Possible application of melatonin treatment in human diseases of the biliary tract

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List of abbreviations: (AADC) Aromatic L-amino acid decarboxylase; (AANAT) Aralkylamine N-acetyltransferase; (ALT) alanine transaminase; (AKT) protein-kinase B; (AREs) antioxidant response elements; (As2O3) Arsenic trioxide; (ASMT) Acetyl serotonin O-Methyltransferase; (AST) aspartate transaminase; (ATP) adenosine triphosphate; (BDL) bile duct ligated; (cAMP) 3',5'-cyclic adenosine monophosphate; (Cd) Cadmium; (cGMP) cyclic guanosine monophosphate; (CCA) cholangiocarcinoma; (CFTR) cystic fibrosis transmembrane conductance regulator; (CNS) central nervous system; (COX2) cyclooxygenase-2; (ER) endoplasmic reticulum; (GGT) gamma-glutamyl transferase; (GSH-Px) glutathione peroxidase; (HCC) hepatocellular carcinoma; (HO-1) heme oxygenase-1; (IAP) inhibitor of apoptosis protein; (ICU) intensive care unit; (IKKα) IkB kinaseα; (IL1) interleukin-1; (IL6) interleukin-6; (IP)

intraperitoneally; (IRI) ischemia-reperfusion injury; (JNK) c-Jun N-terminal kinase; (XIAP) X-linked inhibitor of apoptosis protein; (MyD88) Myeloid differentiation factor 88; (MMP) matrix metalloproteinases; (MT1) melatonin receptor 1A; (MT2) melatonin receptor 1B; (MT3) melatonin receptor 1C; (NASH) nonalcoholic steatohepatitis; (NF-kB) nuclear factor kB; (Nrf2) Nuclear factor erythroid-related factor 2; (OCA) obeticholic acid; (PBC) primary biliary cholangitis; (PI3K) phosphatidylinositol 3-kinases; (PKA) protein kinase A; (PKC) protein kinase C; (PO) per os; (PSC) primary sclerosing cholangitis; (SAME) S-adenosyl-methionine; (SCN) suprachiasmatic nucleus; (Sirt3) sirtuin 3; (SOD) superoxide dismutase; (SR) secretin receptor; (TACE) trans arterial chemo embolization; (TIMP-1) tissue inhibitor of metalloproteases; (TLR4) toll-like receptors 4; (TNF-β) tumor necrosis factor-β; (TPOH) tryptophan hydroxylase; (UDCA) Ursodeoxycholic acid.

Summary

Melatonin was discovered in 1958 by Aaron Lerner; its name comes from the ability of melatonin to change the shape of amphibian melanophores from stellate to roundish. Starting from the 80s, the role of melatonin in the regulation of mammalian circadian and seasonal clocks has been elucidated. Presently, several other effects have been identified in different organs. For example, he beneficial effects of melatonin in models of liver damage have been described. This review gives first a general background on experimental and clinical data on the use of melatonin in liver damage. The second part of the review focuses on the findings related to the role of melatonin in biliary functions suggesting a possible use of melatonin therapy in human diseases of the biliary tree.

Melatonin synthesis and excretion

Melatonin (N-acetyl-5-methoxytryptamine) is a hormone found in animals, plants, and microbes (62). In mammals, melatonin it is synthesized from the amino acid tryptophan by the pineal gland as well as peripheral organs including the intestine and liver (72). The biosynthesis of serotonin and melatonin share some common pathways starting from tryptophan. The specific steps in melatonin synthesis, together with the corresponding enzymes involved, are summarized in Figure 1. Tryptophan hydroxylase catalyzes the rate-limiting step of serotonin synthesis, the oxidation of tryptophan to 5-hydroxy-tryptophan. Then, aromatic L-amino acid decarboxylase catalyzes the decarboxylation of 5-hydroxy-L-tryptophan to generate serotonin. The enzyme, aralkylamine N-acetyltransferase (AANAT) catalyzes the N-acetylation of serotonin to N-acetyl serotonin, the rate-limiting step in the synthesis of melatonin. Finally, the enzyme, acetyl serotonin O-Methyltransferase (ASMT), catalyzes the last reaction in melatonin synthesis. AANAT is the enzyme that regulates the circadian rhythm of melatonin synthesis by

the pineal gland (19). Indeed, the diurnal fluctuation of AANAT expression is related to the diurnal melatonin synthesis in vertebrates (67). Since the pineal storage of melatonin is undefined, melatonin serum levels are considered indices of the dynamic synthesis of melatonin by the pineal gland (64). Enhanced melatonin secretion has been demonstrated during night hours (peaking between 3-4 AM), whereas serum melatonin levels are virtually undetectable during light hours; nocturnal melatonin blood levels range between 10 and 80 mcg/night. After secretion, melatonin diffuses easily in both aqueous and lipidic phases and circulates in blood bound to albumin, for the 70% of its total amount. Circulating melatonin easily reaches all body tissues, and can cross the blood-brain-barrier as demonstrated by a positron emission tomography study (47).

Liver is the main organ involved in melatonin catabolism (90%) through the typical glucuronidation-sulfation pathways. Elimination occurs by urine either as sulfated or unchanged in small amount (26). Rhythmic melatonin synthesis is sustained by fibers located in the hypothalamus at the level of suprachiasmatic nuclei; light–dark cycle is the main determinant of melatonin cyclic secretion. Information regarding light- or dark-regulated melatonin synthesis is transmitted to the hypothalamus by the retino-hypothalamic fibers. In support of this view, the exposure to significant artificial light during darkness has been demonstrated to reduce or completely abolish the secretion of melatonin according to the intensity of the illumination. The dark–light cycle of melatonin may stimulate other region of the central nervous system (CNS) giving information on the 24 hours shift. Melatonin synthesis has been also demonstrated in several extrapineal sites including retina, brain, skin, and the gastrointestinal tract including the liver (1, 65, 75).

General melatonin physiologic effects

Melatonin regulates several seasonal endocrine and reproductive activities in mammals. However, at least in humans, the main function of this hormone is associated with the regulation of circadian clock with a direct link with the CNS (27). Two subtypes of melatonin receptors have been identified and cloned in mammalian tissues (MT1 and MT2) (21), whereas an MT3 receptor has been identified in amphibian (70). Melatonin receptors have been identified in the nervous (21) and vascular tissue (22), liver (34, 66, 75) and lung (58). Receptor expression seems to be regulated by circulating melatonin levels and circadian cycle (32). In humans, melatonin is beneficial in several sleep disorders including difficult sleep initiation (69), shift work sleep impairment (9) as well as blindness (7). Also, reduced circulating levels of melatonin have been demonstrated in patients undergoing pinealectomy (49). Nevertheless, patients undergoing this type of surgery did not show different sleep impairment from individuals undergoing craniotomy. A “pinealoprive syndrome” has been linked to headache, mood and vision disorders or even seizures in the affected subjects (18).

