementary cDNA with All-In-One 5X RT MasterMix (Applied Biological Materials (abm) 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 AriaMx Real-Time PCR System (Agilent) through the SYBR Green fluorophore. Relative abundance of target genes was normalized to Peptidylprolyl isomerase B

(Cyclophilin B) as internal control. Oligonucleotide sequences of primers used for real-time PCR are listed in the table above-Table 3:

Gene name	Sense 5'→ 3'	Sense 3' → 5'
Rat Il- 1α	ACTACTTCACATCCGCAGCT	TGCGAGTGACTTAGGACGAG
Rat Il- 1β	CAGGAAGGCAGTGTCACTCA	AAAGAAGGTGCTTGGGTCCT
Rat Igf-1	GCTATGGCTCCAGCATTCG	TCCGGAAGCAACACTCATCC
Rat LC3-II	GGACCTGCTGCCTTCTCTAAAA	GGAGACCTCTGCTAGGCAAC
Rat p16 ^{INK4a}	ATGGAGTCCTCTGCAGATAGA	ATCGGGGGTACGACCGAAAGTGTT
Rat p21 $^{CIP/WAF1}$	CTGGTGATGTCCGACCTGTTC	CTGCTCAGTGGCGAAGTCAAA
Rat p53	CCTATCCGGTCAGTTGTTGGA	TTGCAGAGTGGAGGAAATGG
Rat Twf1	GTCAGTTCATCCCCATTGTCT	TATTCAATGCGCGGATACACA

Table 3. **Sequences of primers used for real-time PCR**

3.2.4 Western Blotting

Total protein lysates were obtained by NRC using a RIPA lysis buffer (Santa Cruz Biotechnology, Texas, USA) supplemented with protease inhibitor cocktails, PMSF and sodium orthovanadate (Santa Cruz Biotechnology, Texas, USA). The protein fractions recovered from cell lysis homogenization was 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 80 μg of protein lysates was analyzed 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 sulfatepolyacrlyamide 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 protein 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, catalyzed 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.

3.2.5 Autophagy evaluation

Investigating the autophagic process is difficult, due to the lack of absolute criteria applicable to all biological or experimental contexts. Indeed, owing to its complex and dynamic nature, some assays are unsuitable, problematic or may not work at all [193]. Transmission electron microscopy (TEM) is the only tool that depicts structures in their natural environment and position and support quantitative studies [193].The disadvantages of TEM studies are related to the specialized expertise required for sample handling in all stages of specimen preparation, from fixation to sectioning and staining. Moreover, accurate identification of autophagic structures is essential and requires considerable experience. Such problems can be addressed by using approaches that allow monitoring autophagy, such as fluorescence microscopy and biochemical methods. While there are different publications summarizing the wide set of experiments traditionally used in autophagy research, the main goal is the analysis of the autophagy flux by Western Blot [214] - Figure 5. In order to investigate the autophagic flux and the possible influence of Twf1, we evaluated the dynamic status of the protein LC3. The conversion of LC3-I (cytosolic form) to LC3-II (PE-conjugated form) can be evaluated by immunoblotting or by the LC3 turnover assay, where LC3-II degradation in the lysosome is estimated by comparing samples exposed and not exposed to lysosomal

inhibitor treatment. Despite its greater molecular weight, LC3-II migrates faster than LC3-I in SDS-PAGE due to its hydrophobicity. The unconjugated (approximately 16-18 kDa) and PE-conjugated (approximately 14-16 kDa) forms should be indicated on western blots whenever both are detectable [5,215]. Another marker, sequestosome 1 (SQSTM1)/p62, is an autophagy receptor that links ubiquitinated proteins to LC3 and accumulates in cells when autophagy is inhibited. Since SQSTM1 changes can be cell-type or context-specific, its use requires the utmost caution. Other proteins that can be used as autophagy markers include Atg9/ATG9A, ATG12-ATG5, ATG14 and BECN1/Vsp30/Atg6. A useful and detailed guide to autophagy, especially for researchers new to the field, has been published by Klionsky *et al* [193].

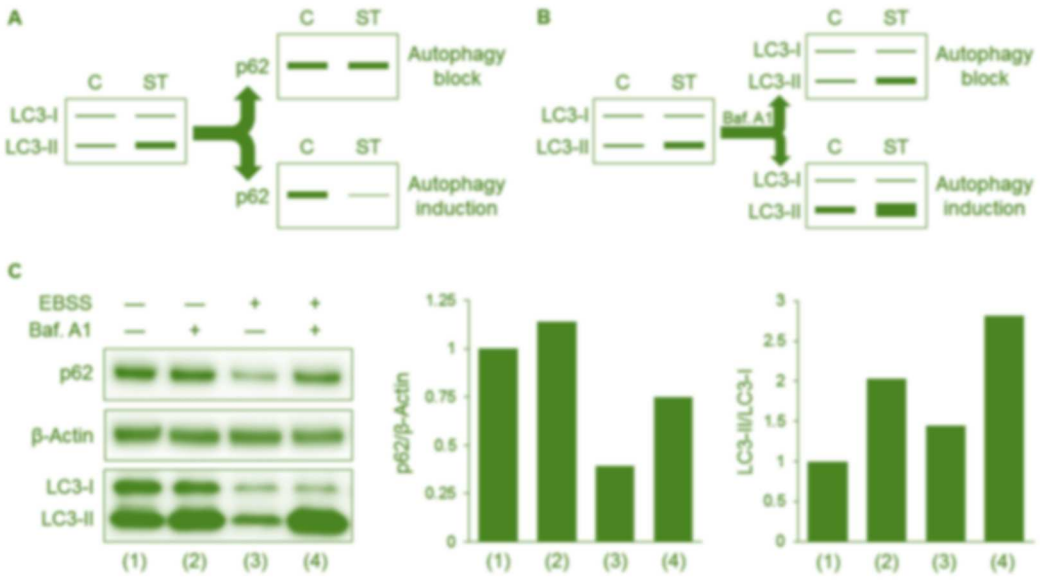

Figure 5. **Interpreting the autophagy flux analysis by Western blot** (Image modified from Fernández *et al* [214]). An accumulation of LC3-II can mean a block in the last step of the process that diminishes its degradation. To avoid this mistake, an additional analysis of p62 is always recommended to discern these two possibilities (a). A complete study of the autophagy flux using inhibitors like bafilomycin A1, accumulated (b). A real case is displayed in (c), where a complete study in HeLa cells treated with EBSS and bafilomycin A1 shows how these cells can trigger a correct autophagy process. In this example, the ratio LC3-II/LC3-I turns out to be necessary, as LC3-II levels decreased after starvation due to its intense degradation

3.2.6 Fluorescence microscopy

To evaluate the autophagic flow, a live cell analysis by fluorescence microscopy provided us a convenient approach for the analysis of the regulation of autophagy at the cellular level (Autophagy Assay Kit (ab139484)-abcam). NRC cells were seeded (2×10^4) in DMEM/F12 supplemented with 5% FBS at 37°C in a humidified atmosphere of 5% CO_2, on a 4-well plate containing cover slips (Nun Thermano Coverslips) previously coated with collagen I. Cells were incubated with Green Detection Reagent and Nuclear Stain, following protocol instruction. Following washing three times with PBS, cells were fixed with 4% paraformaldehyde for 20 min and then washed three times with PBS. Cells were treated with 200 nM Rapamycin for 3h for autophagy induction (positive control cells). To confirm the autophagy flux, positive control cells were pretreated with Chloroquine (CQ) for 18 h, at the concentration of 60 μM, before stimulation with Rapamycin. Response to Rapamycin and CQ is time and concentration dependent and may also vary significantly depending upon cell type and cell line. Negative control cells were cells treated with a vehicle (DMSO used to reconstitute or dilute an inducer or inhibitor) for an equal length of time under similar conditions. Analysis was conducted on Nikon Eclipse 80i, and image analysis were conducted with the program NIS ELEMENTS version 4.0.

4. RESULTS

4.1 Twf1 is involved in cholangiocyte senescence

Our research group has recently shown that the Twinfilin-1 protein (TWF1) modulates the response to damage of cholangiocytes to aging [216]. 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 6, the lack of *Twf1* expression influences the induction of senescence as evidenced by the significant increasing of mRNA levels of $p16^{INK4a}$ in *Twf1* knocked-down cells exposed to a proproliferative stimulus. However, no changes in the alternative senescence marker $p21^{WAF/CIP1}$ 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 level, 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 increased compared to control cells. These data suggest

that *Twf1* expression is induced in response to cellular senescence possibly as a compensatory mechanism stimulating cholangiocyte proliferation.

