The challenges of Primary Biliary Cholangitis

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The Challenges of Primary Biliary Cholangitis: What is new and what needs to be done

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- personalized medicine

Highlights

- The role of histology in PBC should be reconsidered
- Is disrupted bile acid homeostasis cause or consequence of the autoimmune attack?
- The role of the microbiome is linked to its close physiological interaction with bile acids

- High-risk patients probably would benefit from early combined treatment, including biologics

Abstract

Primary Biliary Cholangitis (PBC) is an uncommon, chronic, cholangiopathy of autoimmune origin and unknown etiology characterized by positive anti-mitochondrial autoantibodies (AMA), female preponderance and progression to cirrhosis if left untreated. The diagnosis is based on AMA- or PBC-specific anti-nuclear antibody (ANA)-positivity in the presence of a cholestatic biochemical profile, histologic confirmation being mandatory only in seronegative cases. First-line treatment is ursodeoxycholic acid (UDCA), which is effective in preventing disease progression in about two thirds of the patients. The only approved second-line treatment is obeticholic acid.

This article summarizes the most relevant conclusions of a meeting held in Lugano, Switzerland, from September 23th-25th 2018, gathering basic and clinical scientists with various background from around the world to discuss the latest advances in PBC research. The meeting was dedicated to Ian Mackay, pioneer in the field of autoimmune liver diseases. The role of liver histology needs to be reconsidered: liver pathology consistent with PBC in AMA-positive individuals without biochemical cholestasis is increasingly reported, raising the question as to whether biochemical cholestasis is a reliable disease marker for both clinical practice and trials. The urgent need for new biomarkers, including more accurate markers of cholestasis, was also widely discussed during the meeting. Moreover, new insights in interactions of bile acids with biliary epithelia in PBC provide solid evidence of a role for impaired epithelial protection against potentially toxic

hydrophobic bile acids, raising the fundamental question as to whether this bile acid-induced epithelial damage is the cause or the consequence of the autoimmune attack to the biliary epithelium. Strategies are needed to identify difficult-to-treat patients at an early disease stage, when new therapeutic approaches targeting immunologic pathways, in addition to bile acid-based therapies, may be effective. In conclusion, using interdisciplinary approaches, groundbreaking advances can be expected before long in respect to our understanding of the etiopathogenesis of PBC, with the ultimate aim of improving its treatment.

1. Introduction

Primary Biliary Cholangitis (PBC) is an uncommon, chronic, cholestatic liver disease of autoimmune origin and unknown etiology characterized by anti-mitochondrial autoantibodies (AMA) and female preponderance. In absence of treatment, it progresses to cirrhosis and liver failure (1). The diagnosis is made in the presence of AMA or PBC-specific anti-nuclear antibodies (ANA) coupled with a cholestatic biochemical profile, a histologic confirmation being mandatory only in seronegative cases (2,3). Treatment is based on ursodeoxycholic acid (UDCA), which is effective in preventing disease progression in about two thirds of the patients (2,3). The only approved second-line treatment is obeticholic acid, fibrates representing an effective but off-label alternative (4). This article summarizes the main conclusions of a research meeting held in Lugano, Switzerland, on September 23th to 25th gathering basic and clinical scientists from around the world with various backgrounds to share knowledge and discuss the latest advances and new challenges in PBC research. The meeting was dedicated to Professor Ian Mackay, a pioneer in the field of autoimmune liver diseases.

2. Historical aspects

PBC was first described in 1851 in a case report of a 42-year-old woman with jaundice and xanthomata (5) (Figure 1). The name “primary biliary cirrhosis” was proposed by Dauphinée and Sinclair in 1949 (6), and in the same year the classical description of the clinical phenotype was published by Ahrens et al. (7). Ian Mackay first reported in 1958 the association of PBC with autoantibodies to human liver and kidney tissue detected by complement fixation test (8). This report was followed in 1965 by a landmark paper by Walker, Doniach, Roitt and Sherlock describing the association of AMA, detected by indirect immunofluorescence, with the clinical PBC phenotype (9). Thanks to this serological hallmark, it became possible to diagnose PBC at much earlier, pre-cirrhotic, stages, leading eventually to a nomenclature change to primary biliary cholangitis in 2015 (10). A major step forward in our understanding of PBC was the identification and cloning of the AMA target antigen by M. Eric Gershwin and Ian Mackay in 1987, i.e. the pyruvate dehydrogenase E2 subunit (PDC-E2) (11). The establishment of large national and international cohorts of PBC patients has significantly contributed to the identification of phenotypic risk factors associated with more severe disease and to the development and validation of prognostic models (12–14). Large, population-based epidemiological studies also contributed significantly to the description of the phenotype and identification of risk factors (15–17).

It has been known for more than two decades that patients with positive AMA and normal alkaline phosphatase (ALP) serum levels, a condition referred to as isolated AMA, may have liver pathology consistent with PBC and are at high risk of developing overt disease during follow up

(18–20). Indeed, recent data from China confirm that a high proportion of patients with isolated AMA have histologic changes consistent with PBC (21). These data suggest that the use of ALP as the only disease marker in AMA-positive patients likely misses patients with active disease. In a recent study from France, 15% of patients with isolated AMA developed biochemical cholestasis (ALP > 1.5 ULN) after five years, highlighting again that AMA-positivity predates overt PBC by many years, the relatively short follow-up period of this study probably explaining the low proportion of patients developing clinical disease (22). Of note, a diagnosis of PBC was retained only in patients with ALP levels > 1.5 ULN, whereas according to the guidelines from both the European Association for the Study of the Liver (EASL) and the American Association for the Study of Liver Diseases (AASLD) any level of cholestasis accompanied by AMA-positivity is diagnostic of PBC, without the need for histologic confirmation (2,3). This concept that ALP is not a perfect surrogate marker has likewise been questioned in a recent very informative case report (23).

Until 1983, the standard treatment for advanced PBC was liver transplantation (LT); UDCA, the first pharmaceutical treatment, was approved in 1997 (24). In 2016, obeticholic acid became available as approved second-line therapy for patients with insufficient response or intolerance to UDCA (25,26). In 2018, a randomized, double-blind, controlled trial demonstrated the efficacy of bezafibrate as add-on therapy to UDCA in patients with insufficient UDCA response (4).

Two recent papers suggest that changes may occur in the PBC phenotype over time. One paper evaluated PBC patients on the United States LT waitlist according to ethnicity and race: although the number of Caucasians on the waitlist decreased from 2000 to 2014, the number of Hispanics

remained stable, this latter subgroup having the worst outcomes among all ethnicities in terms of waitlist mortality, rates of LT and waitlist removal for clinical deterioration (27). It is unclear whether the worse outcomes in Hispanics are due to race-associated more aggressive disease, or to socioeconomic factors and racially-based healthcare disparities.

The second paper stems from the Global PBC Study Group, including 4805 patients diagnosed between 1970 and 2014 and aimed at describing over time the clinical features of patients diagnosed with PBC: in recent decades, PBC was diagnosed at an older age and at milder disease stages in comparison with patients diagnosed before 1990 (28). Possible explanations for these changes in the PBC population may be linked to epigenetics, changing environmental exposures, changes in microbiome, or exposure to infections. Increasing awareness coupled with the aging of the population may lead to higher numbers of elderly patients diagnosed with PBC who have mild disease, whereas the number of younger patients with more aggressive, clinically apparent disease remains stable over time (14). AMA are increasingly tested, substantially contributing to the diagnosis of PBC at early stages and remains the immunological platform used to understand loss of tolerance (29–31).

What has to be done:

- Investigate if isolated AMA-positivity – or even PBC-specific ANA – is sufficient for the diagnosis of PBC
- Develop prognostic models to establish risk-stratified follow-up care and personalized management

- Reconsider the role of ALP as a surrogate disease marker and therapeutic endpoint
- Liver histology is required in the context of research studies

3. Histology

PBC histologic hallmarks are chronic non-suppurative destructive cholangitis, characterized by lymphocytic infiltration of the biliary epithelium and biliary epithelial cells (BECs) senescence, and, most importantly, bile duct loss, with areas of macrophage-rich fibrosis replacing bile ducts in portal tracts (Figure 2). Interface hepatitis, the characteristic histologic picture of chronic hepatitis, particularly of autoimmune hepatitis (AIH), develops universally in untreated PBC, and is associated with disease progression (32,33). The recent Nakanuma histologic grading and staging system (Table 1) has the major advantage of providing a numerical score correlating with disease progression, despite its disadvantage of being cumbersome for clinical use, even in the revised version published in 2010 (32,34). The grading of the necro-inflammatory activity correlates well with UDCA response, while the deposition of orcein-positive granules, a marker of cholestasis, correlates with progression to cirrhosis (35). A recent paper reports that ductular reaction, not included as a specific parameter in the Nakanuma scoring system, also correlates with UDCA response (36).

4. Pathophysiology

4.1 Autoantibodies

4.1.1 Autoantibodies generation

Autoantibodies are generated through complementary pathways:

- a) during germline DNA rearrangement of VDJ genes, thus before antigen stimulation, by escaping clonal deletion or DNA editing mechanisms
- b) during affinity maturation, thus after antigen stimulation, by fortuitous somatic mutations (37). In this case, the original antigenic target does not need to be structurally similar to the antigen targeted after somatic hypermutations, in contrast to autoantibodies generated by the mechanism referred to as molecular mimicry (38). Somatic hypermutations can also lead to redemption of autoantibodies, redirecting the immune reaction away from self toward foreign antigens: this has been elegantly demonstrated in malaria (39).