Melatonin effects during liver damage

Several beneficial effects of melatonin have been described during liver injury. For a complete review on the intracellular mechanisms related to melatonin functions see reference (78). In brief, as described in the following sub-paragraphs, the positive effects of melatonin have been observed in ischemia-reperfusion injury (IRI), non-alcoholic steatohepatitis (NASH), alcoholic steatohepatitis as well as in liver cancer models. The favorable properties of melatonin have been related to: (i) inhibition of liver cells death by necrosis or apoptosis (44); (ii) direct antioxidant actions (20); and (iii) attenuation of mitochondrial damage (33).

a) Melatonin and IRI

With regard to IRI, melatonin administration has been shown to be beneficial in several organs (54). Melatonin exerts favorable effect against mitochondrial dysfunction, which is an important regulator in the onset of IRI. In fact, free radicals generated by IRI are responsible for respiratory chain damage and determine leakage of polar charge and enzymes by mitochondria, thus maintaining an extensive production of free radicals (81). In this setting, melatonin exerts a free-radical scavenger activity up-regulating the expression of the antioxidative enzymes catalase, glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD) (25). The positive effects of melatonin on mitochondrial functions were also observed in the liver. In this study (60), rats were submitted to vascular clamp of liver vessels for 70 minutes (ischemia time) followed by 2 hours of relapse of clamping (reperfusion time). In the treatment group, melatonin was administered (10 mg/kg BW, IP) 15 minutes before clamping and at reperfusion. By evaluation of isolated mitochondria, melatonin maintained an adequate ATP synthesis reducing lipid peroxidation (after IRI) to values similar to those of control values. At the ultra-structural level, mitochondria coming from IRI rats were swollen with loss of cristae, whereas several mitochondria with a normal structure were seen in the melatonin-treated animals. With regard to the specific mechanisms associated with melatonin attenuation of liver IRI, one study in rodents demonstrated the role of c-Jun N-terminal kinase (JNK) and IkB kinase alpha (IKKα) (50). These signaling pathways were activated, in the course of IRI, by stimulation of tumor necrosis factor-α (TNF-α) hepatocyte receptor. In this setting, the increased activity of JNK and IKKα was reduced by melatonin treatment. Further studies (39, 41) linked enhanced toll-like receptors 4 (TLR4) expression (due to stress condition such as infection, inflammation and ischemia) to melatonin effects on a similar model of liver IRI. TLR4 regulates IRI-induced liver damage trough myeloid differentiation factor 88 (MyD88) dependent downstream activation of nuclear factor kB (NF-kB) and consequent release of pro-inflammatory cytokines. Melatonin administration (10 mg/kg BW, IP) inhibited TLR4 expression enhancing the activity of heme

oxygenase (HO) 1. Supporting this mechanism, the HO1 inhibitor, Zinc protoporphyrin, reversed the beneficial effect of melatonin, suggesting a HO1-dependent melatonin modulation of TLR4.

A study has evaluated the effects of melatonin in the setting of human liver IRI (15). Specifically, since liver damage is characterized by delayed apoptosis of neutrophils (38), the possible effects of melatonin on neutrophils from resected livers was evaluated. In this study (by evaluation of apoptosis by flow-cytometric assessment of DNA breaks), melatonin restored a normal level of apoptosis in neutrophils, thus suggesting a reduced neutrophilic activity as a possible mechanism for reducing IRI damage in humans. On the basis of these findings, a double-blind clinical study evaluated the possible clinical effects of a single dose of melatonin (50 mg /Kg BW) on outcome after major liver resection in humans (59). The study lacked mechanistic insights, but demonstrated: (i) complete safety and good absorption for the administered dose; and (ii) a trend for improved liver enzymes, reduced intensive care unit and hospitalization. These finding, however, did not reach the statistical significance likely due to the limited number of patients included in the trial (n=50).

2) Melatonin and NASH

NASH is defined in humans as the occurrence of more than 5% of hepatic steatosis in association with liver inflammation and hepatocyte injury in the absence of other known causes of liver diseases or alcohol abuse (12). This condition, frequently related to metabolic alterations including diabetes and obesity, is gaining importance as a cause of cirrhosis in humans and is becoming one of the major indications for liver transplantation in USA (73). Melatonin effects were studied in two rat models of NASH. The first study evaluated the effect of melatonin (2.5, 5 or 10 mg/Kg BW daily, IP) in animals on high fat (10% fat) diet (61), and demonstrated reduced levels of cholesterol and triglycerides in liver homogenates of melatonin-treated animals compared to controls. In addition, the levels of the antioxidant enzymes SOD and GSH-Px were

192 increased by melatonin treatment. By H&E staining, melatonin treatment (5 or 10 mg/kg BW)
193 decreased liver steatosis in comparison with control animals (animals with severe steatosis: 0%
194 with melatonin vs 60% control; $p<0.01$). In the second study, NASH was induced in rats by a
195 methionine- and choline-deficient diet (71). Melatonin, that was administered at higher dose (50
196 mg/kg BW/daily compared to the aforementioned study) (61): (i) significantly reduced serum
197 levels of liver enzymes and inflammatory cytokines [Interleukin-1(IL-1)β, Interleukin-6 (IL-6) and
198 Tumor Necrosis Factor-β, (TNF-β)]; (ii) restored the concentration of glutathione and superoxide
199 dismutase at values similar to those observed in rats under normal diet; and (iii) reduced cellular
200 apoptosis evaluated by DNA fragmentation on liver sections. Other studies (11, 30, 31)
201 described the treatment of NASH patients with melatonin and demonstrated that: (i) 5 mg
202 melatonin tablet twice a day was safe; (ii) melatonin administration, coupled with a physical and
203 dietary treatment, reduced aspartate transaminase (AST), alanine transaminase (ALT) and
204 gamma-glutamyl transferase (GGT) levels compared to diet and physical exercise only; and (iii)
205 melatonin treatment decreased the serum levels of the inflammatory cytokines, IL1, IL6 and
206 TNF-β after 14 months of treatment. In addition, melatonin improved some metabolic
207 parameters such as insulin, adiponectin and leptin blood levels (29). However, all of these
208 studies, even if they represent an encouraging advancement, did not show a marked effect of
209 melatonin on the parameters observed. For instance: (i) the number of patients displaying
210 normal liver transaminases accounted only for 10-20% of the total number of patients; (ii) the
211 decrease of inflammatory cytokines was not corroborated by histological parameters of liver
212 inflammation; and (iii) changes in metabolic parameters were not evaluated in long-term
213 treatment. Furthermore, all studies related to the potential therapeutic effects of melatonin on
214 NASH in rodents and humans did not pinpoint the possible molecular mechanisms by which
215 melatonin protects against NASH, but rather only focused on the general antioxidant and
216 cytoprotective properties of melatonin in this setting.

*3) **Melatonin and toxic hepatitis***

The potential therapeutic effects of melatonin on the prevention of toxin-related and sepsis-mediated hepatic damage are summarized in a recent review article (23). In a rat model of liver injury induced by arsenic trioxide (As^2O^3) melatonin ameliorated liver inflammation and serum chemistry, effects that were associated with enhanced nuclear factor erythroid-related factor 2 (Nrf2) and OH1 (member of antioxidant response elements, AREs) expression through activation of phosphatidylinositol 3-kinases/protein kinase B (PI3K/AKT) pathway (79). Another study has also demonstrated the protective role of melatonin against cadmium (Cd)-induced hepatotoxicity in the tumor cell line, Hep-G2 cells (63). The study focused on the inhibitory role of melatonin on mitochondrial-derived O2-stimulated autophagic cell death, which was enhanced during Cd-induced liver toxicity through a transduction pathway involving the Sirtuin3-SOD2-mROS axis. The limitation of this study is based on the use of Hep-G2 cells rather than normal hepatocytes. A clinical study evaluated the possible role of melatonin in toxic hepatitis in humans, enrolling subjects with statin-induced liver damage (17). In this study, 60 subjects (with increased serum liver enzymes) who underwent therapy with statins were randomized to placebo or melatonin (5 mg twice a day for 6 months) treatment. After six months of melatonin treatment liver enzyme levels were decreased (nearly a 40% decrease in comparison with baseline values, 25% normalized their levels versus no-change in controls), suggesting a protective effect of melatonin for toxic-induced human liver damage.