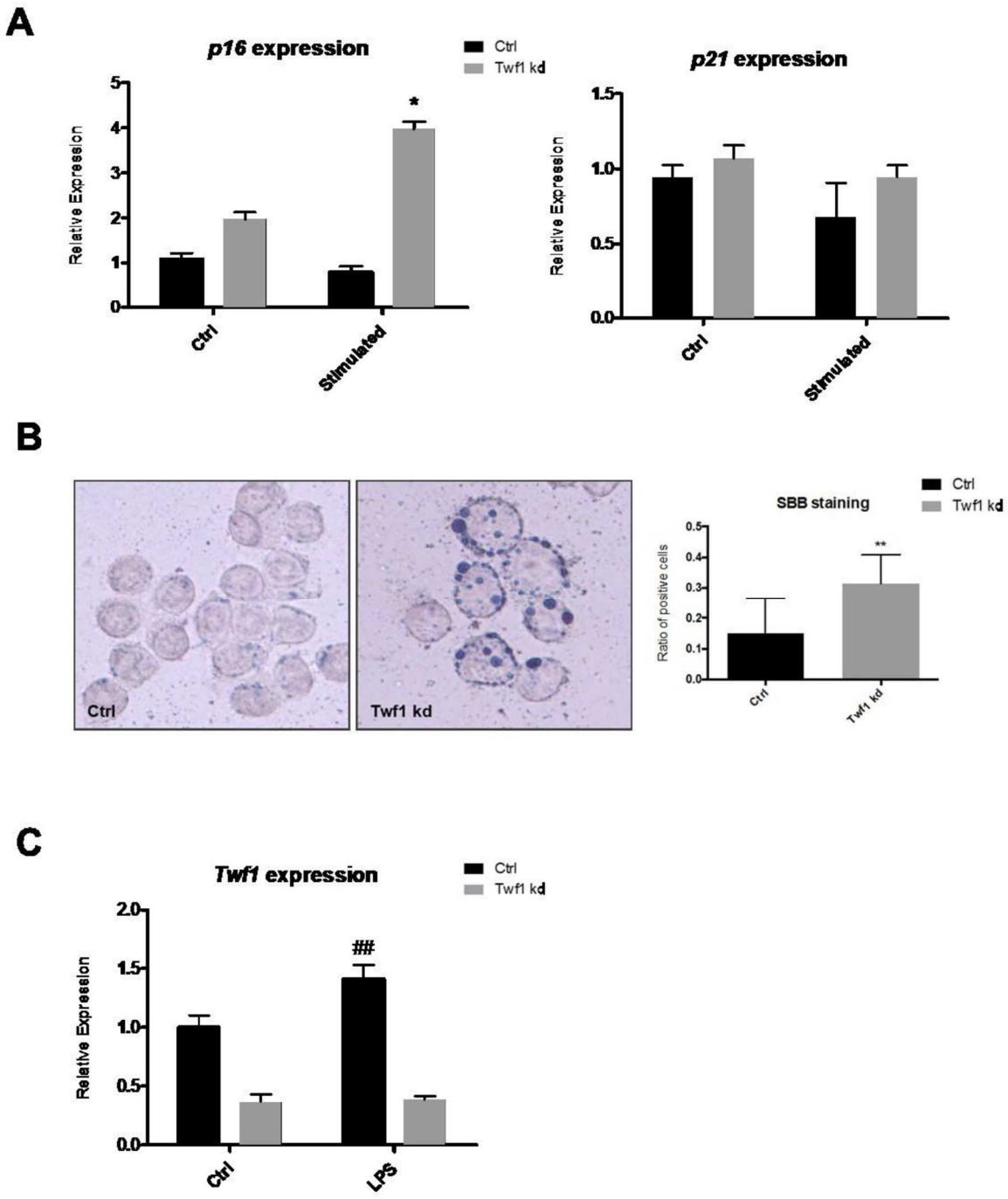

Figure 6. **Senescence is induced in Twf1 knocked-down cholangiocytes *in vitro*.** (A) The expression levels of the senescence markers *p16* and *p21* were evaluated in *Twf1* knocked-down cholangiocytes

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 statistically significant- Figure 7. 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.

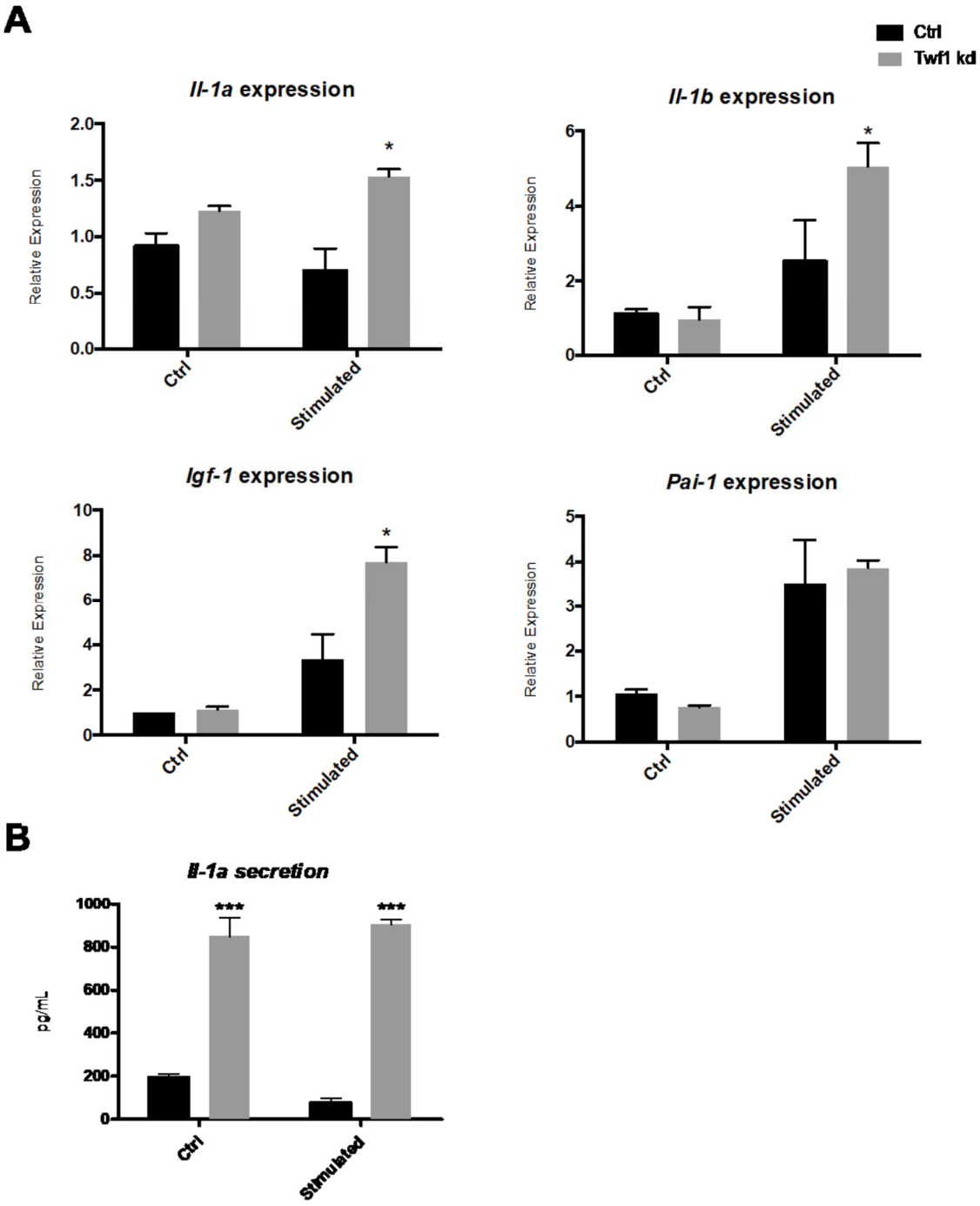

Figure 7. **Senescence associated secretory phenotype is induced in Twf1 knock down cholangiocytes *in vitro***. (A) The expression of SASP markers was evaluated in *Twf1* knocked-down cholangiocytes subjected to a pro-proliferative stimulus, with respect to relative controls. Levels of *Il-1α*, *Il-1β* and *Igf-1* were increased in *Twf1* knocked-down cholangiocytes, whereas no difference in *Pai-1* expression could be evidenced. (B) Levels of *Il-1α* released in cell culture media were significantly increased in *Twf1* knocked-down cholangiocytes either subjected or not to proproliferative 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 8. No differences could be found in Il-1α and Pai-1 expression.

Overall, 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.