4.1.2 Anti-nuclear antibodies

AMA are considered the most disease specific autoantibodies in human pathology, being detected in up to 95% of PBC patients, and being rare outside PBC (40,41) (Figure 3). However, AMA are not the only autoantibodies detected in PBC: disease-specific ANA are found in about one third of PBC patients and are typically characterized by multiple nuclear dots (MND) or rim-like/membranous (RLM) pattern in indirect immunofluorescence (IIF) on HEp2 cells (42) (Figure 4). These patterns are considered diagnostic of PBC even in the absence of AMA, the presence of anti-MND and/or anti-RLM antibodies in patients with cholestasis facilitating the diagnosis of AMA-negative PBC. More recently, identification of target antigens has led to the establishment of molecular-based assays and better understanding of the clinical significance of these ANA subtypes (43-46). The primary target antigens of the antibodies giving the RLM pattern are the nuclear envelope proteins gp210 and p62 (40). A number of cross-sectional studies have

consistently demonstrated that RML antibodies are detected in PBC patients with more advanced disease (40). A retrospective single center study of prospectively collected sera from 127 newly diagnosed PBC patients demonstrated that anti-gp210 and anti-p62 antibodies were associated with a higher mortality rate, even in patients with a normal bilirubin level at diagnosis (47). These findings have been confirmed by subsequent studies (48,49). The antibodies giving the MND pattern target mainly the nuclear antigens sp100 and promyelocytic leukemia protein, and, despite being highly specific for PBC, their prognostic value has been controversial (48). There is a third ANA-subtype with clinical significance in PBC, namely anti-centromere antibodies, which are found in about 10% of PBC patients, and have been associated with portal hypertension and more severe disease in most, but not all, studies (48,50,51). These observations led to the Japanese proposal of an association of ANA-subtypes with different clinical phenotypes, anti-gp210 being found more frequently in patients with hepatic failure, while the anti-centromere specificity being associated with a portal hypertension phenotype (45). Despite the clinical value of ANA in PBC, their potential pathogenic role has been poorly investigated so far. The few available data include two papers, one reporting higher gp210 expression in BECs of PBC patients as compared to BECs of patients with other liver diseases, and one reporting positivity for anti-gp210 and anti-sp100 in the dominant negative (dn)-transforming growth factor (TGF)- β R11 mouse model (52,53). Preliminary data on CD4 (cluster of differentiation 4) T-cell response to nucleoporin p62 requires further research (54). As with other autoimmune diseases, there appears to be a long latency time between the onset of autoantibodies and the development of clinical symptoms (20).

What has to be done:

- Investigate the mechanisms leading to AMA and PBC-specific ANA generation
- Characterize CD4 T cells recognizing the antigenic targets of PBC-specific ANA
- Study similarities of PBC-specific ANA target antigens and microbial proteins, as reported for AMA, which would suggest molecular mimicry as a potential mechanism leading to their generation (55)

4.2 Immunological enigmas**4.2.1 Innate immunity**

The involvement of innate immunity in PBC is underlined by single nucleotide polymorphisms (SNPs) identified by genome-wide association studies (GWAS), located in several genes encoding proteins critical for the proper functioning of mononuclear phagocytes, i.e. dendritic cells, monocytes and macrophages, including interferon regulatory factors 5 and 8, and interleukin (IL)-12A (56). Macrophages represent a key cell type of innate immunity. Two parallel macrophage systems exist: one established before birth from primitive precursors, giving rise to tissue-resident macrophages such as microglia and Kupffer cells, and one from the hematopoietic system, giving rise to classical monocyte-derived macrophages (57–59). Both systems express CD68. Despite the fact that CD68+ cells have been detected in/around injured bile ducts in PBC and that peripheral blood monocytes from PBC patients produce more pro-inflammatory cytokines as compared to controls, the role of monocytes/macrophages in PBC remains unclear (60–62). Chemokine receptor type 2 (CCR2) is required for monocyte migration to inflammation sites (63). Using the 2-octynoic acid-bovine serum albumin (2OA-BSA) immunized PBC mouse

model (see below) knocked out for CCR2, it has been shown that sinusoidal-resident Kupffer cells do not replace monocytes in portal tracts, suggesting that these two cell types have different functions. In addition, these mice exhibit markedly less portal tract inflammation and fibrosis compared to mice expressing CCR2 (64). Cenicriviroc, a dual CCR2/CCR5 antagonist, improves portal tract fibrosis and inflammation in this PBC mouse model, raising the question as to whether this compound, which is currently tested in phase III clinical trials in non-alcoholic steato-hepatitis (NASH), could be effective in PBC as well (64). Furthermore, a role of macrophages and a beneficial effect of Cenicriviroc in cholestasis mouse models has recently been reported (65,66). Of note, this compound is already under investigation in primary sclerosing cholangitis (PSC) (PEREUS trial, NCT02653625).

Fractalkine (CX₃CL1) is a membrane-bound protein expressed on endothelial and epithelial cells promoting transmigration of leukocytes expressing its receptor CX₃CR1; in its soluble form, fractalkine acts as a chemoattractant (67,68). There are multiple data on the role of this molecule in PBC, including the observation that BECs from PBC patients have higher fractalkine expression than BECs from patients with other liver diseases. In addition, serum fractalkine levels correlate with disease activity in PBC (69–71). In the above-mentioned PBC mouse model, a high expression of fractalkine was seen in the portal tracts: however, CX₃CR1 and CX₃CL1 knock out did not lead to any significant difference in histologic severity score, and in tumor necrosis factor (TNF)- α or interferon- γ expression (E. Zigmond, personal communication).

A subtype of bone marrow-derived dendritic cells called BATF3, expressing CD8 α and CD103, is very efficient in cross-presentation and is the major IL-12 source in animal models (72–74). These cells are found in the human liver, but only in portal tracts. 2OA-BSA mice lacking BATF3 cells demonstrate attenuated disease severity, with increased CD4/CD8 hepatic T cell ratio. These interesting preliminary results deserve further research on the role of BATF3 dendritic cells in PBC.

Liver resident natural killer (NK) cells represent approximately 30% of hepatic lymphocytes, being among the major players of the hepatic innate immune system, and being able to distinguish self from non-self. This function is regulated by a large family of inhibitory NK cell receptors, including killer-cell immunoglobulin-like receptors and C-lectin type molecules, which recognize major histocompatibility complex (MHC) class I present on autologous cells. This mechanism is key to tune the threshold of NK cell immunologic tolerance in autoimmune liver disorders (75). Circulating NK cells are different from liver-resident NK cells, which constitutively express the homing receptors CCR5 and CXCR6 (76). In contrast to other autoimmune liver diseases, potential autoreactive liver resident NK cells are enriched in PBC livers and show a remarkably high cytotoxic activity against autologous BEC (77–79). Although several working hypotheses on the generation of autoreactive liver-resident NK in PBC have been postulated, the mechanisms regulating the threshold of immunologic tolerance of hepatic innate immune responses are still being debated. Moreover, the possible detection of autoreactive liver resident NK cells recirculating in the peripheral blood would substantially facilitate research on the role of NK cells in PBC.

4.2.2 Adaptive immunity

Using liver infiltrating mononuclear cells obtained from explanted PBC livers co-cultured with autologous BECs, it has been demonstrated that NK cell cytotoxicity against BECs is initiated only after toll-like receptor (TLR)3-mediated NK stimulation and TLR4-mediated monocyte stimulation (79). Small numbers of NK cells surrounding BECs do not attack them, but secrete a small quantity of interferon- γ , leading to human leukocyte antigen type I (HLA-I) expression by BECs, which in this way are protected from NK-cytotoxicity (80). In contrast, when NK cells surround BECs in high number, they attack autologous BECs, with consequent exposure of intact PDC-E2, which in turn is recognized, upon antigen-presentation, by T-cells as an autoantigen, leading to perpetuation of the bile duct injury (80). These data suggest that NK cells play a key role in bridging innate with adaptive immunity in PBC. Upon stimulation with TLR ligands, BECs from PBC patients produce chemokines in comparable amounts to BECs from non-PBC patients: however, PBC-BECs have more potent chemotactic effects, mediated by CX3CL1, as compared to non-PBC BECs, suggesting that the bile duct injury is produced by liver-infiltrating mononuclear cells, rather than by the pro-inflammatory activity of BECs (60,81). In addition, BECs exposed to hydrophobic bile acids, particularly glycochenodeoxycholic acid, produce inflammatory cytokines and chemokines, suggesting that altered bile composition may be an early event in PBC pathology (82). Autoreactive T cells recognizing the PDC-E2 immunodominant epitope, i.e the lipoyl domain PDC-E2₁₆₃₋₁₇₆, are found only in PBC patients, and are massively enriched in the liver and hilar lymph nodes as compared to peripheral blood, highlighting the disease- and organ-specificity of these cells (83). Identification of autoreactive T-cells recognizing the same epitope as B-cells, i.e. PDC-E2₁₆₃₋₁₇₆, represented a major advance in our understanding of the immunological mechanisms in

PBC (84). BECs, despite expressing HLA-II molecules, do not act as antigen-presenting cells: due to programmed death-ligand 1 (PD-L1) and PD-L2 expression and prostaglandin E2 production, they inhibit T-cell activation (84). Immune cells co-cultured with BECs produce high amounts of the anti-inflammatory cytokine IL-10; by contrast, in the presence of TLR-ligands, this anti-inflammatory effect is abolished by interferon- γ production (85,86). These data further support the key role of the innate immune system in initiating the immune attack towards BECs in PBC. Immune regulation has been poorly investigated in PBC so far, and further studies are expected to result in significant advances (87–89). In conclusion, the immune response in both human and murine models of PBC is promiscuous, involving multiple lineages of cells and likely dependent on the stage of disease activity (29,87,90–97). These stage-related and individual differences in immune response suggest that personalized medicine may be required to successfully treat patients with PBC.

4.2.3 Alloimmunity and autoimmunity

Bile ducts are a preferential site of immune-mediated conditions, being targeted by autoimmune diseases, namely PBC and PSC, and by allogenic diseases, namely graft versus host disease, allograft rejection and recurrent post-transplant PBC and PSC, though it is still unclear if the latter two conditions should be classified as autoimmune or alloimmune. However, the pathological bile duct injury seen in PBC has unique features. The typical histologic findings in PBC are lymphocytic cholangitis, granulomata and tertiary, i.e. ectopic, lymphoid follicles. The latter are encountered also in other autoimmune diseases, such as rheumatoid arthritis and Sjögren syndrome (98,99). This well-organized inflammation, focused on bile ducts and therefore

referred to as portal-associated lymphoid tissue, is not seen in chronic allograft rejection and graft versus host disease; in contrast, the vanishing bile duct phenotype is shared between all three conditions, but probably has a different pathogenesis in each, being mediated in PBC by epithelial cytolytic and senescence mechanisms. Lack of response to immunosuppressive treatment of the vanishing bile duct syndrome is probably due to chronicity, since this condition represents late-stage disease.