*4) **Melatonin and alcoholic hepatitis***

Melatonin effects have been assessed in experimental model of alcoholic hepatitis. For example, mice fed a liquid diet with ethanol (5%) developed liver injury characterized by increased liver enzymes as well as hepatic steatosis, necrosis and inflammation. In this setting,

melatonin (from 5 to 20 mg/kg BW daily by gavage) improved liver damage decreasing the total hepatic content of triglycerides and levels of inflammatory cytokines such as TNF, IL6 and IL1-β (37). In another model, alcoholic hepatitis was studied as a function of matrix metalloproteinases (MMPs) activity. MMPs are responsible for the degradation of extracellular matrix protein during damage, as MMP-9 over-expression has been advocated as a possible mechanism of damage in alcoholic hepatitis (43). In rats, the administration of ethanol (a variable dose 2-8 ml/Kg BW, 50% ethanol twice a day, for three days), increased: (i) MMP-9 activity (eightfold increase in liver tissue and fivefold increase in serum at the maximum ethanol exposure); and (ii) the levels of the pro-inflammatory cytokines, TNF-α, IL1-β and IL6, compared to control-treated rats. When rats were treated with melatonin (15 mg/kg BW IP, twice a day for three days) before the induction of alcoholic damage, the levels of MMP-9 (in both liver and serum) and metallopeptidase Inhibitor 1 (that was decreased in rats exposed to alcohol) returned to values similar to that of control rats (56).

*5) **Melatonin and hepatocellular carcinoma***

The protective effects of melatonin on hepatocellular carcinoma (HCC) are summarized in a recent review (57). HCC progression is in part related to the capacity of tumoral cells to inhibit apoptosis through the synthesis of specific inhibitors of Apoptosis Proteins (IAPs) (24), that downregulate the activity of caspases, specialized proteases required for cellular apoptosis. Human HCC tissue specimens displayed enhanced immunoreactivity of the IAPs, XIAP, cIAP-1, cIAP-2, and survivin, suggesting that these moieties may play a role in the survival of tumor cells. In neoplastic HepG2 and SMMC-7721 cell lines (treated *in vitro* with melatonin, 10^{-3}-10^{-5} mol/L) there was enhanced endoplasmic reticulum stress-induced apoptosis, whereas the protein expression of XIAP and survivin significantly decreased compared to control cells (77).

In a clinical study focusing on the treatment with IL 2 (3 million UI/daily, subcutaneously for 4 weeks) plus melatonin (50 mg/day, oral administration, starting 1 week before IL 2) for advanced gastro-intestinal cancers, six subjects underwent the treatment for HCC (52). This type of tumor was the one showing the best complete response rate (17%) having one subject with complete healing from the neoplasm after treatment. In another study (76), one hundred patients with unresectable HCC, were randomly assigned to receive treatment with Trans Arterial Chemo Embolization (TACE) or TACE plus melatonin (20 mg/day in the 7 days before procedure). The results demonstrated improved survival (3 years survival TACE vs. TACE + melatonin = 26% vs. 40%, $p<0.05$); and reduced liver damage after the procedure as demonstrated by the decrease of liver enzymes in the melatonin group after TACE). On the basis of these results on HCC and other hepatic diseases, a recent review suggested melatonin diet supplementation as a good strategy to prevent and treat liver diseases (8). Despite the fact that few clinical data are available, the anti-oxidant and mitochondria-preserving effects of melatonin would be considered a potential therapeutic approach for managing human liver diseases. Large scale, well designed, randomized trials are needed to validate this hypothesis. Main results and references regarding studies on melatonin and liver injury are reported in Table 1. Figure 2 summarizes some mechanisms of melatonin positive effect in different models of liver injury as described in the previous paragraphs.

Melatonin effects on cholangiocytes and its possible therapeutic use in human cholangiopathies

The biliary epithelium is lined by cholangiocytes of different sizes and functions. Studies on rodents identified different subpopulations of cholangiocytes: small (mean diameter ~8.4 μm) and large (mean diameter ~14.5 μm) lining small and larger bile ducts, respectively (3). The

291 difference in cholangiocyte size is closely related to functionality diversity, since large 3',5'-cyclic
292 adenosine monophosphate (cAMP)-dependent cholangiocytes are more differentiated and
293 participate in the secretion of water and bicarbonate (3), whereas small, undifferentiated inositol
294 trisphosphate/Ca^{2+}-cholangiocytes are considered a quiescent, progenitor subpopulation (more
295 resistant to liver injury) that differentiate into large cholangiocytes when these cells are
296 damaged (48). In normal conditions, the most important function of large cholangiocytes is to
297 support the so-called bile acid-independent bile flow, which occurs through the interaction of
298 secretin with a specific secretin receptor (SR, expressed only by cholangiocytes) (5) located on
299 the basolateral membrane of cholangiocytes (2). The interaction of secretin with SR induces an
300 increase in intracellular cAMP levels, activation of cystic fibrosis transmembrane conductance
301 regulator (CFTR) with extracellular extrusion of Cl^- and subsequently activation of the Cl^-/HCO_3^-
302 exchanger AE2 stimulating a bicarbonate-rich choleresis while Cl^- is recovered back in
303 cholangiocytes (2).
304 Extensive examination of the molecular mechanisms related to the interplay between melatonin
305 and the biliary epithelium is summarized in a recent review (28). In an early study, the authors
306 compared the effects of S-adenosyl-methionine (SAME, 10 mg/kg BW/day) or melatonin (750
307 μg/kg BW/day) administration (both IP for 10 days) on liver functions in the cholestatic model of
308 bile-duct ligation (BDL) (53). The antioxidants and hepatoprotective effects of melatonin were
309 superior to those of SAME since melatonin decreased the levels of liver enzymes and
310 malondialdehyde and glutathione at higher rate compared to SAME, levels that were similar to
311 those of sham-operated control rats. We recently extended these findings demonstrating the
312 expression of melatonin receptor subtypes (MT1 and MT2) in bile ducts (by
313 immunohistochemistry in liver sections) and isolated cholangiocytes (by real-time PCR and
314 FACS analysis), with MT1 expression that increased in cholestatic rodents (66). Treatment of
315 BDL rats with melatonin in drinking water (20 mg/L, estimated assumption 2 mg/g BW per day)