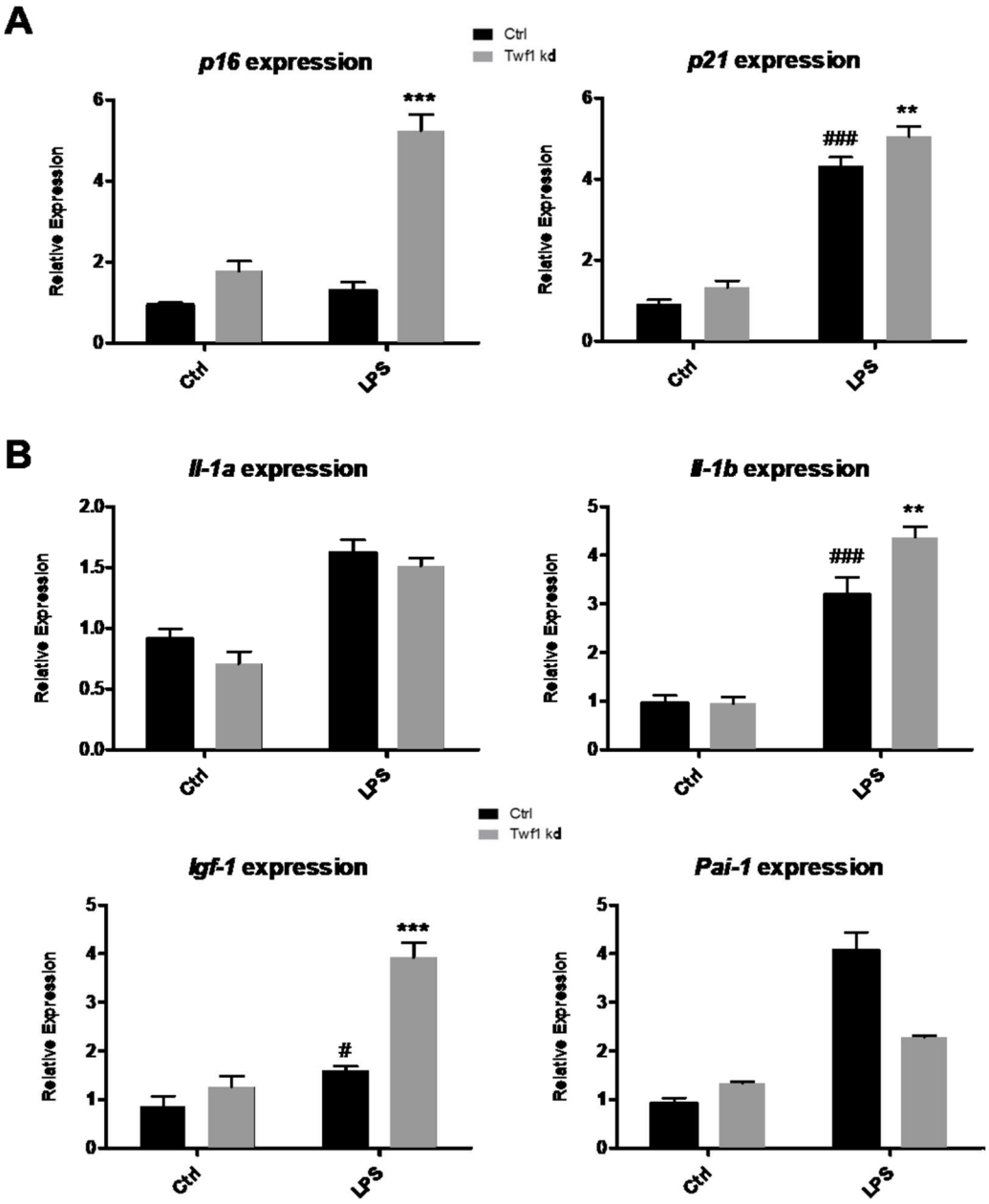

Figure 8. **Twf1 may dampen cholangiocyte senescence *in vitro*.** (A) The expression of senescence markers p16 and p21 was evaluated in Twf1 knocked-down cholangiocytes subjected to a pro-senescence stimulus. Levels of p16 and p21 were increased in Twf1 knocked-down treated cholangiocytes, (B). SASP 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. LPStreated control;***: $p < 0.001$ vs. LPS-treated control; #: $p < 0.05$ vs. respective control; ###: $p < 0.001$ vs. respective control (ANOVA)

4.2 Autophagy activation in vitro

The next step of the project has had as objective the understanding of the molecular mechanisms responsible for cellular alterations related to the aging process. In this regard, the research project plan was to evaluate whether autophagy plays a role in regulating cellular aging processes at the level of the biliary epithelium. During cellular stress, autophagy facilitates cell survival by eliminating damaged molecules that are isolated from the rest of the cell within a double-membrane vesicle known as an autophagosome. Cells can respond to different types of stress by 1) autophagy and healing; 2) senescence, thus irreversibly leaving the cell cycle; 3) apoptosis with consequent cell death [217]. Autophagy, senescence and apoptosis are therefore distinct types of stress response, but their signaling pathways are often overlapped. To investigate the possible correlation between autophagy and senescence, cholangiocytes were stimulated with increasing concentrations of Everolimus, an immunosuppressant that exerts most of its effect by blocking the mammalian target of rapamycin (mTOR), as previously described. The conversion of LC3-I (cytosolic form) to LC3-II (PE-conjugated form), evaluated by Western Blotting, was greater at 3h of treatment with Everolimus, mostly at the concentration of 200 nM. To confirm these data, we also used another autophagy inducer, Rapamycin, at different concentrations and times, always evaluating the autophagy markers. Also, in this case the conversion of LC3-II / LC3-I to 3h of treatment with Rapamycin, has significant values in a concentration range between 100nM and 500nM, with an increase in the conjugated form LC3II indicating an increase in the number of autophagosomes -Figure 9.

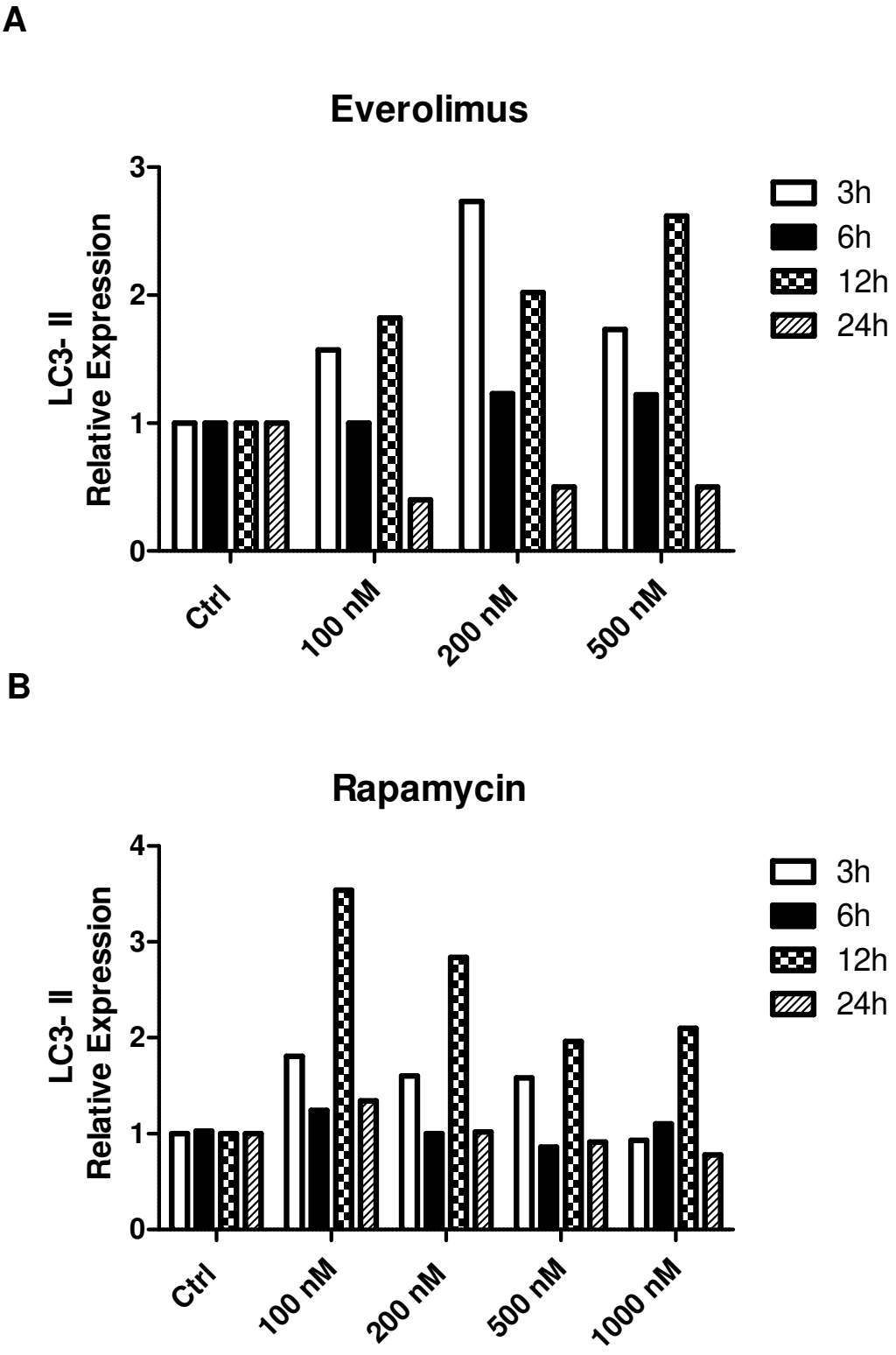

Figure 9. **Autophagy activation *in vitro*.** Autophagy is induced in cholangiocytes cells incubated with Everolimus (A) and Rapamycin (B) in different concentrations of 100, 200, 500 and 1000nM. Significant LC3 conversion was greater in a concentration range between 100nM and 500nM in a time interval of 12h.