BECs express many surface proteins allowing their interaction with the immune system, including CD1d, which activates NK T cells, class I-related molecule (MR1), which activates mucosal-associated invariant T (MAIT) cells, and delta-4, which is a ligand for the Notch receptor (100–102). Moreover, BECs, in response to cytokines like IL-22 and TNF α secreted by Th17 cells, can change the milieu in the liver, skewing T cell response away from regulatory response towards effector response (103). Activated BECs play an important role in maintaining the well-organized chronic inflammation characteristic of PBC also by producing chemokines such as CCL19 and cytokines, and by expressing vascular cell adhesion molecule-1 (104). The resulting highly organized inflammation is resistant to immunosuppression. The role of the epithelium in perpetuating inflammation is highlighted by the concept of the epi-immunome: traumatized epithelium initially produces cytokines such as IL-33 and IL-25 driving the T-cell response towards regulatory T-cells (Tregs) and Th2, but as the severity of the epithelial trauma increases, cytokines driving Th1 and Th17 T cell responses are produced, including IL-1 β , IL-6, TNF α , CCL20 and type 1 interferons, representing potential therapeutic targets in PBC, using the right combination of already available drugs (105). These characteristics of BECs contribute to explain why they are targeted by both auto- and allo-immunity.

There is clear evidence that PBC recurs after LT, the incidence of recurrent PBC increasing with the time elapsed from surgery, and being as high as 30% after only 3.5 years (106). A wide range of risk factors for recurrent PBC have been reported, including younger age at transplant and absence of cyclosporine-based immunosuppression (106,107). Recurrence of autoimmune diseases occurs in all types of solid organ transplantation and also after stem cell transplantation performed to treat autoimmune diseases. Recurrent PBC is a chimeric condition involving both allo- and autoimmunity: while the host genetic predisposition to autoimmunity is unchanged, the host microenvironment undergoes profound changes after transplantation involving the microbiome, exposure to immunosuppressive drugs and tissue damage due to surgery. The allograft, as a new target organ, is HLA-unmatched, has been damaged by preservation-reperfusion injury and is the site of immune activation owing to graft recognition. The observation that the incidence of PBC recurrence after LT from a related living donor is the same as from cadaveric donor, suggests that HLA matching does not prevent PBC recurrence: why does this happen? Lessons from experiments performed in autoimmune diabetes are helpful to answer this question. There are two distinct pathways of antigen presentation after organ transplantation: the direct pathway, characterized by donor antigen-presenting cells (APCs) presenting donor-derived peptides to recipient T-cells and predominating in acute cell-mediated rejection, and the indirect pathway, characterized by recipient APCs presenting donor-derived peptides to recipient T-cells and predominating in chronic rejection (108). As demonstrated using the non-obese diabetic (NOD) mouse model of autoimmune diabetes, post-transplant recurrent disease does not require donor HLA II presentation, because the indirect pathway predominates and the same tissue-specific antigens are presented to host CD4 T-cells before and after

transplantation, leading to autoimmune disease recurrence via activation of both allo- and auto-reactive T-cells (109,110). Post-transplant recurrent PBC is, therefore, probably mediated by indirect presentation of donor-derived, tissue-specific antigens to host CD4 T cells, thus preserving MHC restriction and replicating what happened before LT.

4.2.4 T cell homing

CD4+ T cells are highly heterogeneous, each subset functioning by different mechanisms tailored to their specific protective functions against viruses, bacteria, parasites and fungi.

Chemokine receptors drive the homing of the different subtypes of CD4+ T cells into the different tissues, and there is a correlation between the effector function and the migratory capacity of T-cells, regulated by the differential expression of chemokine receptors. Furthermore, certain T-cell subsets are defined by the expression of chemokine receptors, particularly Th1* cells and Th22, which are specific for *Mycobacterium tuberculosis* and skin pathogens, respectively (111). Inflammation of autoimmune origin releases defined chemokines, thus attracting specific subsets of T cells expressing their receptors. This could be exploited for new therapeutic approaches, but has been hampered by redundancy of chemokine receptors and pleiotropy of their ligands (112).

What has to be done:

- Investigate further the role of macrophages in PBC, which may lead to new therapeutic approaches
- Investigate fractalkine as a biomarker and potential therapeutic target in PBC
- Study the role of NK cells and of immune regulation

- Assess the therapeutic potential of blocking the chemokine-driven recruitment of autoreactive T cells to the liver
- Examine why *de novo* development of PBC following LT has not been described whereas *de novo* AIH has

4.3 Cholangiocyte biology

4.3.1 Bile acids and autoimmunity: chicken or egg?

Despite the clear autoimmune features of PBC, i.e. AMA and autoimmune phenomena targeting cholangiocytes, this condition does not respond to immunosuppressive treatment, and the first line therapy, improving prognosis, is UDCA, a choleric bile acid (113). These observations suggest a key role for bile composition in the etiopathogenesis of PBC and particularly for biliary bicarbonate secretion (see below), regulated mostly by the bicarbonate-chloride exchanger protein located in the large cholangiocyte apical membrane and referred to as anion exchanger 2 (AE2) (114). Cholangiocytes live in an unfriendly environment, being exposed at their apical membrane and cilia to millimolar, i.e. extremely high, concentrations of hydrophobic bile acids, which are buffered in micelles with cholesterol and phospholipids, but are still present as monomers, having detergent effects on the cell membrane. Hydrophobic bile acids are sensed by the G-protein-coupled bile acid receptor Gpbar1 (TGR5) and other receptors located on the apical and ciliary cholangiocyte membrane, leading to an increased secretion of chloride and bicarbonate, and, consequently, to stabilization of the biliary bicarbonate umbrella, an alkaline barrier crucially protecting cholangiocytes from bile acid toxicity (115). AE2 also tightly regulates the intracellular pH of cholangiocytes (116,117).

It has been demonstrated that in BEC from early-stage PBC patients there is a failure in secretin-stimulated biliary secretion of bicarbonate, as well as a reduction of AE2 expression and activity (118). UDCA restores the secretin response and the combination of UDCA with steroids upregulates AE2 expression (119–122). These observations suggest that altered intracellular pH regulation, mediated by AE2 downregulation, alters cholangiocyte functions and antigen expression, favoring the autoimmune attack.

The AE2 ^{-/-} mouse model develops AMA and cholangitis, but not cirrhosis (123). Of note, AE2 ^{-/-} mice have a reduced frequency of Tregs: considering that post-transplant PBC recurrence is frequent in patients on tacrolimus or mycophenolate, which are potent immunosuppressors, the question arises as to whether autoimmunity is linked to immunodeficiency (124). A link between autoimmunity and immunodeficiency is being increasingly investigated, particularly in the context of genetic defects (124,125).

There are important differences between human and murine bile, which should be considered in the interpretation of results from mouse models of cholestasis. Murine bile is produced mainly (>90%) by hepatocytes, whereas 40% of human bile is produced by BEC, and only 60% by hepatocytes (126). In addition, murine bile salts are 90% taurine-conjugated, compared to only one third of human bile salts, the remainder being glycine-conjugated: since bile acid toxicity is pH-dependent, the acid being more toxic than its salt counterpart, and since glycine-conjugated bile acids have a higher pKa than the taurine-conjugated bile acids, the mild bile-duct injury seen in AE2 ^{-/-} mice can be explained by the lower bile acid toxicity in mice.

In PBC, decreased bicarbonate secretion leads to an increased biliary concentration of protonated bile acids, which can invade cholangiocytes without transporters, inducing apoptosis (127,128) (Figure 5). Only unconjugated and glycine-conjugated bile acids have this property, but not those that are taurine-conjugated, due to their lower pKa (127,128). AE2-knockout H69 cholangiocyte cell lines are more sensitive to apoptosis induced by lower biliary pH, highlighting the protective role of bicarbonate-rich bile. Interestingly, untreated PBC patients have in their bile higher amounts of taurine-conjugated hydrophobic bile acids than healthy controls, suggesting that protective mechanisms against biliary toxicity are developed (129). Intracellular glycine-conjugated hydrophobic bile acids contribute to further reduce AE2 expression, to induce production of reactive oxygen species, as well as that of IL-6, IL-8 and chemokine (C-X-C motif) ligand 10 (CXCL10), to upregulate the expression of CD40 and HLA-DR and to stimulate the migration of effector cells (82).

MicroRNA 506 (miR-506), encoded on chromosome X, plays a central role in regulating the expression of AE2. It is upregulated in PBC, leading to downregulation of AE2 and also of type III inositol 1,4,5-triphosphate receptor, another important component of cholangiocyte secretory capacity (116,130–132). Cholangiocytes invaded by hydrophobic bile acids become senescent and apoptotic, and express intact PDC-E2 on their cell membrane, potentially enabling an autoantibody-mediated immune attack to occur. According to this hypothesis, bile duct injury in PBC initiated by increased bile acid toxicity, mediated by an impaired protective function of the bicarbonate umbrella, leads to autoimmunity. A pH-independent additional mechanism leading to cholangiocyte apoptosis mediated by the bicarbonate-sensor soluble adenylyl cyclase has been recently reported (133,134).

The key role of bicarbonate secretion is highlighted by the fact that all available and investigational PBC pharmaceutical treatments attempt to stabilize the biliary bicarbonate umbrella (113).

The cholangiocyte glycocalyx, a 20-40 nm structure located on the apical membrane, is important in stabilizing the bicarbonate umbrella, since it forms a microenvironment trapping bicarbonate molecules and thus preventing protonation of bile acids and subsequent transporter-independent invasion of hepatocytes and cholangiocytes (135,136). It is unknown whether LT restores a normal biliary profile in PBC patients.