316 decreased ductular reaction (DR), serum bilirubin and transaminases levels, the expression of
317 clock genes, cAMP levels, and protein kinase A (PKA) phosphorylation in cholangiocytes by
318 interaction with MT1 (66).
319 In another study we examined the: (i) expression of AANAT (the limiting-step enzyme for
320 melatonin synthesis) in normal and proliferating cholangiocytes; and (ii) the effects of AANAT
321 biliary downregulation on changes in DR and ductal secretory activity (65). By
322 immunohistochemistry in liver sections and real-time PCR in isolated cholangiocytes, the study
323 demonstrated the immunoreactivity/expression of AANAT and secretion of melatonin in
324 cholangiocytes, parameters that increased following BDL; minimal expression of AANAT was
325 detected in hepatocytes (65). Downregulation of biliary AANAT (by administration of In Vivo
326 AANAT Morpholino) was associated with enhanced DR in liver sections and increased SR,
327 CFTR, and Cl^-/HCO_3^- AE2 expression (65), functional indices of biliary hyperplasia (4, 5).
328 In a recent study, the role of the melatonin brain-liver axis was evaluated by exposing
329 cholestatic BDL rats and $Mdr2^{-/-}$ mice to total darkness, a condition that increases melatonin
330 secretion from pineal gland (36, 75). When BDL rats were exposed to complete dark for 1 wk,
331 there was: (i) enhanced expression of AANAT in the pineal gland and melatonin serum levels;
332 (ii) improved liver morphology, serum levels of liver enzymes and reduced DR; and (iii)
333 decreased deposition of collagen as well as biliary expression of the clock genes, PER1,
334 BMAL1, CLOCK, and Cry1 (36). When $Mdr2^{-/-}$ mice [that mimic some of the features of human
335 primary sclerosing cholangitis (PSC)] were exposed to total darkness for 1 wk, there were
336 higher serum melatonin levels and reduced DR, collagen deposition and angiogenesis
337 compared to $Mdr2^{-/-}$ mice exposed to 12:12 hr dark:light cycles (75). The study also
338 demonstrated enhanced expression of miR-200b in both $Mdr2^{-/-}$ mice and human PSC samples,
339 expression that was reduced in $Mdr2^{-/-}$ mice subjected to dark exposure or melatonin treatment
340 (75). Also, by *in vivo* and *in vitro* downregulation of miR-200b in $Mdr2^{-/-}$ mice and human biliary

cells, respectively, there was reduced DR, collagen deposition and angiogenesis in liver sections from Mdr2$^{-/-}$ mice and angiogenesis and fibrosis mRNA expression in biliary lines (75). The role of melatonin brain-liver axis was further evaluated in BDL cholestatic rats undergoing pinealectomy or prolonged light exposure, maneuvers that reduce melatonin secretion from pineal gland as well as peripheral organs (16). In BDL rats plus pinealectomy or prolonged light exposure, there were increased levels of liver enzymes serum chemistry, ductular reaction, biliary senescence, liver fibrosis, inflammation, angiogenesis, ROS generation and expression of miR-200b (that is increased in cholestatic cholangiocytes) compared to BDL rats exposed to 12 hr:12 hr light/dark cycles (16). Another study (55) has shown that intracerebroventricular (ICV) infusion of melatonin to BDL rats reduces ductular reaction and liver fibrosis through inhibition of expression/secretion of hypothalamic gonadotropin-releasing hormone release (GnRH) from cholangiocytes and reduced expression of its receptor (GnRHR); the GnRH/GnRHR axis has been shown to stimulate biliary proliferation and liver fibrosis (45). Enhanced bioavailability of melatonin in the brain may improve the outcome of cholestatic liver diseases. Main results and references of studies regarding melatonin and the biliary tree are summarized in Table 2. A chronological timeline of the major discoveries with regard to melatonin effect on the biliary tract is depicted in Figure 2.

Cholangiocytes are the target of cholangiopathies including Primary biliary cholangitis (PBC), PSC and cholangiocarcinoma (CCA), diseases that characterized by biliary damage/senescence, liver inflammation and fibrosis (13, 51). Currently, several therapeutic options are evaluated for these diseases including immunologic approach or use of stem cells (for details we refer to a recent review) (14); however, new therapeutic approaches are needed since these cholangiopathies still represent an important cause of liver decompensation and death. A number of experimental studies demonstrated that melatonin might be beneficial for the management of chronic cholestatic liver diseases as it: (i) regulates biliary homeostasis; and

366 (ii) decreases collagen deposition in the liver. In both PBC and PSC, biliary proliferative activity
367 is important for disease progression. In fact the increase in the number of bile ducts (the so
368 called ductular reaction, DR) is a common finding in these diseases (68). DR in PBC and PSC
369 is characterized by functionally ineffective, truncated, bile ducts expanding through portal areas
370 in the parenchymal region (6). A recent study in human liver sections comparatively evaluated
371 DR in patients with PBC or PSC (10). In both diseases there was a statistically significant linear
372 relationship between DR and extent of fibrosis with a correlation coefficient of 0.6 ($p<0.01$). The
373 concept that changes in DR regulate the activation of hepatic stellate cells and liver fibrosis is
374 supported by several studies (42, 68, 80). Downregulation of DR (for example by blockage of
375 the secretin/SR axis) was associated with reduced liver fibrosis mediated by decreased
376 secretion of biliary senescence-associated secretory phenotypes (SASPs such as TGF-β1),
377 factors that activate hepatic stellate cells (42, 80); up-regulation of the secretin/SR/TGF-β1 axis
378 was also seen in liver sections of PSC patients (75). Another study has shown that BDL-
379 induced increases in: (i) serum enzyme levels; (ii) liver inflammation and ROS levels; (iii) DR
380 and liver fibrosis; and (iv) liver angiogenesis were exacerbated by both pinealectomy and
381 prolonged exposure to light, maneuvers that decrease melatonin levels (16). The effects of
382 pinealectomy and prolonged exposure to light on these phenotypes were associated with: (i)
383 enhanced expression of TGF-β1 and biliary senescence (42, 74); and (ii) increased expression
384 of the clock genes, CLOCK, ARNTL, Cry1, and Per1, and miR-200b, which were reduced by the
385 administration of melatonin. From these findings, it is apparent that melatonin is able to
386 coordinately modulate key phenotypes of cholangiopathies such as liver inflammation, DR,
387 cellular senescence and liver fibrosis.
388 There is growing information with regard the role of melatonin in the growth of CCA (34, 35). A
389 recent study demonstrated the antiproliferative effects of melatonin on CCA using six different
390 CCA cell lines (Mz-ChA-1, HuH-28, TFK-1, CCLP1, SG231, and HUCC-T1, and the normal

391 human cholangiocyte line, H69) and male BALB/c nude mice with CCA established by injection
392 of Mz-ChA-1 cells (34). The study demonstrated an autocrine loop by which the
393 ASMT/AANAT/melatonin/MT1 axis inhibits CCA growth. Specifically, there was decreased
394 immunoreactivity (in sections from liver biopsies) and expression (by FACS analysis and real-
395 time PCR) of ASMT/AANAT but enhanced MT1 expression in CCA tissue/lines compare to
396 normal controls; there was decreased melatonin bile (but not serum) levels in human samples.
397 In the same study, in BALB/c nude mice (with established CCA) treated for 34 days with
398 melatonin (4 mg/kg BW, IP daily injections) there was a significant decrease of tumor volume
399 that was coupled with enhanced number of apoptotic cholangiocytes. Another study
400 demonstrated that the rhythmic expression of core clock genes (modulated by melatonin) was
401 disrupted in CCA cell lines, since a marked decreased of the clock gene, Per1, was observed in
402 liver biopsies and human CCA lines (35). Overexpression of Per1 was coupled with decreased
403 CCA proliferation but enhanced biliary apoptosis both *in vitro* in CCA cell lines and *in vivo* in
404 athymic mice. The study also demonstrated that Per1 is a target of miR-34a, since inhibition of
405 miR-34a (overexpressed in CCA) reduced the proliferation and invasiveness of CCA cells
406 compared to normal controls (35). Another study evaluated the effect of melatonin on the
407 apoptosis of the human CCA cell lines, KKU-M055 and KKU-M214, that were treated with
408 melatonin (0.5-2 mM for 48 h). In these CCA cell lines, melatonin inhibited cell viability,
409 increased intracellular ROS levels leading to increased oxidative DNA damage and 8-oxodG
410 formation (46). The main biological determinants of human biliary disease are reported in
411 Figure 3 together with the possible melatonin modulatory effects.
412
413 **Conclusions**
414
415 Several important effects of melatonin have been identified in the last decades. Original studies
416 focusing on beneficial effect on sleep disorder have been implemented by the observation that
417 melatonin is favorable in several pathological conditions involving different organs. Regarding