The senescence marker p53 was then evaluated at 12 and 24h: we can see how this marker, involved in the autophagy / senescence / apoptosis pathways, undergoes a decline- Figure 10, corroborating the thesis that the different pathways are critical in deciding the fate of the cell, and that the activation of a certain path hinders the continuation of another [218,219].

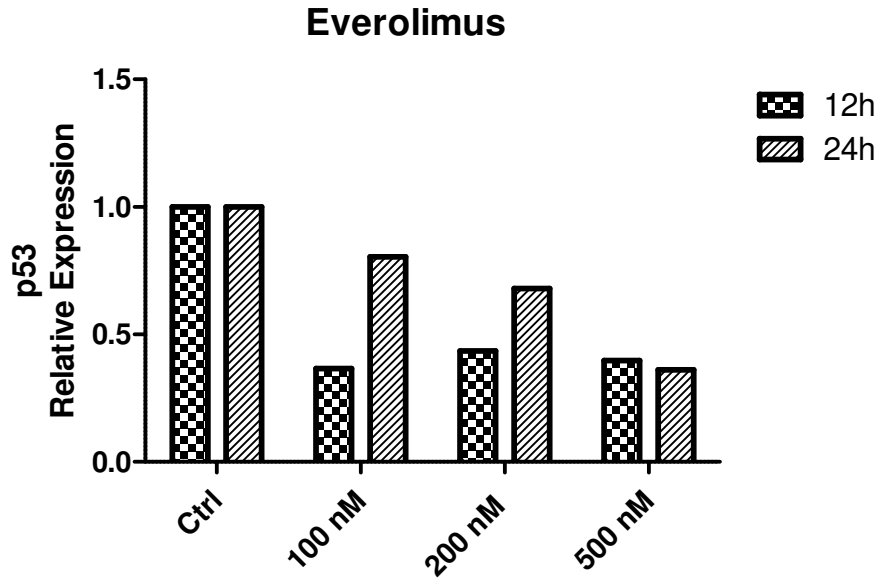

Figure 10. **p53 decline.** The expression level of the senescence marker p53, undergoes a decline in a concentration range between 100 and 500nM, in cholangiocyte cells after treatment with Everolimus during 12-24h; these data corroborate the hypothesis that autophagy and senescence overlap, in deciding cell fate.

4.3 Autophagy evaluation through inhibitor

Subsequently, we evaluated the autophagic flow in the presence of the autophagic inhibitor Chloroquine (CQ). Autophagy inhibitors, such as chloroquine and hydroxychloroquine, have already been clinically approved, promoting drug combination treatment by targeting autophagic pathways as a means of discovering and developing more novel and more effective cancer therapeutic approaches [220]. CQ inhibits the fusion of the autophagosome to the lysosome, thus allowing the quantization of autophagosome formation as a measure of the degree of autophagy by stopping the autophagic flow before lysosomal degradation can occur. Furthermore, p62 (Sequestosome 1), another important marker for a correct evaluation of autophagic flow, was also taken into consideration. p62 is mainly degraded by the autophagic process, and if the lysosomal degradation of the autophagosome and its contents is blocked, there is an accumulation of p62. In fact, an increase in the conversion of LC3-II does not necessarily correspond to an increase in autophagic flow and the evaluation of p62 allows us to establish it with greater precision- Figure 11. The expression of LC3-II increases with autophagic induction of 3h by Rapamycin, while in the 18h pre-treatment with CQ, there is a decrease in conversion parallel to an increase in the marker p62 indicating a block of autophagic flow due to the accumulation of autophagosomes but not autophagolysosomes.

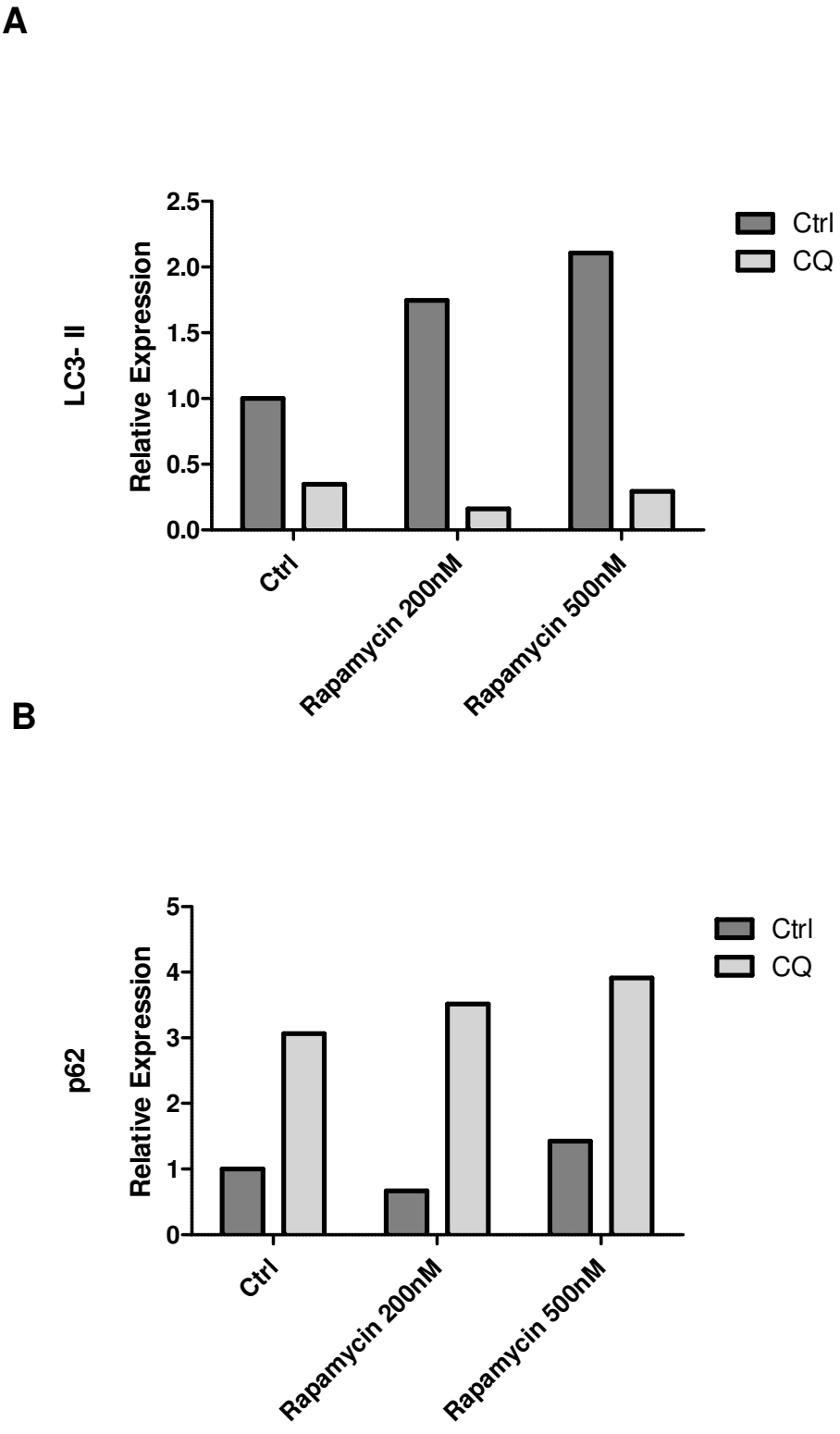

Figure 11. **Evaluation of autophagic flow**. Autophagy induction was further confirmed in cholangiocyte cells, after pre-treatment with the autophagic inhibitor Chloroquine (CQ). Decrease of LC3 was concurrent to an increase in the marker p62, indicating a block of autophagic flow due to the accumulation of autophagosomes but not autophagolysosomes.