What is the mechanism underlying the downregulation of AE2 observed in PBC? As alluded to above, miR-506 is a key regulator of AE2 expression. miR-506 is upregulated in BECs from PBC patients leading to decreased AE2 expression through an miR-506 binding site in the 3' untranslated region of AE2 mRNA (116,130). Inhibition of miR-506 partially restores AE2 function (116). The type III isoform of the inositol 1,4,5-trisphosphate receptor, regulating calcium-dependent choleresis, is also a target of miR-506, leading to further worsening of cholestasis via the acetylcholine pathway (Figure 5) (116,132). Pro-inflammatory cytokines upregulate miR-506 as well, raising the question as to whether PBC is triggered by impaired immunological functions or by bacterial products reaching the liver via the portal circulation (137). Moreover, upregulation of miR-506 leads to cholangiocyte de-differentiation, senescence, DNA damage, increased susceptibility to glycochenodeoxycholic acid-induced apoptosis, abnormal mitochondrial metabolism and PDC-E2 overexpression (137). The key question which remains open is: why is miR-506 overexpressed in PBC cholangiocytes? Is the autoimmune attack to BEC secondary to a

defect of the biliary bicarbonate umbrella, thus challenging the hypothesis that disrupted biliary homeostasis is secondary to autoimmunity (Figure 6)? To further complicate the question is the observation that in cystic fibrosis, where biliary bicarbonate secretion is impaired by the lack of Cl⁻ gradient needed to operate AE2, biliary damage is consequent to an activation of innate immune responses, not to changes in biliary alkalinity (138–140). Furthermore, in this model a PBC-like immune attack is not observed.

4.3.2 Ductopenia

In PBC, chronic portal inflammation leads to ductopenia, in contrast to what happens in portal inflammation of other etiologies that are also associated with chronic lymphocytic cholangitis, such as chronic viral hepatitis C. Normal cholangiocytes become activated by pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) arriving through the portal circulation, leading to inflammasome production of a proinflammatory microenvironment, with recruitment of inflammatory cells from the circulation. The progressive ductular inflammation displaces the basement membrane of the biliary epithelia and promotes obliteration of the vessels of the peribiliary plexus, adding ischemia as a contributor to biliary duct damage (141). Due to unknown mechanisms, repair and recovery do not occur, resulting in ductopenia.

Preliminary data suggest an important role of congregation of MAIT cells adjacent to interlobular bile ducts. One role of peribiliary MAIT cells could be to protect cholangiocytes from bacterial infections (142). In contrast, activated MAIT cells could also contribute to deleterious chronic peribiliary inflammation (142). MAIT cells are a subset of T cells expressing a T cell receptor (TCR)

composed of an invariant α chain coupled with a limited number of variable β chains, leading to a semi-invariant repertoire. The MAIT cell TCR recognizes only bacterially processed vitamin B metabolites presented by MR1, as opposed to classical TCR-MHC I/II interaction (143). Thus, it is possible that peribiliary MAIT cells, chronically stimulated by gut microbiome production of vitamin B metabolites, leads to ductopenia (142). Since MAIT cells can also be activated by IL-12 and IL-18 in an antigen-independent way, activation of cholangiocytes inflammasomes could contribute to chronic MAIT cell activation. Liver MAIT cells are activated, exhausted and depleted in PBC (95,144).

4.3.3 Ductular reaction

Cholestatic disease progression is regulated by ductular reaction, a histologic entity that involves several different cell types, including hepatic progenitor cells, reactive ductular cells and intermediate hepato-biliary cells; the latter being progenitor cells on their way of becoming hepatocytes. Reactive ductular cells, in contrast to progenitor cells, do not have regenerative potential: in fact, they pave the way to fibrosis, senescence and carcinogenesis. Activated cholangiocytes secrete angiogenic factors and express their receptors, so that newly forming bile ducts promote the growth of their vascular supply (145). The biliary vascular plexus, for its part, exerts very powerful mitogenic effects on cholangiocytes by paracrine mechanisms: therefore, ductopenia may be seen as a failure of neoangiogenesis. As mentioned above, ductular reaction leads to fibrosis, i.e. extracellular collagen deposition. The origin of the mesenchymal collagen-producing cells seen in the ductular reaction remains a matter of debate, and probably differs according to the type of the initial injury, contributing to disease-specific histologic fibrosis

patterns. Examples of disease-specific repair mechanisms leading to characteristic histologic patterns are provided by genetic cholangiopathies. For instance, in congenital hepatic fibrosis, the defective fibrocystin/polyductin protein complex leads to increased β -catenin signaling, activation of the inflammasome, secretion of IL-1 β and CXCL10, resulting in portal tract fibrosis and portal hypertension (146).

4.3.4 Cholangiopathies as a model to study cholangiocyte biology

The biliary tree is composed of large, interlobular, and small bile ducts, which have different functions. Small bile ducts have proliferative capabilities, interlobular and large bile ducts modify the bile produced by hepatocytes under the modulatory action of a variety of receptors including those for secretin and acetylcholine (Figure 5) (147).

Cholangiopathies provide an excellent model to study epithelial inflammation, epithelial reaction to damage and tissue repair in the liver. As a group, they are relatively common diseases, which feature a chronic progressive course, lack established effective therapies and are common indication for LT in children. Cholangiopathies encompass genetic, immune-mediated, infectious, drug-induced, malignant and idiopathic conditions. Whatever the initial injuring trigger, bile ducts produce an inflammatory response, which, in the presence of predisposing immunogenetic factors, may become chronic, leading to cholestasis, ductopenia, fibrosis and malignancy (147).

The injured epithelium sustains the inflammatory pathology by directly activating innate and adaptive immune responses, leading to the proposal of the “epi-immunome” concept (148). A recently recognized trigger of inflammation, in addition to classical triggers such as infections, auto- and allo-immune attacks, xenobiotics, mechanical obstruction, genetic defects and

endogenous toxins, is that which features the disruption of homeostasis as an adaptation to stress, as exemplified by the accumulation of misfolded proteins in α 1-antitrypsin deficiency (149). This model may, at least in part, explain why cholestasis, which is a disruption of BEC homeostasis, can trigger autoimmunity.

4.3.5 Signaling

During tissue repair, a number of growth factors and signal molecules drive the cells to change their function (150). Among signaling molecules, Notch has the unique feature of working via cell-cell contact, whereas the remainder of the signaling molecules work via autocrine/paracrine mechanisms. Alagille syndrome, a rare genetic cholestatic disease with paucity of intrahepatic bile ducts, is due to defects involving the *JAG1* and *NOTCH2* genes, resulting in impaired function of the Notch signaling pathway. Ductular reaction in Alagille syndrome is characterized by a high number of intermediate hepato-biliary cells lacking the expression of the Notch-regulated hepatic nuclear factor 1 β and consequently being unable to switch to a biliary phenotype, suggesting that a normal functioning Notch pathway is needed for cholangiocyte differentiation and tubulization of proliferating ducts (151). While loss of function of the Notch signaling pathway leads to Alagille syndrome, its gain of function is associated with hepatocellular carcinoma and cholangiocarcinoma (152). Proteins of the Notch pathway are overexpressed in PBC, and the PBC ductular reaction has unique features, suggesting a role for this pathway in PBC, which deserves further investigation (153,154). Of note, however, cholangiocarcinoma is not typically observed in patients with PBC.

4.3.6 Future approaches

Induced pluripotent stem cells derived from skin fibroblast and mononuclear blood cells, and 3D organoids derived from liver tissue are new technologies that promise to overcome the current lack of adequate cellular models to investigate signaling mechanisms and test new therapeutic approaches (155).

What has to be done:

- Intensive studies of the biology of biliary epithelium
 - What is the role of chromosome X? Does failure of one copy silencing explain the overexpression of miR-506 and the female preponderance?
 - Can miRNAs be biomarkers in PBC?
 - Biomarkers discriminating between hepatocellular, cholangiocellular and obstructive cholestasis are needed
 - It remains to be established whether the biliary damage is initiated by an impaired protection against hydrophobic bile acids (e.g. due to a defective biliary bicarbonate umbrella) or by an autoimmune attack to the biliary epithelium
 - Investigate the role of MAIT cells in peribiliary inflammation, and the pathogenic pathways to biliary damage initiated by DAMPs and PAMPs and the role of altered bile acid homeostasis in PBC

4.4 Genetics: why have Genome Wide Association-Studies failed?

The important role of genetics in PBC is underscored by the high concordance rate in monozygotic twins and by familial clustering (156). Taking advantage of the unique demography of Iceland, a recent population-based study found an increased risk for PBC among first, second and third degree relatives of PBC patients, supporting a major role of genetic factors in the PBC pathogenesis (157).

GWAS were expected to provide major advances in our understanding of the PBC pathogenesis and in developing new therapeutic strategies (158). PBC GWAS include patients with a precise clinical phenotype, defined as AMA-positivity and cholestasis, are hypothesis-free and identify only single nucleotide polymorphisms (SNPs). GWAS supported the view that PBC is an autoimmune disease, since the identified SNPs are located in genes encoding proteins of the immune system, such as HLA, IL-12A, IL-12RB2 and STAT4 (159,160). A GWAS from Japan revealed PBC-associated loci different from those identified by GWAS in Caucasian populations, i.e. POU2AF1, the B-cell specific transcriptional factor; and TNFSF15, which promotes effector T cell expansion, apoptosis of cells overexpressing DR3 as well as cytokine production: both loci also encode proteins of the immune system (161). A Chinese GWAS found six new PBC risk loci, all of them once again in genes encoding proteins of the immune system (162). These data indicate that the same phenotype may result from different immunopathologic pathways. PBC risk loci are shared with many other autoimmune diseases, environmental factors and cell-specific responses likely determining the clinical autoimmune phenotype (163). GWAS data have been confirmed by hypothesis-driven studies showing an enhanced expression of IL-12 subunits around damaged bile ducts in livers of PBC patients (164). Data from animal models also reflect

a role for IL-12 in triggering unremitting inflammation in the liver (165), as well as for interferon- γ as a critical component in the pathogenesis of PBC (166).

In conclusion, GWAS studies have contributed to our knowledge of PBC by revealing the polygenic basis of the genetic risk, by being replicable across different populations, and by showing widespread pleiotropy among multiple autoimmune diseases.

As a translational outcome of GWAS, a clinical trial investigated the therapeutic potential of ustekinumab in PBC, a monoclonal antibody targeting IL-12 and IL-23, which demonstrated no benefit, possibly because, in contrast to GWAS, the trial focused on phenotypes at risk for progressive disease (2,167).