418 the liver, melatonin determines improvement in several experimental model of damage. Positive
419 effects are observed after toxic, ischemic and oxidative hepatic injury. Our knowledge on
420 melatonin is now extending on its interaction with specific cells within the liver such as
421 cholangiocytes. These cells are the main target of cholestatic chronic or neoplastic liver
422 diseases in human, affections that still are in waiting for a conclusive therapy. Experimental
423 results demonstrate advantages in the use of melatonin in model of damage involving the biliary
424 tree, allowing to speculate on the possible application of this hormone in human therapy. Well-
425 designed clinical studies in the future will address this issue.
426

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Figure legends

Figure 1 Synthesis of melatonin from tryptophan. The specific steps are reported together with the corresponding enzymes (black frames) involved.

Figure 2 Mechanisms of melatonin positive effect in different models of liver injury. A: Melatonin counteracts IRI damage both decreasing the activity of INK and IKKα and by direct repression of TLR4 synthesis. B: Melatonin reduces As2O3 toxicity stimulating of PI3K/AKT pathway and protects against Cd injury trough SOD2 removal of mROS. C: Alcohol/MMP-9 mediated cell damage is reversed by melatonin. For details see text. Abbreviations: (As2O3) Arsenic trioxide; (AKT) protein-chinasi B; (Cd) Cadmium; (IKKα) IkB kinase alpha; (IRI) ischemia-reperfusion injury; (JNK) c-Jun N-terminal kinase; (MMP) matrix metalloproteinase; (mROS) mitochondrial reactive oxygen species; (PI3K) Phosphoinositide 3-kinases; (SOD) superoxide dismutase; (TNF) Tumor necrosis factor; (TLR) toll-like receptor.

Figure 3 Time course of experimental findings on melatonin and the biliary tree. Chronological timeline of the major discoveries with regard to melatonin effect on the biliary tract is reported. Abbreviations: (BDL) bile duct ligated; (MT1) melatonin receptor 1; (AANAT)=Aralkylamine N-acetyltransferase.

Figure 4 Biological determinants of main human bilary diseases and the corresponding findings regarding melatonin. The main biological determinants of PBC,PSC and CCA are reported together with the respective melatonin experimental data suggesting a possible therapeutic application. For details see text. Abbreviations: (AANAT) Aralkylamine N-acetyltransferase; (ASMT) Acetyl serotonin O-Methyltransferase; (CCA) cholangiocarcinoma; (PBC) primary biliary cholangitis; (PSC) primary sclerosing cholangitis;

Table 1 Experimental and clinical use of melatonin in liver injury

Type of liver damage	References	Model	Melatonin treatment (route)	Results
IRI	(60)	Rats (vessel clamp)	10 mg/kg BW (IP)	ATP synthesis maintained ↓ lipid peroxidation
	(50)	Rats (vessel clamp)	50 mg/kg BW (gavage)	↓ necrosis /inflammation ↓JNK and IKKα
	(40)	Rats (vessel clamp)	10 mg/kg BW(IP)	↓ liver enzymes ↓ TLR response
	(41)	Rats (vessel clamp)	10 mg/kg BW (IP)	↓ liver enzymes ↑HO-1 ↓TLR4-MyD88
	(59)	Human with liver resection	50 mg /kg (PO)	↓liver enzymes ↓ICU stay ↓hospital stay
NASH	(61)	Rats (high fat diet)	2.5, 5 or 10 mg/kg BW daily, IP	↓lipids accumulation ↑SOD and GSH-Px
	(71)	Rats (methionine-choline deficient diet)	50 mg/kg BW, daily (IP)	↓liver enzymes ↓inflammatory cytokines ↑SOD ↓apoptosis
	(30)	Human with NASH	10 mg/daily (PO)	↓liver enzymes
Toxic damage	(79)	Rats (As^2O^3)	20 mg/kg BW (IP)	↓histologic inflammation ↓liver enzymes ↑PI3K/AKT- Nrf2-AREs
	(63)	Hep-G2 cells (Cd)	1 μM	Regulation of autophagy by Sirt3-SOD2 pathway
	(17)	Human (statins)	5 mg twice a day (PO)	↓liver enzymes
Alcoholic	(37)	Mice (ethanol 5%	5 -20 mg/kg daily (gavage)	↓lipids accumulation ↓inflammatory cytokines ↓liver inflammation, steatosis and apoptosis
	(56)	Rats (4-16 ml/Kg BW, 3 days)	60 mg/kg (IP, 3days)	↓inflammatory cytokines ↑ TIMP-1 ↓MMP-9
HCC	(24)	HepG2 and SMMC-7721 cells	10^{-3}- 10^{-5} M	↓XIAP and Survivin ↑ apoptosis
	(77)	HepG2 cells	10^{-3} M	↓ER stress and COX2 ↑ apoptosis
	(76)	Human on TACE treatment	20 mg /day (PO, 7 days before procedure)	↓liver damage ↑ survival

Abbreviations: (AREs) antioxidant response elements; (As^2O^3) Arsenic trioxide; (AKT) protein-kinase B; (ATP) adenosine triphosphate; (Cd) Cadmium; (COX2) cyclooxygenase-2; (ER) endoplasmic reticulum; (GSH-Px) Glutathione peroxidase; (HO-1) heme oxygenase 1; (ICU) intensive care unit; (IKKα) IkB kinase alpha; (IP) intraperitoneally; (IRI) ischemia-reperfusion injury; (JNK) c-Jun N-terminal kinase; (XIAP) X-linked inhibitor of apoptosis protein; (MyD88) Myeloid differentiation factor 88; (MMP) matrix metalloproteinase; (Nrf2) Nuclear factor erythroid 2-related factor 2; (PI3K) Phosphoinositide 3-kinases; (PO) per os; (Sirt3) sirtuin 3; (SOD) superoxide dismutase; (TACE) trans arterial chemo embolization; (TIMP-1) tissue inhibitor of metalloproteinase 1; (TLR-4) toll-like receptor 4.

Table 2 Results of main experimental studies of melatonin effects on the biliary tree.