4.4 Autophagy evaluation by fluorescence microscopy

These results were further confirmed via a live cell analysis by fluorescence microscopy. Cholangiocytes were incubated with Green Detection Reagent and Nuclear Stain, following protocol instruction; merge figures were obtained using NIS ELEMENTS program. The fluorescent signal observed in cells treated with Rapamycin -figure 12C confirmed that the signal was specifically produced due to autophagy induction. Since autophagy is activated under physiological conditions, there is no surprise of background fluorescence – figure 12A. As stated above, the use of autophagic inhibitor – Figure 12B and 12D allow us to distinguish between basal level -Figure 12A and induction of autophagy -Figure 12C.

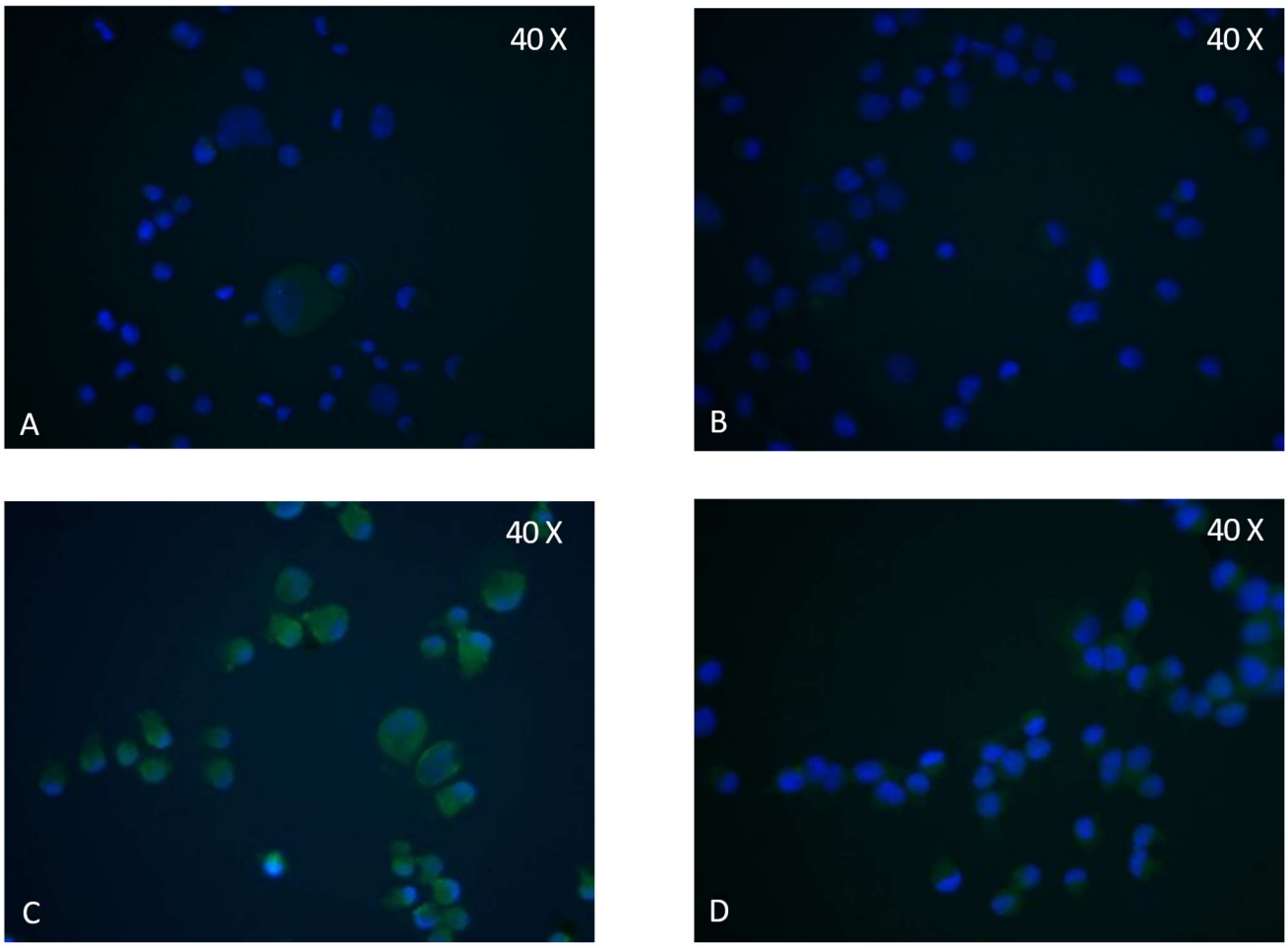

Figure 12 **Evaluation of autophagic flow by fluorescent microscopy.** Fluorescent microscopy analysis showing nucleus (blue nuclear stain; DAPI filter) and autophagic vesicules (green, FITC filter) in control cholangiocyte cells (A) or cells treated with 200nM of Rapamycin for 3h (C) to induce the formation of autophagic vesicles. Cholangiocyte cells were also treated with 60 µM Chloroquine for 18 hours (B) and with Rapamycin and Chloroquine (D) at the same conditions, to further confirm the presence of autophagic flow.

4.5 Role of Twf1 during autophagy

Based on earlier findings, possible Twf1 expression changes were evaluated, in order to understand whether Twf1 regulates cellular senescence at the level of the biliary epithelium, during autophagy induction. This data provides characterization of basal autophagy flux and response to classical inducedrs – EBSS (Earle′s Balanced Salt Solution), Rapamycin and Everolimus and inhibitors. These data were evaluated by Western Blotting. The most significant changes of Twf1 are in the concentration range between 100 nM and 500 nM treatment with Everolimus, concurrently with the increase of the autofhagic flow- Figure 13. To investigate whether Twf1 plays a role during autophagic flow, cholangiocyte cells were treated with CQ as previously described in section 4.3. The expression of Twf1 increases with autophagic induction, with respect to cells pretreated with CQ, when autophagy flux is blocked -Figure 14A, while the senescence marker p53 has a parallel decrease in conversion to an increase of Twf1- Figure 14B. These data suggest that Twf1 may have a marginal role in autophagy and senescence processes, in a time range between 12 and 24h. During this time range, autophagy and senescence pathways could overlap in deciding cell fate.

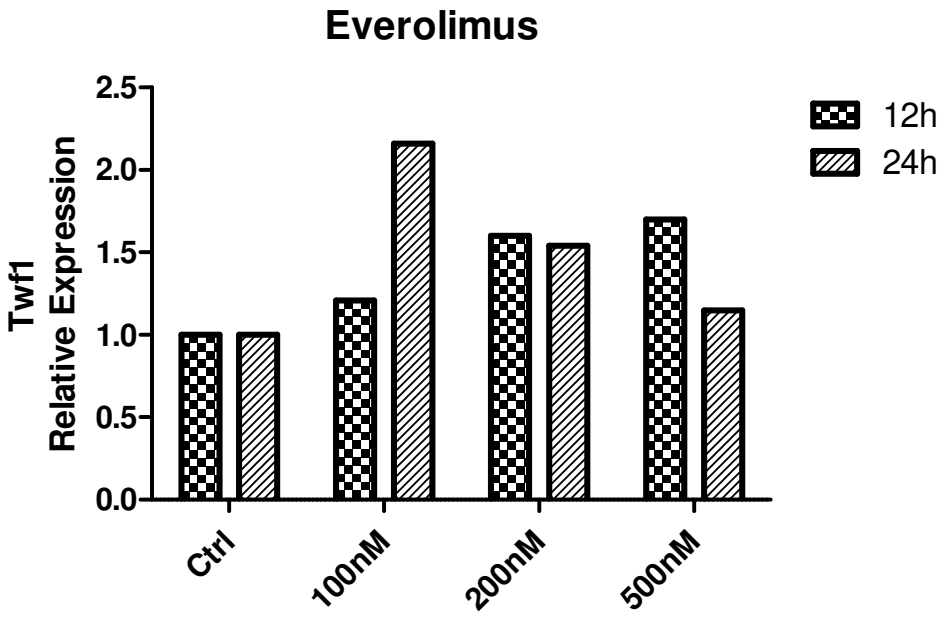

Figure 13. **Evaluation of Twf1 expression during autophagy in vitro.** The most significant changes of Twf1 are in the concentration range between 100 nM and 500 nM treatment with Everolimus, concurrently with the increase of the autophagic flow.