What has to be done:

- GWAS are carried out using sequencing of peripheral blood cells: the study of gene expression in the affected tissues is likely to be more informative, as each tissue has its own way to respond to immune-mediated injury
- A precise and detailed description of the clinical phenotype is essential to identify subgroups to be studied, as this information is often missing in large international data bases
- Understanding the role of the environment is crucial, since genetic predisposition without environmental triggers does not lead to disease
- GWAS only identify SNPs, whereas structural variations, gene-gene interactions, and epigenetic effects are not addressed by this approach and need to be investigated with different methods

- A further emphasis on why there is a female predominance in PBC is required, possibly using the new toolset XWAS (90).
- As biological mechanisms linking risk loci with clinical phenotype are not addressed by GWAS, the omnigenic model that takes into account an infinity of small effects may deserve cautious appraisal (168).

4.5 The role of the microbiome

4.5.1 Overview

Clinical studies on microbiome in pathological conditions have to deal with several problems, including lack of knowledge about what is the normal microbiome, presence of confounders such as diet, lack of assessment of treatment influence, and problems related to sampling.

With this important premise, emerging human data demonstrate the close relationship between diet, age, general health, response to dietary or pharmaceutical interventions, and microbiome.

A few examples are summarized here.

The microbiota of elderly people is less diverse than the one of younger individuals. An interesting study investigated the microbiome composition of five different proband groups, i.e. elderly healthy subjects, subjects seen at day hospital, subjects in rehabilitation, long-term inpatients, and younger healthy controls (169). Moving from healthy to hospitalized people, the quality of the diet in terms of diversity and of fat and fiber content decreased dramatically, the dietary diversity correlating with the microbiome diversity, and inversely correlating with higher inflammatory parameters and with poorer health outcomes, highlighting the intersecting influences of the diet and the microbiome on general health (169). In another study

improvements in glucose metabolism induced by high-fiber diet in healthy subjects were greatest among those with a higher baseline *Prevotella/Bacteroides* ratio, indicating that the microbiome composition can predict the response to a therapeutic intervention, in this case, a high-fiber diet (170).

Treatment with antibiotics impacts the microbiome, e.g. clindamycin treatment for only seven days produces changes that persist for as long as two years (171).

Low-dose antibiotics are used in animal husbandry to increase body weight, rather than to treat infections; the mechanisms leading to animal weight gain are largely unknown. However, the potential contribution of the microbiome was explored in murine experiments designed to mimic our exposure to the low doses of antibiotics ingested in our diet: young mice fed low-dose antibiotics developed an altered body composition featuring an increase in body fat which was associated with an altered microbiome composition, possibly inducing increased digestion of complex dietary carbohydrates (172). Rifaximin, an antibiotic with very low bioavailability after oral administration, is an effective treatment of irritable bowel syndrome with diarrhea, traveler's diarrhea and hepatic encephalopathy. Counterintuitively, rifaximin does not change the quantity of known species and strains of the gut microbiome in patients with these conditions, suggesting that the benefit derives from either a selective influence on yet unidentified species or strains, direct action on the small intestine, alterations of bacterial metabolism or anti-inflammatory effects (173,174).

Beyond analyzing microbiome differences between health and disease, or between treated and untreated subjects, it is essential, in order to manipulate the microbiome for therapeutic purposes, to identify which commensal bacteria are beneficial, and by which mechanisms.

Bifidobacterium longum subs. *longum* 35624, a commensal bacterium with clinical efficacy in irritable bowel syndrome, has anti-inflammatory properties (175). Thanks to the full characterization of its genome, it has been possible to knockout the gene encoding the exopolysaccharide coat of this *Bifidobacterium longum*: this manipulation eliminates its anti-inflammatory properties in mice, pointing out that even minor changes in the bacterial genome can lead to highly significant functional changes (176). Moreover, a human study with healthy volunteers fed *Bifidobacterium longum* 35624 demonstrated increased IL-10 blood levels, confirming the anti-inflammatory properties of this commensal bacterium (177). Another placebo-controlled study investigated the immune effects of *Bifidobacterium longum* 35624 in patients with psoriasis, irritable bowel syndrome/chronic fatigue syndrome and ulcerative colitis, showing a significant decrease of blood inflammatory markers (178). These studies demonstrate that the microbiome and/or microbiome-host interactions can be modulated through the administration of probiotics possessing anti-inflammatory properties. However, the response to probiotics is not universal, as pointed out by an interesting study where healthy individuals were treated with an 11-strain probiotic combination or placebo: mucosal and luminal colonization with probiotics occurred only in a subgroup, and was predicted by genotypic features such as positive regulation of antigen processing and presentation, regulation of dendritic cell dendrite assembly and of ion transport (179).

Fecal microbiota transplantation is an attractive therapeutic approach for inflammatory conditions, bearing in mind that inflammation itself changes the microbiome (180). This is well exemplified by two studies carried out in patients with ulcerative colitis and *Clostridium difficile* infection, reporting a more profound dysbiosis in ulcerative colitis patients and less changes in their microbiome after fecal microbiota transplantation as compared to patients without ulcerative colitis (181,182).

4.5.2 Microbiome in primary biliary cholangitis

Recent and highly relevant data on the role of the microbiome in PBC arise from Chinese studies. The main PBC features described in Western populations are shared by the Chinese population. Epidemiological data on PBC in Asia are very recent, reporting a yearly incidence of 0.84-0.86 cases per 100,000 inhabitants, and a prevalence of 4.75-5.64 per 100,000 inhabitants, both lower than in Europe and North America (17,183). Compared to Western and Japanese cohorts, Chinese patients have a higher prevalence of anti-gp210 and of advanced disease (49,184,185). The recently published continuous PBC prognostic scoring systems referred to as the GLOBE score and the UK-PBC score, derived in Western populations, have proved to be good prognostic predictors also in Chinese PBC patients (49). A Chinese GWAS study confirmed genetic risk loci identified in North American and European populations, and added six novel risk loci (162), highlighting the potential role of T follicular helper cells and IL-21 in the immunopathogenesis of PBC (186,187).

One of the most informative reports on the role of microbiome in PBC is a Chinese cross-sectional study comparing the gut microbial profile of 79 treatment-naïve PBC patients to that of 114 healthy controls, reporting a reduced species richness and alterations in 12 genera in PBC patients (188). Furthermore, recognition of disease status was possible by analyzing these 12 genera, suggesting a unique microbiome signature of PBC (188). PBC-associated dysbiosis was partially reverted by UDCA treatment (188).

In genetically predisposed subjects, exposure to environmental factors, including xenobiotics and microbiota, may lead to clinical PBC: however, it is still unclear whether imbalances in gut microbiome are a cause or a consequence of a polygenic disease (189). Moreover, an altered microbiome has been found in a variety of diseases, including PBC, but mechanistic explanations are lacking (190). Some light has been shed by a recent paper reporting that the gut commensal *Enterococcus gallinarum* translocates to the liver inducing autoantibodies, interferon- α synthesis and high Th17 levels in a lupus-prone mouse model, whereas in a healthy mouse model translocation and pro-inflammatory features occur only if the mouse is mono-colonized by *E. gallinarum* (191). Antibiotic treatment attenuated *E. gallinarum*-induced immunopathological features in the murine model (191). Interestingly, in humans, *E. gallinarum* was detected in liver biopsies of systemic lupus erythematosus and AIH type 1 patients, but not in healthy donor livers (191), suggesting that the integrity of the gut epithelial barrier, which is disrupted in autoimmunity-prone organisms, is a key factor in maintaining a physiological interaction between the gut microbiome and the host (192).

There is a close physiological interaction between the gut microbiome and bile acids: hepatocytes synthesize the primary bile acids, i.e. cholic acid and chenodeoxycholic acid, which are subsequently conjugated with either taurine or glycine, and secreted into the bile (193). In the intestinal lumen, conjugated bile acids undergo dihydroxylation and removal of taurine/glycine by intestinal bacteria, giving rise to secondary bile acids (193). Both primary and secondary bile acids interact with their receptors, including farnesoid X receptor (FXR) and TGR5, among others, affecting the expression of a variety of target genes (194). Bile acids, on the other hand, are able to shape the gut microbiome composition. These mutual interactions, which are altered in cholestasis, have led to the proposal of the bile acids – intestinal microbiota – cholestasis triangle, offering attractive novel therapeutic approaches (195) (Figure 7). Preliminary data show that fecal and serum bile acids of PBC patients correlate with PBC-associated bacterial genera (196).

What has to be done:

- Define normal microbiota and changes associated with disease
- Define precise targets of interventions impacting the microbiota
- Define clear endpoints for clinical trials of interventions on the microbiota in well-defined study populations
- Further explore the role of microbiota as diagnostic and prognostic tools
- Refine therapeutic approaches, including those involving pharmabiotics (small molecules with therapeutic benefits harvested from the microbiota)
- Further investigate the role of disease-associated bacterial genera

4.6 Animal models

PBC animal models recapitulating the clinical features of human disease are essential to investigate the early events involved in the induction of tissue inflammation and autoimmunity, since patients are diagnosed at more advanced stages, when biochemical cholestasis and symptoms appear (197). Females are autoimmunity-prone, but in PBC female preponderance is particularly striking; therefore, a reliable animal model has to mirror this key feature, in addition to AMA production, lymphocytic liver infiltration, bile duct autoimmune attack and cholestasis (198). Since the gene pool is shared by both sexes, differential gene expression in response to stimuli in males and females may contribute to explain female preponderance in PBC: thus, interferon-related genes are overexpressed in healthy women, and even more in women with autoimmune diseases (199). Interferon- γ has multiple immunocyte targets and bridges innate to adaptive immunity, which are both involved in PBC. Prolonged exposure to high amounts of interferon- γ , which is the case in females, is key to activate inflammatory pathways and autoimmunity.