References	Model	Approaches to study melatonin effect	Results
(53)	BDL rat	IP administration 750 μg/kg/day	↓liver enzymes Preserved levels of malondialdehyde and glutathione
(66)	Normal and BDL rats	PO administration 2 mg/g daily in water	Identification of MT1 and MT2 receptor on cholangiocytes. Melatonin in BDL rat determines: ↓MT1 and MT2 expression ↓proliferation ↓secretin stimulated bile flow ↓intracellular cAMP
(65)	Normal and BDL rats	Administration 10^{-11} M on isolated cells	Identification of AANAT expression in cholangiocytes Melatonin exposure increases AANAT expression in BDL cholangiocytes Identification of melatonin synthesis by BDL cholangiocytes (autocrine loop) AANAT downregulation in BDL induces: ↑proliferation ↑secretin stimulated bile flow
(36)	Normal and BDL rats	Prolonged darkness exposure (1week)	↑ANAT and melatonin expression in pineal gland In BDL rats: ↓proliferation ↓secretin stimulated bile flow
(75)	$Mdr2^{-/-}$ mice	Prolonged darkness or PO administration 2 mg/g BW daily	↓fibrosis ↓expression fibrosis genes ↓vascular endothelial growth factor A/C ↓angiopoietin ½ Miicro RNA-200b
(16)	Normal and BDL rats	Pinealectomy or prolonged light exposure	↑fibrosis ↑proliferation ↑Micro RNA-200b

Abbreviations: (BDL) bile duct ligated; (IP) intraperitoneal; (PO) per os; (cAMP) cyclic adenosine monophosphate; (AANAT) Aralkylamine N-acetyltransferase.