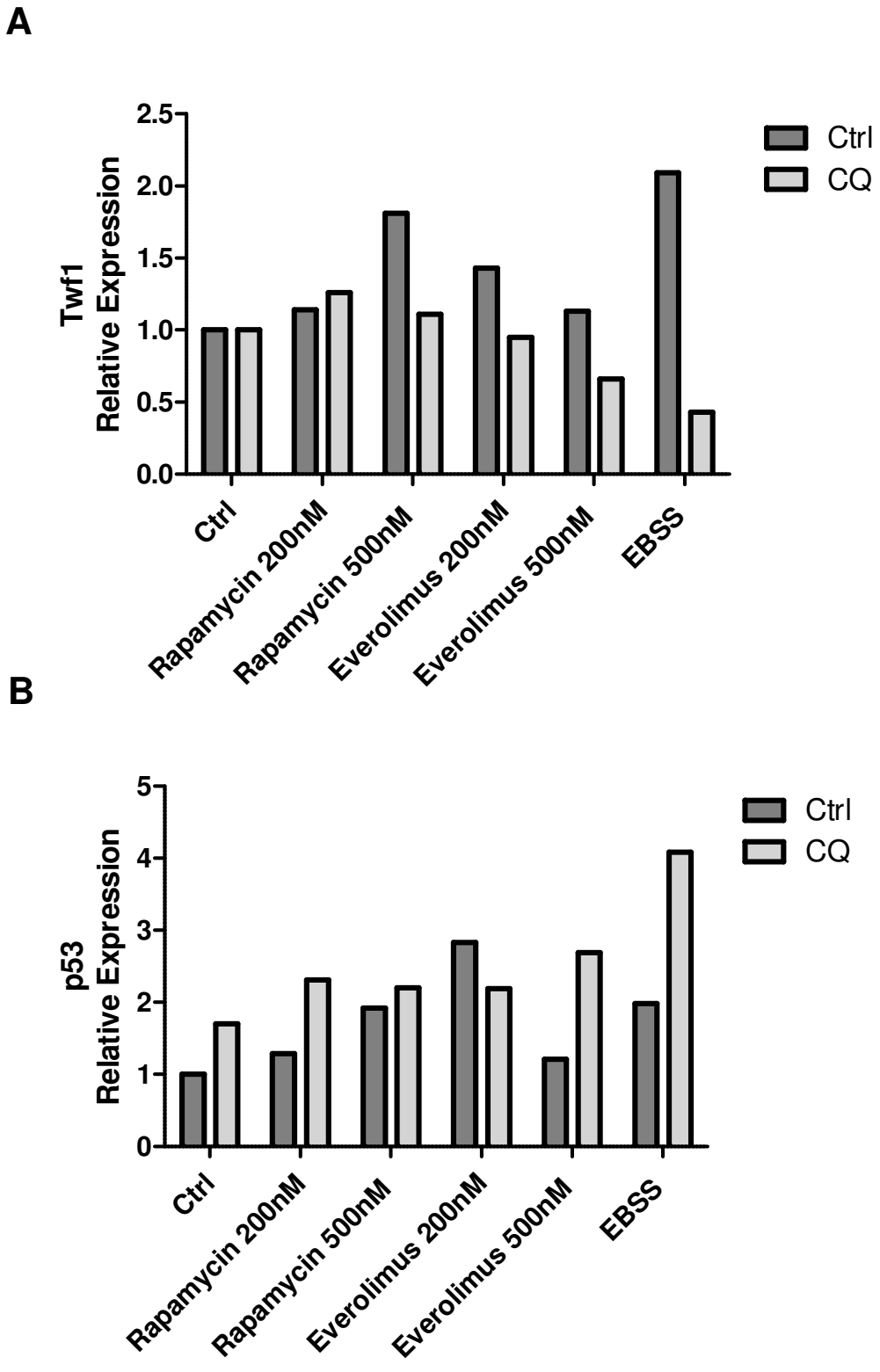

Figure 14. **Autophagy and senescence evaluation with respect to Twf1**. The expression levels of Twf1 and senescence marker p53, suggest as well that autophagy and senescence pathways seem to overlap in deciding cell fate, and that Twf1 could mediate the entry into senescence.

4.6 Relation between Twf1/autophagy/senescence

To understand the possible role of Twf1 in the modulation of the pathways of autophagy and senescence, cells were treated with persistent LPS stimulus (10 days), as described in section 4.1.

On a mRNA level, results of section 4.1 were confirmed- Figure 15 and Figure 16B, while the marker p53 does not undergo significant changes; this different behavior could be since p53 exerts its action in the first phase of response to damage, before senescence is established.

Consistently, autophagy seems to be induced in response to a persistent pro-senescence stimulus as evidenced by an increase in LC3-II mRNA level- Figure 16A. Stressed cells activate autophagy, which prevents damage and maintains metabolism though lysosomal turnover of cellular components. Autophagy can facilitate senescence or limit damage and delay apoptosis to allow recovery and repair of normal cell function [217]. Remarkable, the mRNA expression of LC3-II is decreased in LPS induced senescence cholangiocytes compared to control cells, in absence of Twf1. These data suggest that the lack of Twf1 may determine a slowdown of autophagy, facilitating the senescence mechanism, as stated in section 4.1 -Figure 16A.

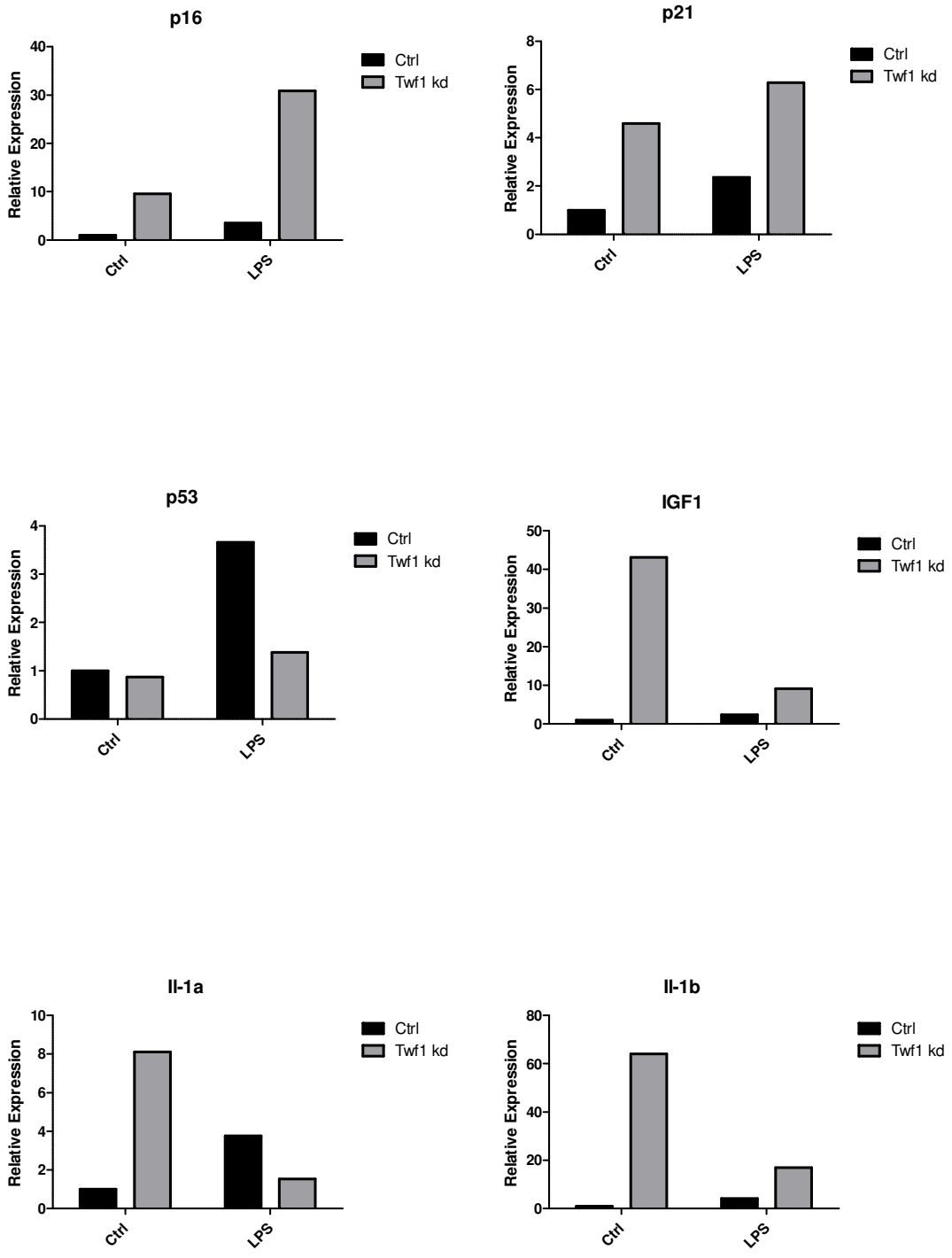

Figure 15. **Senescence is induced in Twf1 knock down cholangiocytes.** The expression of senescence markers and SASP component expression was investigated with respect to a pro-senescence stimulus, in the same experimental setting. Any variation of p53 expression could be evidenced.