A mouse strain with a deletion in the interferon- γ gene involving the adenylate-uridylate-rich element (ARE) of the 3'-untranslated region, leading to chronic expression and over production of interferon- γ , faithfully mirrors human disease, including upregulation of total bile acids, spontaneous production of AMA, periductal inflammation, age-related disease and, in contrast to previous models, a clear female predominance (Table 2) (166). Male ARE-Del-/- mice demonstrate mild histologic changes and transient AMA-positivity, whereas females have moderate to severe portal tract lymphoid cell infiltration, bile duct destruction, granuloma

formation, and persistent AMA-positivity. The pivotal role of interferon- γ in PBC is highlighted by marked upregulation of this pathway in areas of chronic non-suppurative cholangitis in human livers (166). Increased interferon- γ production in female ARE-Del/- mice is thought to be mediated by estrogens. Since the cytokine messenger system is strongly interconnected, interferon- γ upregulation leads to upregulation of other cytokines, including IL-6, IL-10, IL-13 and TNF- α (166).

An ideal animal model should also recapitulate histopathologic findings, which in human PBC include chronic non-suppurative destructive cholangitis, epithelioid granulomas and bile duct loss. While PBC animal models reported before 2005 were unsatisfactory in this respect, recently reported models are more useful to study PBC. There are six mouse models that spontaneously develop lymphocytic cholangitis, and two models that develop PBC features upon xenobiotic immunization (Table 2). However, bridging fibrosis or cirrhosis is not seen in any of the so far reported animal models. The NODc3.c4 mouse has multiple B6- and B10-derived insulin-dependent diabetes-resistant alleles on chromosomes 3 and 4 (200): despite showing more pronounced histologic PBC features in females, these mice develop biliary cystic disease leading to liver failure in up to 50% of the animals. The dnTGF- β RII mice show pathological features more close to human disease as compared to NODc3.c4 mice (201). These mice are transgenic for the directed expression of a dominantly negative form of TGF- β receptor type II, under the direction of the CD4 promoter, restricted to T cells. Thanks to the versatility of this model, related models have been generated (202–218). The mouse model lacking functional IL-2 receptor (R) α , which is essential for regulatory T cells to differentiate, has been generated based on the observation

of AMA positivity and liver disease in a child with congenital IL-2R α deficiency (219). Despite the presence of portal lymphocytic infiltration and destructive cholangitis, this model is less attractive owing to severe extrahepatic diseases leading to short life span. Nevertheless, several models were generated based on the IL-2R α -/- (97,220–225). The scurfy mouse carries a genetic mutation in the Foxp3 transcription factor, which is necessary for T cells to differentiate into Treg cells (226): therefore, these mice have impaired Treg function and develop marked portal tract lymphocytic inflammation coupled with destructive cholangitis, these pathological features being the most similar to human disease among the PBC animal models. Unfortunately, their short life span has hampered their use for longitudinal experiments (227). The role of environmental factors in breaking tolerance to PDC-E2 is highlighted by the 6-bromohexanoate-immunized guinea pig PBC model, and by the 2-octynoic acid-immunized mouse model (228,229).

The dnTGF- β RII mouse model was used to demonstrate the pivotal role of CD8+ T cells in inducing autoimmune cholangitis: adoptive transfer of CD8+ T cells, but not of CD4+ T cells, from dnTGF- β RII mice into recombina1-deficient mice (a mouse model lacking a diversified B and T cell receptor repertoire, Rag-1-/-) induced PBC-like liver pathology (217). The presence of high-titer AMA and elevated serum IgM levels in PBC suggest that autoreactive B cells play a role in the pathogenesis (230,231). However, B-cell deficient (Ig μ -/-) dnTGF- β RII mice demonstrated an exacerbation of cholangitis with ductopenia in some inflamed portal tracts, suggesting that besides effector B cells there is a subpopulation of regulatory B (Bregs) cells playing an important role in autoimmune cholangitis (215). Bregs, in contrast to Treg, lack specific markers and exert their regulatory function mainly by IL-10 and TGF- β production, as well as by Treg induction (232).

Liver inflammation caused by adoptive transfer of CD8⁺ T cells from dnTGF- β RII mice into Rag-1^{-/-} mice was attenuated by co-transfer of B cells from the peritoneal cavity as compared to CD8⁺ transfer alone or in combination with spleen CD8⁺ cells, suggesting that Bregs are a subpopulation of peritoneal cavity-derived B cells (215). Moreover, anti-CD20 therapy ameliorates liver inflammation in dnTGF- β RII mice aged 4-6 weeks, but not in those aged 20-22 weeks, suggesting a potentially beneficial role of anti-CD20 therapy in early PBC (212). By knocking out IL-12p40 in dnTGF- β RII mice, but not by knocking out interferon- γ , a substantial improvement of liver inflammation was observed, underscoring the key role of IL-12 signalling in the pathogenesis of liver damage in this model (213). The dnTGF- β RII mouse model was used also to demonstrate a pivotal role of natural killer T (NKT) cells in the pathogenesis of PBC at its early stages (218). PBC animal models usually do not develop progressive liver fibrosis: however, by knocking out the IL-2R α ^{-/-} mouse model for IL-12p40, more severe portal inflammation, including fibrosis and portal hypertension, was unexpectedly observed (222).

What has to be done

- Investigate mechanisms of the universal production of AMA in animal models
- Using the ARE mouse model investigate:
 - the estrogen effects on pathology
 - the effects of spleen and liver immune cells transfer from male to female and vice versa
 - the female microbiome and its differences with that of males
 - the mechanisms leading to the break of tolerance in germinal centres

5 Clinical aspects

5.1 Disease definition

As mentioned above, PBC is defined as a chronic, cholestatic liver disease of autoimmune origin and unknown etiology with positive AMA and/or PBC-specific ANA, and female preponderance. If left untreated, it often progresses to cirrhosis and liver failure (1). The diagnosis is based on AMA-positivity or PBC-specific ANA positivity associated with a cholestatic biochemical profile. Histologically, the disease is characterized by chronic non-suppurative destructive cholangitis, epithelioid granulomas and ductopenia (2,3). Treatment is based on UDCA, which is effective in preventing disease progression and improving survival in about two thirds of the patients (2,3). Second-line treatments include obeticholic acid and fibrates (4,25).

5.2 Symptoms

PBC patients are often symptomatic, with a negative impact on quality of life. Fatigue and pruritus account for the most frequent symptoms, affecting more than half of the patients (2). Additional symptoms include sicca complex, abdominal pain, arthralgia, restless legs, sleeplessness, depression and cognitive dysfunction (233,234). Symptomatic patients have a worse prognosis (2). Management of symptoms is difficult, since there is no effective treatment due to a limited understanding of their pathophysiology (2).

5.3 Variant forms

A subgroup of PBC patients have clinical features of AIH, which can be present from the time of initial presentation or develop during follow up (235). According to the EASL clinical practice guidelines the term 'variant syndrome' is preferable to 'overlap syndrome', which would imply

the coexistence of two distinct diseases in the same patient (235–237). In the absence of a consensus definition, this condition is at times referred to as ‘hepatitic form of PBC’ or ‘AIH secondary to PBC’ (238,239).

Variant syndrome diagnostic criteria have been published in 1998 (Paris criteria), and state that a diagnosis of overlapping AIH in a patient fulfilling the diagnostic criteria for PBC is made if liver histology demonstrates moderate or severe interface hepatitis and at least one of the two following criteria are met: alanine aminotransferase (ALT) levels above five times the upper limit of normal (ULN); positive anti-smooth muscle antibody (SMA) or total immunoglobulin G (IgG) levels above twice ULN (2,240,241). However, less than half of AIH patients fulfill the latter criterion, leading to the suggestion of lowering the IgG level cut off (241,242). Moreover, if the variant syndrome is diagnosed following the Paris criteria, its prevalence is only about 2%, thus much lower than the observed prevalence in clinical practice, which is around 10% (2). In this context, it must be underlined that in clinical practice there is a risk of over-diagnosing the variant syndrome, exposing patients to potentially severe side effects of immunosuppressive therapy (235). Therefore, a careful evaluation of PBC patients with an unsatisfactory UDCA response should include liver histology, assessed by an expert liver pathologist, before making a diagnosis of variant syndrome (2).

Four hypothetical scenarios may explain the still obscure etiopathogenesis of the PBC/AIH variant syndrome:

- two distinct, coexisting diseases
- a single disease with features of both PBC and AIH
- a midpoint of a continuum spectrum between AIH and PBC

- AIH complicating the course of PBC, as AIH patients developing PBC are much rarer than PBC patients developing AIH

A few small studies have investigated the outcomes of patients diagnosed with the PBC/AIH variant syndrome, demonstrating a more severe disease course, a higher risk of liver decompensation, and a poorer survival in comparison with AIH and PBC in isolation (243–245). Randomized trials to guide treatment decisions of PBC/AIH patients are lacking, and recommendations are based on small retrospective series: it is recommended to add immunosuppressive treatment (steroids $\pm$ azathioprine) to UDCA in PBC patients presenting with or developing AIH features, and to add UDCA to immunosuppression in AIH patients with PBC features (2,235,241). As mentioned above, the most important AIH feature in PBC patients is histological presence of severe interface hepatitis (241,246): therefore, if liver histology suggests AIH in patients with an insufficient response to UDCA, immunosuppression should be started even if the classical Paris criteria are not fulfilled (2,240,241). Autoimmune liver serology, which would be very helpful in the characterization of patients, is hampered by insufficiently standardized methods of testing. It should be emphasized that in PBC there is an increased prevalence also of extrahepatic autoimmune manifestations including scleroderma, sicca syndrome and related rheumatic diseases (247).

5.4 Autoimmune or infectious origin?

As stated by Sheila Sherlock in her classical hepatology textbook in the '50s, "What triggers off the immunopathological cascade may be a virus, bacterium or some other neo-antigen, or simply

defective immuno-regulation alone”, suggesting that an autoimmune or an infectious etiology are not mutually exclusive. The autoimmune origin of PBC is widely accepted, and is viewed as loss of tolerance towards cholangiocytes, the target antigen being the E2 subunit of PDC (2,248). The immune attack is restricted to BECs despite PDC-E2 being a ubiquitous protein in the human body: the reason for this is probably that intra-hepatic BECs have the unique capacity of maintaining PDC-E2 immunologically intact during apoptosis and thus accessible to AMA and antigen-presenting cells, perpetuating the local autoimmune attack (249,250).