Effects of melatonin in human biliary diseases

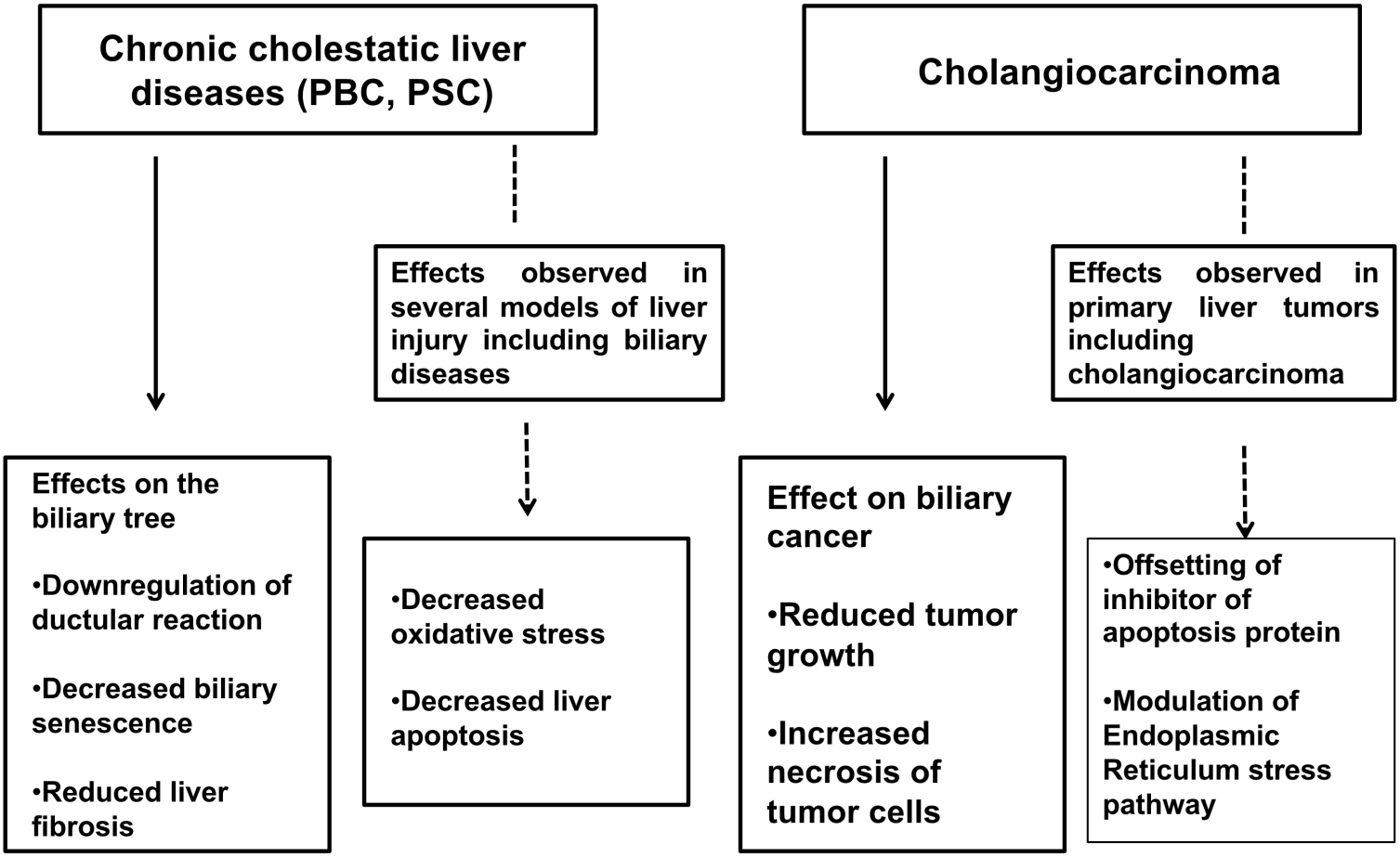


Figure 1

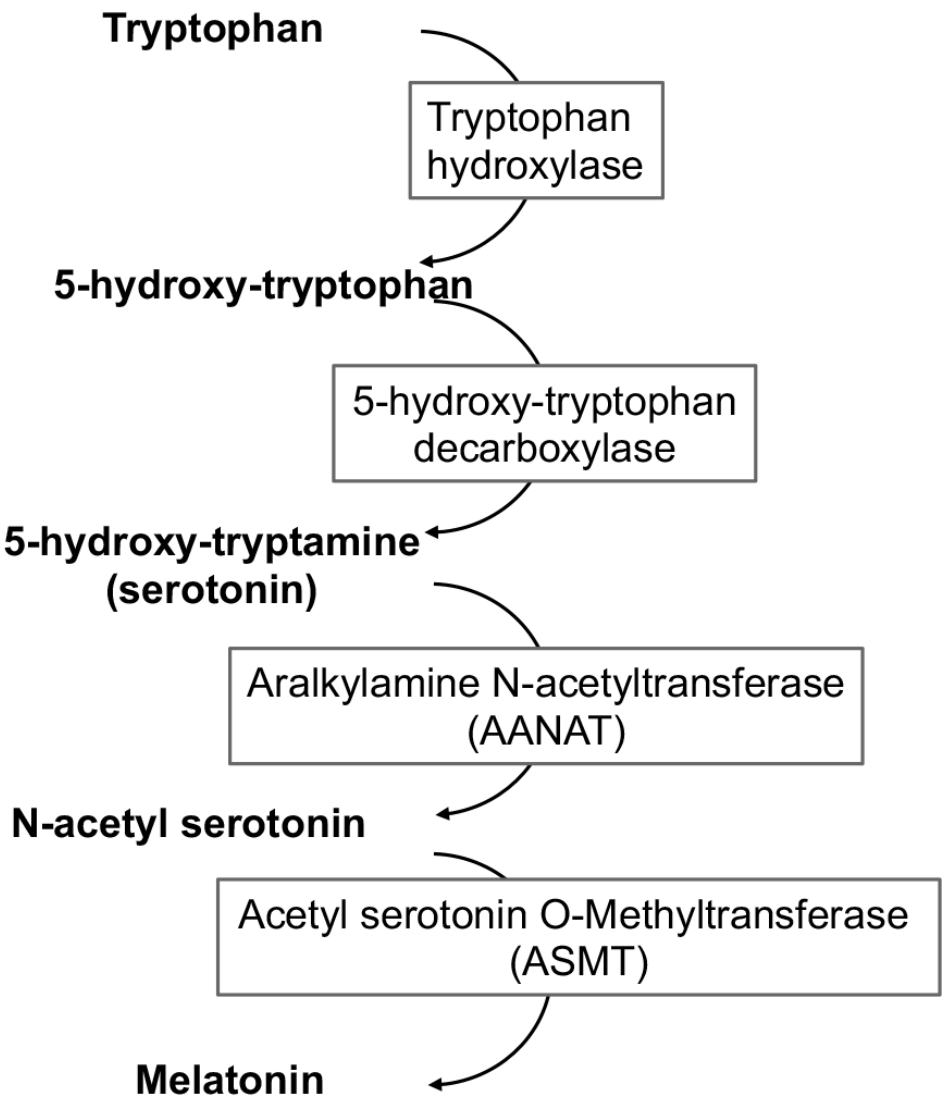

Tryptophan
Tryptophan hydroxylase
5-hydroxy-tryptophan
5-hydroxy-tryptophan decarboxylase
5-hydroxy-tryptamine (serotonin)
Aralkylamine N-acetyltransferase (AANAT)
N-acetyl serotonin
Acetyl serotonin O-Methyltransferase (ASMT)
Melatonin

Figure 2

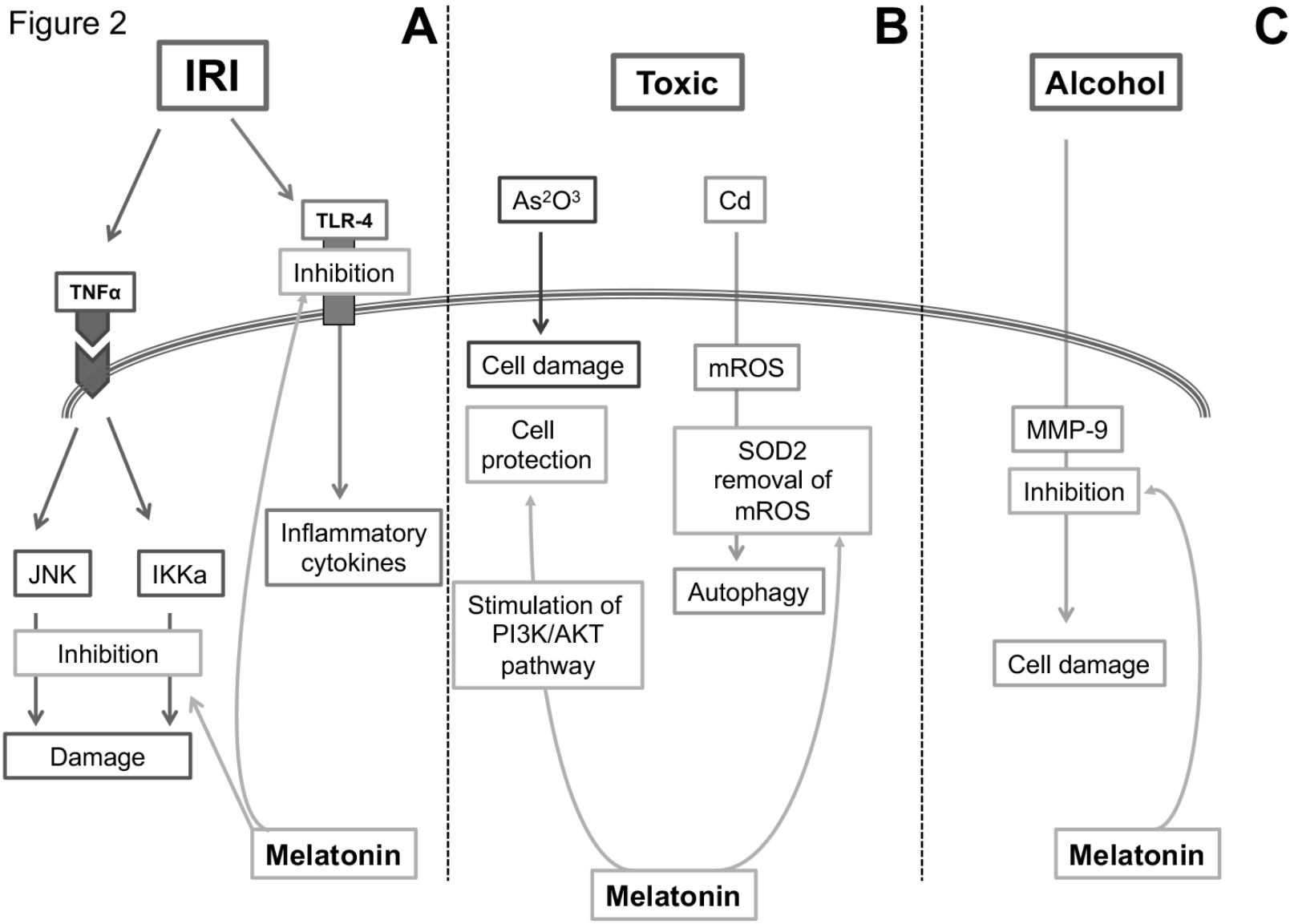

A
B
C
IRI
Toxic
Alcohol
TLR-4
Inhibition
TNFα
As2O3
Cd
Cell damage
mROS
Cell protection
SOD2 removal of mROS
MMP-9
Inhibition
JNK
IKKa
Inflammatory cytokines
Stimulation of PI3K/AKT pathway
Autophagy
Cell damage
Inhibition
Damage
Melatonin
Melatonin
Melatonin