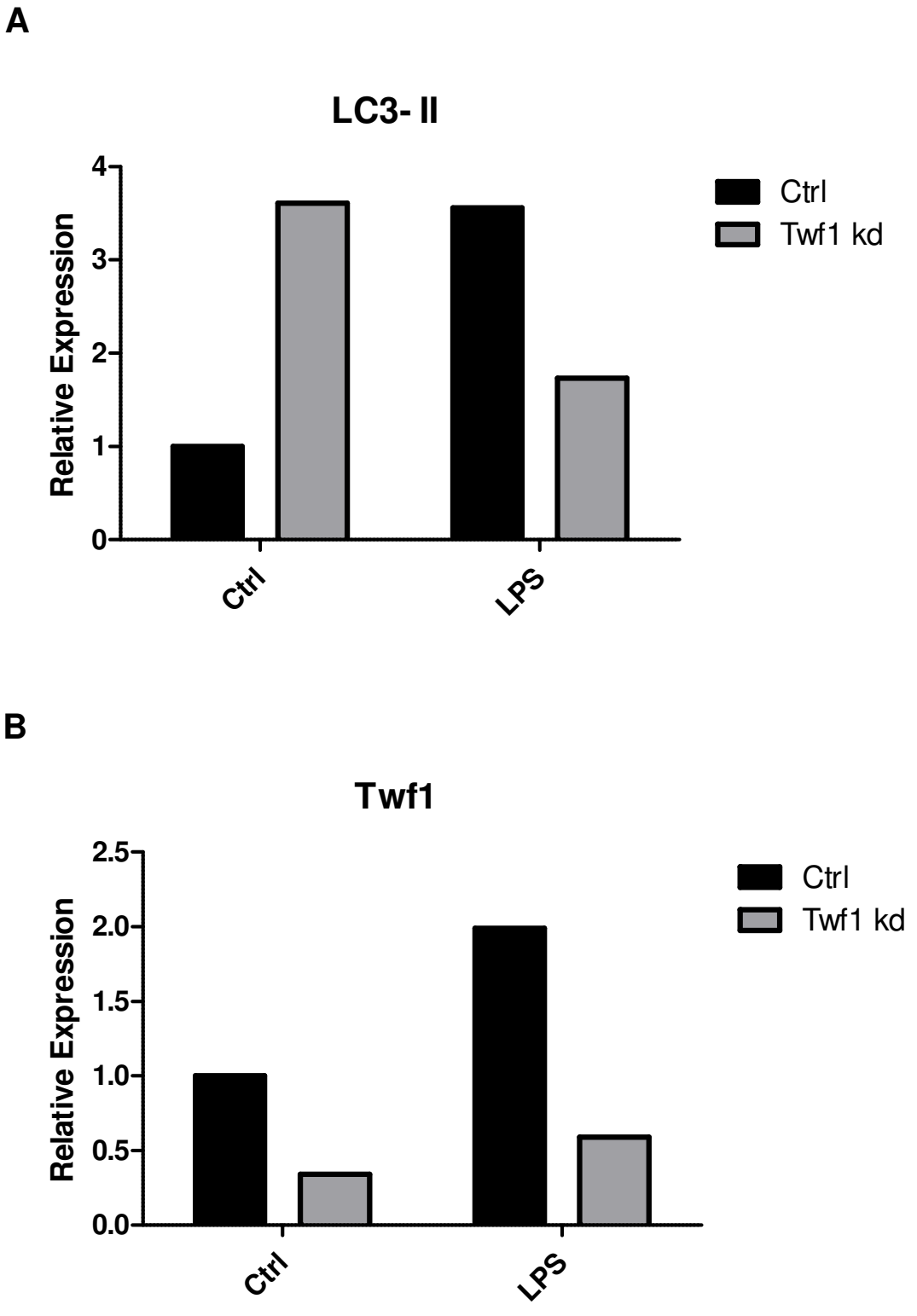

Figure 16. **Relation between Twf1/autophagy/senescence.** (A) The expression level of the autophagy marker LC3-II was evaluated in Twf1 knocked-down cholangiocytes and in control cells, either or not exposed with persistent LPS stimulus (senescence induction) (B)Twf1 expression was induced in LPS-treated cholangiocytes.

To better characterize the dynamics of autophagy in conditions of senescence, the conversion of LC3-I to LC3-II and the autophagy markers ATG 13 and ATG101 were evaluated by Western blotting. The ratio conversion of LC3-II seems to undergo no significant changes, this could be because cells may have already entered senescence -Figure 17A. Furthermore, protein levels of ATG 13 and ATG101, involved in the initiation complex (section 1.1.1), suggest us that autophagy does not appear to be activated - Figure 17 B and 17C. The lack of Twf1, could dampen the activation of autophagy, facilitating senescence instead.

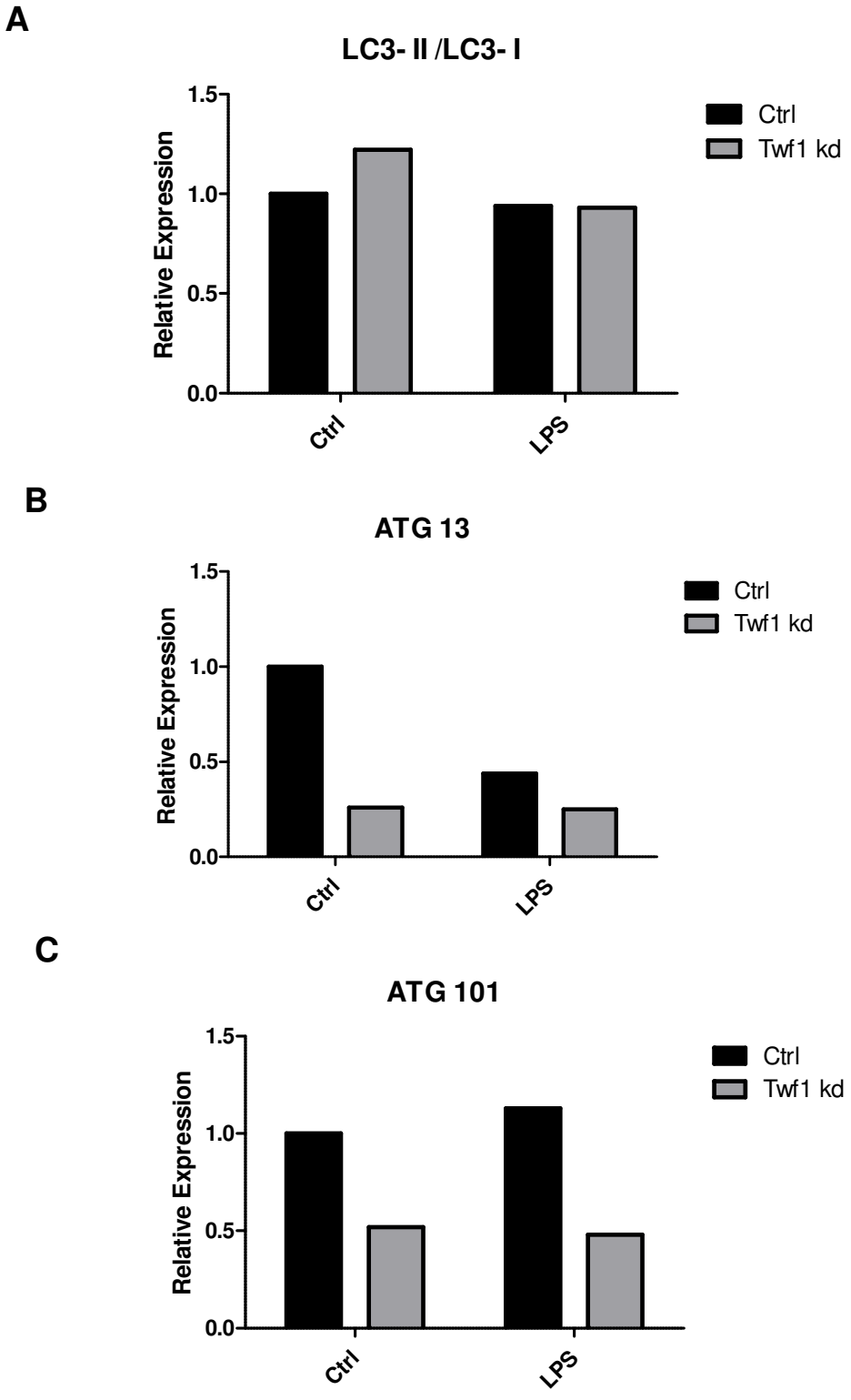

Figure 17. **Autophagy evaluation with respect to senescence induction** (A) The expression levels of the autophagy marker LC3-II was evaluated in Twf1 knocked-down cholangiocytes and in control cells, either or not exposed with persistent LPS stimulus (senescence induction) (B) (C) ATG13 and ATG101 expression, involved in the initiation of autophagy, were decreased in LPS-treated cholangiocytes.