PBC has many of the classical features of an autoimmune disease, including female preponderance, specific autoantibodies and autoreactive T cells, adoptive disease transfer in murine models using CD8+ T cells, evidence for Treg defects, MHC associations and autoimmune comorbidities (251,252). However, other autoimmunity features are missing in PBC, including response to immunosuppression, correlation of disease activity with autoantibody titers, and disease transfer by autoantibody transfer in murine models (251,252). The recurrence rate of PBC after LT was as high as 47% at 10 years in a French study, suggesting that the immune attack to BECs by the host immune system reoccurs against allogeneic BECs, probably targeting the same, tissue-specific antigens (248,253). Interestingly, recurrence in the same study was associated with IgM levels ≥ 1.1 ULN (253). Notably, mycophenolate mofetil exposure, in addition to tacrolimus, was associated with higher PBC post-LT recurrence rate in a recent large international study (107). Although steroids are not recommended in PBC because of their side effects, there is some evidence that they may be of benefit in selected patients (254,255). There are no data on the role of steroids in post-LT recurrence.

Infectious agents as potential triggers of PBC have been extensively investigated, based on the association of PBC with recurrent urinary tract infections (256). Indeed, sequence similarities between PDC-E2 and proteins from several bacterial strains have been reported (251,256–258). Factors in favor of an infectious etiology, particularly of a role of the innate immune system, are presence of granuloma, high IgM levels, increased biliary cathelicidin (259); the seasonal variation of PBC incidence and lower post-LT recurrence rate on cyclosporine, which may have anti-microbial activity, than on tacrolimus, may also be viewed to support an infectious trigger (107,253,260,261). Chronic retroviral infection has been described in PBC patients and has been suggested to be the disease trigger, leading to attempts with anti-retroviral treatment (262–264). A role for retroviruses, however, could not be reproduced in other centers, and the original finding is likely to be fortuitous (262,265). Probably the most important factor when considering infectious agents as triggers of PBC is the intestinal microbiome: there is evidence of dysbiosis in PBC patients, being improved by UDCA treatment (188). Importantly, chronic cholestasis impacts the microbiome and vice versa, leading to a triangular relationship between bile acids, microbiome and cholestasis (195,196) (Figure 7).

In conclusion, evidence supports that PBC is an autoimmune disease, whose precise etiology remains unknown; a role for environmental factors, including microbiota, is probable, without a clearly identified single factor to date, coupled with genetic and epigenetic factors, thus combining “bad genes and bad luck” (248).

5.5 Pruritus and fatigue

It is still unclear if pruritus in cholestasis is due to the accumulation or the generation of pruritogenic compounds. Whatever the origin of these compounds, itch is initiated in the skin via stimulation of sensory pathway signaling transmitted through the spinothalamic tract and the thalamus to the somatosensory cortex, stimulating scratching. A variety of peripheral pruritogens has been reported, including mast-cell derived histamine (mast cells are activated by bile acids, and also secrete serotonin and vascular growth factor), bile acids, autotaxin, gut microbiome, pregnane X receptor (PXR)-agonists, endogenous opioids and cytokines (266). Central pruritus modulators include serotonin, cannabinoids, bright light therapy and endogenous opioids. Peripheral itching impulses are modulated by different cerebral regions: to investigate brain network connectivity, functional magnetic resonance (fMRI) is increasingly used. Non-cirrhotic PBC patients with higher itch scores demonstrate increased resting-state functional connectivity between the amygdala, hippocampus, thalamus and putamen, as compared to healthy controls (233). The thalamus, which is the cerebral region where interoception signals enter the brain, is smaller in early-stage PBC patients than healthy subjects (267). PBC itching patients have higher amplitude of the low-frequency fluctuation in the insula, reflecting higher neural activity (267). The amplitude of the low-frequency fluctuation in the thalamus is normalized in UDCA responders, at variance with non-responders (267). These data demonstrate brain changes in PBC, raising a question about their significance and possible modification.

PBC-related fatigue is a central form of fatigue, rather than a peripheral-muscular fatigue. Fatigued PBC patients, intriguingly, may have lower survival as compared to non-fatigued PBC patients (268). Autonomic dysfunction, which is very common in PBC, correlates with fatigue and

is more pronounced in females than in males (14). As PBC is an organ-specific disease, fatigue may be triggered in the brain by the diseased liver via unknown signaling pathways (234). It has been suggested that liver inflammation produces brain changes via neural, humoral (in parts of the brain lacking an intact blood-brain-barrier) and immune-mediated routes, leading to microglia activation in the brain and eventually to changes in neurotransmission in the basal ganglia, the main drivers of fatigue syndrome (234). Basal ganglia structural defects in PBC patients have indeed been reported (269). However, a number of open questions about fatigue in PBC remains, and complexity is added by the fact that fatigue is a symptom with biological, social, cultural and genetic components, explaining differences in severity and frequency.

Fatigue in PBC patients is associated with a profound psychological burden including depressive symptoms (270). The hippocampus is a brain structure playing a key role in mood, cognition, memory and movement: it is smaller in PBC patients, in a manner analogous to patients with major depression (233). In the latter group, the hippocampus size is restored by exercise and antidepressants, raising the question as to whether the same would happen in PBC. Moreover, there is evidence of neural inflammation in the hippocampus in PBC patients (233).

Curiously, cognitive impairment has been reported in PBC in the UK, but not in Germany or North America (233,270,271).

5.6 Patient reported outcomes

Patient reported outcomes are defined as a method of collecting information directly from the patients, allowing them to quantify their experiences. Their importance relies on offering the point of view of the patient, which may be different from that of the clinician. Disease-specific

and generic questionnaires exist, used both in clinical trials and in the clinical setting, the generic ones being less specific but generalizable (272,273). PBC-40 is a validated, PBC-specific, health-related quality of life measure encompassing six domains; it is validated in UK English, and revalidation is needed for different languages. Detecting clinical improvement is one key feature of patient reported outcomes. Patients prioritize treatments that improve symptoms, whereas physicians tend to prioritize treatments that impact disease progression (274). Patient reported outcomes have demonstrated that symptoms do not correlate with disease severity, but do correlate with disease duration (14). In addition, this approach allows the study of symptom risk factors by comparing symptomatic with asymptomatic patients, and to target symptoms in clinical trials. Symptom improvement is associated with better quality of life (assessed as Quality Adjusted Life Year) and therefore lower costs, contributing to justifying the reimbursement of pharmacological treatments. It has been demonstrated that UDCA therapy significantly improves health utility by a degree that makes it cost-effectiveness in the UK by National Institute for Clinical Excellence (NICE) criteria (<https://www.nice.org.uk/guidance>).

What has to be done:

- Specific scoring criteria for the PBC/AIH variant syndrome are needed, which would also facilitate randomized, controlled trials
- The etiopathogenesis of the PBC/AIH variant syndrome needs to be investigated
- Standardization of autoimmune liver serology would facilitate the diagnosis of variant syndromes
- The role of liver biopsy in the diagnostic work up of PBC should be reconsidered

- Immunohistochemistry, including the study of the cellular expression of cytokines, chemokines and their cognate receptors, will lead to a better definition of PBC liver pathology
- Data suggesting that IgM levels correlate with disease activity warrant further study
- The pathophysiology of pruritus and fatigue is complex and should be addressed using novel technologies such as fMRI, whereby lessons can be learned from neurophysiology and psychiatry
- Peripheral signaling pathways from the liver to the brain should be investigated
- Additional open questions concern: the link between fatigue and psychological and behavioral symptoms; the link between the observed structural/functional changes in PBC and symptoms; and the role of the environment, including microbiome, in explaining regionality of the behavioral phenotype

5.7 Therapy

5.7.1 Bile-acid therapy

As already mentioned, PBC first-line treatment is the natural bile acid UDCA, at a dose of 13-15 mg/kg body weight/day (2,3,24), which improves survival and significantly decreases the risk of LT (2,275). UDCA is a safe drug, and should be given to all patients lifelong, including during pregnancy and breast-feeding (2). UDCA response is defined according to improvement of serum ALP levels, patients not meeting response criteria facing an increasing risk of liver failure and mortality (185). UDCA intolerance is rare. Probable mechanisms of UDCA action include (113,276):

- Lowering bile toxicity by biliary UDCA enrichment
- Post-transcriptional stimulation of biliary bicarbonate, bile acid, organic anion and phospholipid secretion
- Immunomodulatory effects
- Cytoprotective, anti-apoptotic effects

5.7.2 Targeting nuclear receptors in primary biliary cholangitis

Targeting nuclear receptors is the cornerstone of second-line PBC therapies, which currently include obeticholic acid and fibrates (3,277). Nuclear receptors are ligand-activated transcription factors with a ligand-binding domain and a DNA-binding domain. The human nuclear receptor family includes 48 members, which implement a wide range of transcriptional programs, linking metabolism, immune systems, diet and other environmental factors (193,278,279): therefore, they are at times referred to as immunometabolic drugs (279,280) (Figure 8). Moreover, nuclear receptors are key regulators of adaptive mechanisms in cholestasis, explaining the efficacy of nuclear receptors agonists in PBC (193).

The farnesoid X receptor (FXR) is an attractive therapeutic target in PBC for its multiple effects, including decreasing bile acid synthesis and uptake in the liver, and decreasing bile acid absorption in the gut. FXR is expressed by hepatocytes, BECs, enterocytes and Kupffer cells, but not by hepatic stellate cells. Of note, FXR is expressed in immune cells as well, potentially explaining its immunomodulatory effects (276,281). The key role of FXR in cholestasis is most convincingly highlighted by the severe cholestatic phenotype associated not only with FXR-/-

mice, but also with genetic FXR defects in humans (282,283). Moreover, FXR-agonists improve portal hypertension in animal models (284).