Figure 3

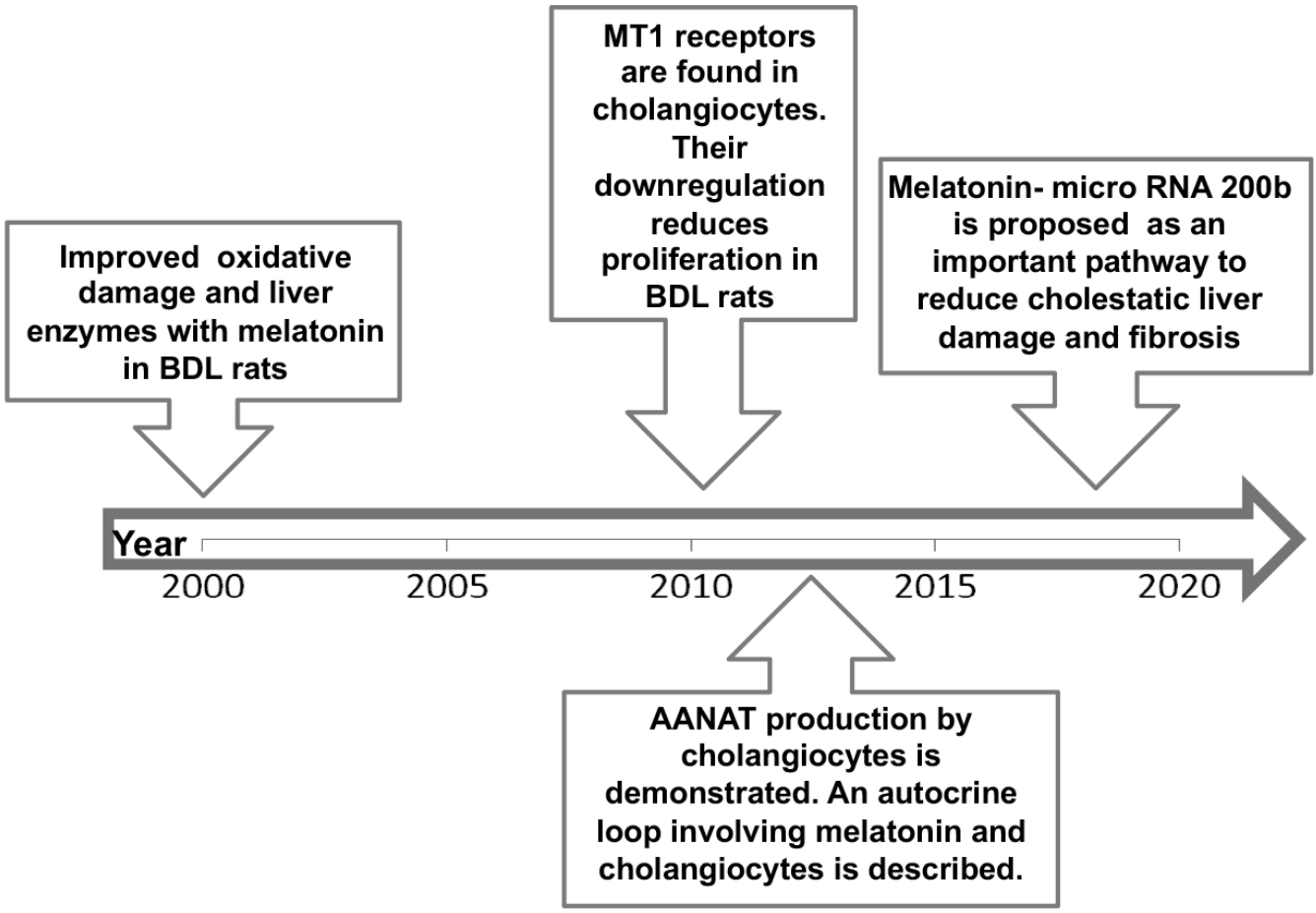
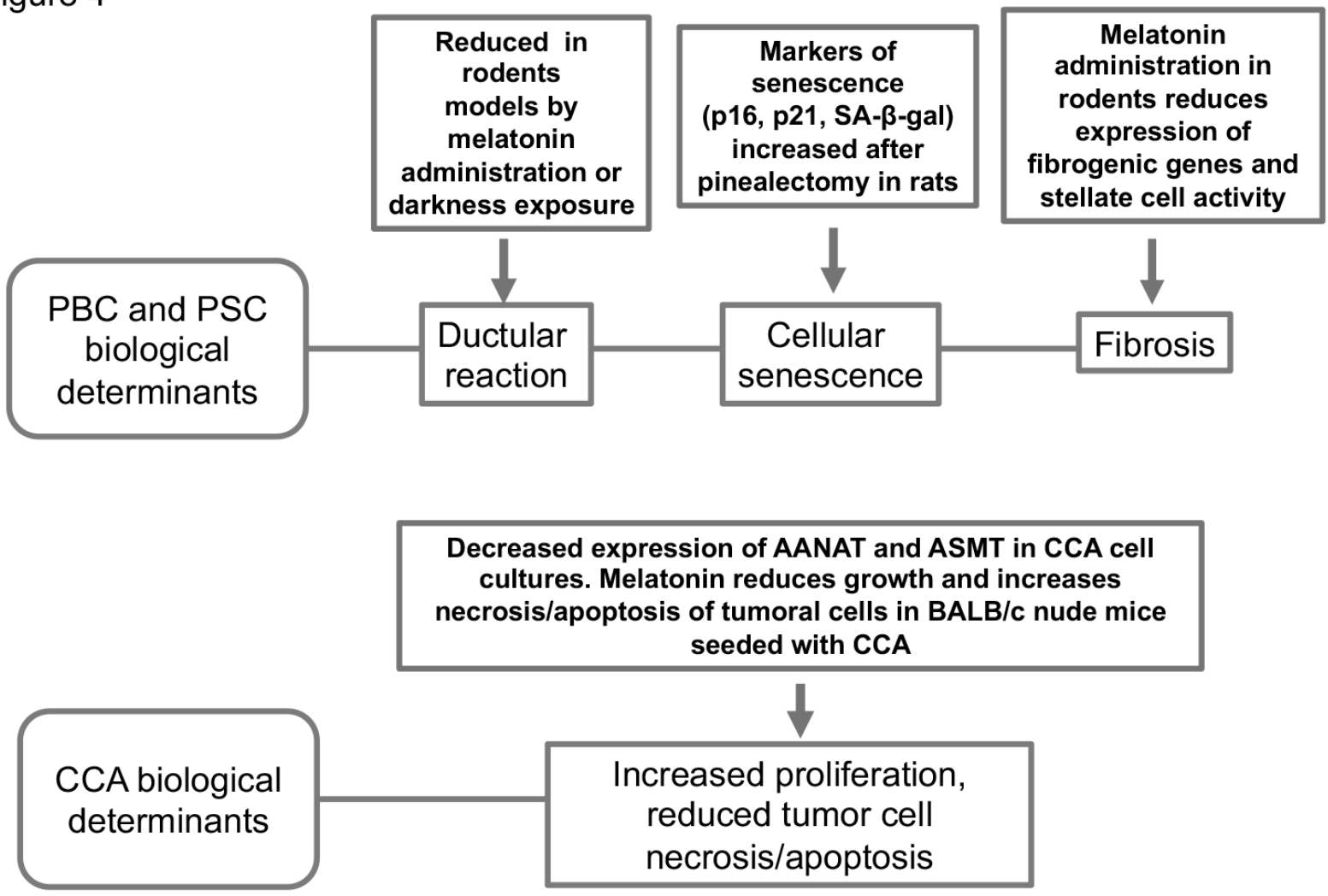

Improved oxidative damage and liver enzymes with melatonin in BDL rats
MT1 receptors are found in cholangiocytes. Their downregulation reduces proliferation in BDL rats
Melatonin- micro RNA 200b is proposed as an important pathway to reduce cholestatic liver damage and fibrosis
Year
2000
2005
2010
2015
2020
AANAT production by cholangiocytes is demonstrated. An autocrine loop involving melatonin and cholangiocytes is described.

Figure 4


Reduced in rodents models by melatonin administration or darkness exposure
Markers of senescence (p16, p21, SA-β-gal) increased after pinealectomy in rats
Melatonin administration in rodents reduces expression of fibrogenic genes and stellate cell activity
PBC and PSC biological determinants
Ductular reaction
Cellular senescence
Fibrosis
Decreased expression of AANAT and ASMT in CCA cell cultures. Melatonin reduces growth and increases necrosis/apoptosis of tumoral cells in BALB/c nude mice seeded with CCA
CCA biological determinants
Increased proliferation, reduced tumor cell necrosis/apoptosis