5. DISCUSSION

In the current study it was possible to evaluate that:

- Twf1 modulates cholangiocyte pathobiology by preventing senescence establishment.
- Autophagy and senescence are therefore distinct types of stress response and their signaling pathways seems to overlap in deciding cell fate.
- Twf1 may play a role in the early stages of cell fate before the cell decides which pathway to take.

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

Originally described in yeast, autophagy is an evolutionary conserved pathway essential for cellular homeostasis and energy balance. Although it is commonly seen as a cytoplasmic event, recent studies have unveiled a transcriptional and epigenetic network that regulates autophagy [11]. Furthermore, dysfunction of this process contributes to the pathologies of many human diseases [13], including cancer, metabolic and neurodegenerative disorders as well as cardiovascular and pulmonary diseases.

Autophagy plays a critical role in the regulation of liver physiology and the balancing of liver metabolism. In conditions of food deprivation (starvation), hepatic autophagy activation provides nutrients via degradation of intracellular materials [29]. A growing body of evidence has shown that liver autophagy contributes to basic hepatic functions, including glycogenolysis, gluconeogenesis and β-oxidation, through selective

turnover of specific cargos controlled by a series of transcription factors. Dysregulation of liver autophagy has been described in severe metabolic disorders such as obesity, hepatic steatosis and diabetes [57]. Yet, it is still unclear whether autophagy favours or prevents the progression of liver injury.

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. In physiological conditions cholangiocytes are mitotically dormant, until biliary tree damage occurs. In response to exogenous/endogenous stimuli, cholangiocytes actively participates in inflammatory and reparative processes within the liver. The mechanisms by which cholestasis induces liver damage require further investigation, but they include at least mitochondrial dysfunction (hence oxidative stress) [109], unbalanced apoptosis, and necrosis [110], which can lead to liver fibrosis [111], and organelle (mainly ER) stress [112]. As noted above, such cellular damage induces an adaptive response that includes autophagy activation. Impaired autophagy, reflected by increased levels of LC3 and p62, has been described in cholestatic liver diseases including PSC and genetic cholestasis [125]. Despite the evidence for a possible protective role of autophagy stimulation in cholestasis, no strategies aimed at its induction have yet been tested in cholangiopathy patients.

The aging of the population, the increased prevalence of chronic liver disease in elderly and the need to broaden the list of potential liver donors suggest to us to better understand the molecular mechanism of the ageing liver [192]. Emerging evidence shows that modulating autophagy seems to be effective in improving the age-related alterations of the liver. These alterations not only impair the metabolic capacity of the

liver but also represent important factors in the in the pathogenesis of malignant liver disease [193].

As well as other cell types, cholangiocytes cytoplasm, possesses 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 [92]. TWF1 is a monomer-actin binding protein highly conserved from yeast to mammals which is involved in motile function and cytoskeleton remodeling [221]. It is localized to cortical G-actin rich-structures, and it is probably regulated by RAC1, a small GTPase. TWF1 can bind sever actin filaments, thus promoting actin structures remodeling and turnover [222,223]. Senescence can be triggered by a series of insults (e.g., telomere shortening, genomic damage, mitogenic signals, epigenomic damage and other forms of stress) which can activate different pathways with tumor suppressor activity, such as p16 ^{INK4a} and p53. From the cellular point of view, the senescent cell is in a state of irreversible growth arrest in the G_1 phase of the cell cycle, metabolically active but with no longer proliferative activity [151]. Morphologically the changes occur in cell volume, cytoskeleton organization and subcellular organelles such as lysosomes, mitochondria, and nuclei [224]. In conjunction with other parts of the cytoskeleton including intermediate filaments and microtubules, actin cytoskeleton is involved not only in regulating the cell shape and mechanical resistance to deformation but is responsible for mediation of signaling pathways involved in ageing and cell death [225]. In this manner actin cytoskeleton further being a possible mediator of aging, seems to be involved in several diseases from cancer to neurodegeneration [226,227]. 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 cholangiocytes physiologic functions [216]. 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 6A). However, no differences have been observed in p21 expression (Figure 6A). Along with the upregulation of genes involved in cell cycle arrest, senescent cell acquires 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) [228]. 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 7A). According to mRNA data, Il-1a secretion is increased in cholangiocytes lacking for Twf1 expression (Figure 7B). 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 6C). 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 8A-B). Since emerging evidence shows that modulating autophagy seems to be effective in improving the age-related alterations of the liver [229], we decided to evaluate whether autophagy plays a role in regulating cellular aging processes at the level of the biliary epithelium. Autophagy is activated *in vitro*, in

cholangiocyte cells due to the action of the inductors Everolimus and Rapamycin (Figure 9). The expression level of the senescence marker p53, undergoes a decline in cholangiocyte cells after treatment with Everolimus during 12-24h; these data corroborate the hypothesis that autophagy and senescence overlap, in deciding cell fate (Figure 10). To fully understand autophagy, we perform experiments to modulate the activity of autophagy itself. Chloroquine (CQ) inhibits the fusion of the autophagosome to the lysosome, thus allowing the quantization of autophagosome formation as a measure of the degree of autophagy by stopping the autophagic flow before lysosomal degradation can occur. Furthermore, p62 (Sequestosome 1), another important marker for a correct evaluation of autophagic flow, was also taken into consideration. Autophagy induction was further confirmed in cholangiocyte cells, after pre-treatment with the autophagic inhibitor CQ (Figure 11). These data were also confirmed with evaluation of autophagy by fluorescence microscopy (Figure 12). Since mounting evidence indicates that actin dynamics and membrane-cytoskeleton scaffolds also play essential roles in macroautophagy [230], and based on earlier findings, possible Twf1 expression changes were evaluated, in order to understand whether Twf1 regulates cellular senescence at the level of the biliary epithelium, during autophagy induction. *In vitro***,** the most significant changes of Twf1 were with treatment of Everolimus (Figure 13). To investigate whether Twf1 plays a role during autophagic flow, cholangiocyte cells were treated with CQ. The expression of Twf1 increases with autophagic induction, with respect to cells pretreated with CQ, when autophagy flux is blocked (Figure 14A). The senescence marker p53 had a parallel decrease in conversion to an increase of Twf1 (Figure 14B). These overall findings suggest that Twf1 may have a marginal role in autophagy and senescence processes.

During this time range, autophagy and senescence pathways could overlap in deciding cell fate. Ultimately, to understand the possible role of Twf1 in the modulation of autophagy/senescence, cells were treated with LPS stimulus. Autophagy seems to be induced as evidenced by an increase in LC3-II mRNA level (Figure 16A). Stressed cells could activate autophagy, to prevent damage and maintain metabolism though lysosomal turnover of cellular components. Since the main goal is the analysis of the autophagy flux by Western Blot, we evaluated the conversion of LC3-I to LC3-II and the autophagy markers ATG 13 and ATG101. The ratio conversion of LC3-II seems to undergo no significant changes, possibly because cells may have already entered senescence (Figure 17A). Furthermore, protein levels of ATG 13 and ATG101, involved in the initiation complex, suggest us that autophagy does not appear to be activated (Figure 17 B-C). The lack of Twf1, could dampen the activation of autophagy, facilitating senescence instead.

Research techniques for measuring autophagy or correlating autophagy with disease status often rely on capturing a snapshot of autophagy regulatory protein expression. Because most autophagy regulatory proteins are not specific for autophagy, multiple biomarkers need to be considered together. Moreover, it is not currently convenient to monitor autophagic flux in clinical samples. Challenges and opportunities remain to identify patients most likely to benefit from this approach[231,232].

6. CONCLUSION

Apoptosis, autophagy, and cellular senescence are distinct cellular responses to stress. Autophagy can facilitate senescence or limit damage and delay apoptosis to allow recovery and repair on normal cell function [217]. Cholestatic liver disease is characterized by a dysregulation of autophagy activity. The use of autophagy modulators (inductors / inhibitors) combined with pharmacological agents appears to be a promising strategy to treat a variety of cholestatic conditions. In this settings, Twf1 modulation of cholangiocyte biology (increase of cholangiocyte proliferation and reducing of senescence phenotype establishment) may play a relevant role when deciding cell fate between autophagy and senescence. Nevertheless, some recent studies have revealed the importance of cytoskeletal elements such as actin microfilaments and microtubules in specific aspects of autophagy [230,233]. The clinical relevance of these findings and the breadth of any potential use of autophagy modulators should be further developed[231,232].

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