FXR was identified in 1995, four years after the demonstration of efficacy for UDCA in PBC (24). In the late '90s, bile acids and obeticholic acid, a chenodeoxycholic acid derivative, were identified as FXR ligands, ultimately leading to FDA approval of the latter as second-line PBC therapy in 2016 (25). Obeticholic acid is the first-in-class steroidal FXR-agonist, its biochemical efficacy as second-line PBC treatment is good, but the histological improvement of the inflammatory activity is less satisfactory (25). In addition, pruritus is a dose-dependent, frequent side effect, leading to discontinuation in up to 10% of patients (25). Non-steroidal FXR-agonists, which probably have less side effects than their steroidal counterparts, are currently being tested in phase II trials; however, some of them show gut-restricted activity, and may be less effective, as compared to FXR-agonists with systemic activity (280).

The 21 years elapsed from FXR identification and the approval of obeticholic acid as the first FXR agonist therapeutic agent, are a relatively short time in terms of drug development, particularly for a rare disease like PBC, and in view of the availability of a first line-therapy, UDCA, which is safe and effective in more than half of the patients. UDCA efficacy is proved by a marked decrease in the number of LT for PBC since its approval, coupled with an increasing PBC prevalence despite stable incidence, suggesting improved survival (275,285–287). In contrast, data are lacking concerning the efficacy of obeticholic acid on hard clinical outcomes, its effectiveness having being proven only using surrogate endpoints. Nevertheless, FXR agonist trials have stimulated the development of additional new drugs for PBC, providing an approval pathway for regulatory authorities, and have contributed to increased physician and patient awareness. Future

directions in PBC treatment include the development of FXR agonists with better tolerability, safety and efficacy, and investigating the potential additive effects of therapies targeting bile acids, though only a minority of PBC patients does not respond to UDCA, FXR and/or PPAR-agonists. This small subgroup of patients probably has advanced disease with ductopenia, which is unlikely to respond to a pharmacological approach and usually requires LT.

Peroxisome proliferator-activated receptors (PPARs) have a relevant impact on biliary homeostasis by regulating bile acid synthesis and detoxification, and modulating phospholipid secretion (288). Three PPAR isoforms exist: PPAR α , PPAR β/δ and PPAR γ (288). While fenofibrate targets the α isoform, bezafibrate is a pan-PPAR ligand with preferential activation of the α isoform. Novel compounds targeting PPARs include elafibranor (PPAR α/δ agonist, NCT03124108) and seladelpar (PPAR δ agonist, NCT03301506), which are currently evaluated as second-line PBC treatments (289,290). PPAR agonism has anti-inflammatory effects, potently represses bile acid synthesis and promotes biliary phospholipid secretion (290). Following small, uncontrolled studies, a large, randomized-controlled trial confirmed the benefit of bezafibrate as second-line treatment in PBC patients with insufficient UDCA response, without major safety issues (3,4). The trial end-point was complete normalization of ALP levels, setting a new standard of treatment end-point in PBC. In addition, fibrates have a beneficial impact on pruritus and hyperlipidemia (4,291). However, long-term efficacy and safety data are not available as yet.

Also the glucocorticoid receptor belongs to the nuclear receptors family (279). One of its ligands is budesonide, which, along with its immunosuppressive effects, impacts favorably on the biliary

metabolism by increasing bicarbonate secretion (122). While data from small clinical studies have demonstrated some benefit of the combination of budesonide and UDCA as second-line treatment in PBC, its use is not currently recommended because of steroids side effects (2,3). Moreover, budesonide is contraindicated in the presence of cirrhosis (292,293).

Rifampicin and phenobarbital target PXR and the constitutive androstane receptor (CAR), respectively (294,295). They potently reduce the bile acid burden by inducing bile acid hydroxylation and conjugation leading to the production of hydrophilic compounds susceptible to renal excretion (294,295). Rifampicin has demonstrated good efficacy in patients with persistent hepatocellular secretory failure and UDCA-resistant intrahepatic cholestasis of pregnancy (296,297). Unfortunately, targeting this pathway has been neglected in PBC, possibly in the fear of the reported hepatotoxicity and pharmacological interactions of this compound. Budesonide is a PXR-ligand as well, and this mechanism may contribute to its reported beneficial effect in PBC. Interestingly, BSEP^{-/-} mice are protected from cholestatic injury by PXR-dependent upregulation of detoxification enzymes, stressing the role of this nuclear receptor in adaptive mechanisms under cholestatic conditions (298). Even more interestingly, conjugated and poly-hydroxylated bile acids produced by the upregulated enzymes in this mouse model are retinoid-acid receptor (RAR) antagonists, with an anti-inflammatory action mediated by decreased IL-17 production (Michael Trauner, personal communication).

Many more nuclear receptors are potential therapeutic targets, including the estrogen receptor, whose agonists have anti-inflammatory and anti-fibrotic effects, the thyroid receptor, whose

liver-specific $\beta 1$ agonists promote biliary phospholipid secretion, and the vitamin-D receptor, whose agonists have immunomodulatory and anti-fibrotic properties (299,300).

In conclusion, targeting nuclear receptors is an already therapeutically exploited pathway in PBC, but there is great potential for further development, considering that the ligands of many nuclear receptors remain to be identified. The main challenges to face are long-term efficacy, safety and drug-drug interactions. Trials combining FXR-agonists with PPAR-agonists are also attractive, bearing in mind potential safety issues, since FXR regulates PPAR transcription, potentially leading to PPAR over activation.

5.7.3 Why did biologics fail in PBC?

As detailed above, an effective and safe first-line therapy, UDCA, is available: patients with early stage disease who respond to UDCA, response being defined according to biochemical criteria, have a normal life expectancy (301). However, therapeutic endpoints are heterogeneous across different studies and different prognostic models (12,13,302–306). Between 25 and 50% of PBC patients are incomplete UDCA responders, depending on the response-criteria used (2). Half of these incomplete responder patients respond to the only registered second-line therapy, i.e. obeticholic acid (25); patients needing new therapeutic options being, therefore, only a minority. Biologics are possibly effective at early disease stages, when break of tolerance towards cholangiocytes takes place and immunopathological phenomena predominate. However, this has not been proven, since biologics have been tried only in UDCA incomplete responders, often at advanced disease stages, with unsatisfactory results in this selected population. Alternatively,

inefficacy of biologics in general as a therapeutic strategy in PBC has to be considered (see discussion on PBC etiology above).

Clinical trials with biologics carried out so far are summarized in Table 3. Rituximab has been investigated in two small clinical trials enrolling patients with insufficient UDCA-response: overall, a decrease of the median ALP levels was observed and in one trial, improvement of pruritus was reported (307,308). One phase IIa open-label trial investigated the efficacy of a monoclonal antibody targeting CXCL10 and failed to demonstrate an improvement of liver function tests (309). One additional, proof-of-concept clinical trial with negative results involved ustekinumab, an anti-IL-12/23 monoclonal antibody, identified as a potential therapeutic molecule after the GWAS results (167). Though this trial failed to achieve the ALP endpoints, modulation of the IL-12/23-related pathways was observed in that subset of patients who achieved a decrease in ALP levels, suggesting that new endpoints could be more appropriate. Other biologics, including JAK-STAT inhibitors, anti-TNF α antibodies and anti-IL-17 antibodies have not been investigated as yet in PBC. Abatacept, a fusion protein composed of the Fc region of the immunoglobulin IgG1 fused to the extracellular domain of CTLA-4, has recently been tried and failed (167). From a pathophysiological perspective, IL-17 inhibition may be the most attractive pathway to target, since IL-17 stimulates chemokine secretion by BECs, thus promoting homing of pro-inflammatory T cells to the liver, and it has been shown that increased Th17 differentiation correlates with disease severity (164).

Possible explanations for the failure of biologics in PBC are suboptimal endpoints and the inclusion of patients with advanced disease, when fibrosis predominates and the immunopathological mechanisms are subdued. Possibly, novel biomarkers of specific immune

pathways would be more appropriate end-points in biologic-based clinical trials. In view of the pathophysiology of PBC, it would be worth considering biologics at early disease stages, with anticholestatic/BEC protecting therapy based on UDCA, obeticholic acid and/or fibrates used at intermediate-stage disease, and anti-fibrotic compounds at more advanced disease stages. Being that UDCA is a safe and effective first line therapy, it will be impossible to carry out clinical trials with biologics on their own as first-line treatment, while a combination of UDCA and biologics may be considered. In this context, identifying at the time of diagnosis patients at risk for disease progression would be very important (36). An orphan disease status for PBC would speed up the trial design. The recent trial demonstrating a powerful effect of bezafibrate in UDCA-incomplete responders sets a new standard in terms of biochemical end-points, as it uses ALP normalization, and not just decrease, as the index of disease response (4). Liver histology should also be considered as an end-point in clinical trials, besides ALP normalization; this was performed so far in two trials investigating the efficacy of budesonide as an add-on therapy to UDCA, with variable results (255,310). Novel approaches are required, including potential nutritional therapy and use of nanoparticles (88).

5.7.4 The secretin/secretin receptor axis

Secretin is a peptide hormone produced by duodenal S cells; its receptor is expressed only in the basolateral membrane of large cholangiocytes (311). Secretin induces bicarbonate secretion, thus acting as a protective mechanism against biliary toxicity, particularly under cholestatic conditions (312) (Figure 5). Indeed, increased secretin levels in serum, bile and supernatant from cholangiocytes and S cells have been demonstrated in mouse models of cholestasis and in the

bile of PSC patients, probably representing an adaptive mechanism to cholestasis (313). Furthermore, secretin induces ductular reaction and consequently fibrosis (313). Experiments using the dnTGF- β RII PBC mouse model have demonstrated that secretin exerts different actions in early versus late stage PBC. While the secretin/secretin-receptor axis is upregulated in early stage PBC, it is downregulated in late stage PBC with ductopenia (118,201). Human studies have confirmed an increased expression of secretin and its receptor in biopsies from early-stage PBC patients, whereas in biopsies from advanced disease patients, the expression of both molecules is reduced as compared to controls (311). Thus, treatment of dnTGF- β RII mice with a secretin antagonist has favorable effects in early stage PBC, decreasing ductular reaction and fibrosis, whereas treatment with a secretin agonist in late stage PBC is under investigation with the hypothesis that it may halt bile duct loss and fibrosis through increased bicarbonate secretion (118,311). These observations suggest that targeting the secretin/secretin receptor axis may be a new and differential therapeutic approach for early and late stage PBC.

What has to be done:

- Identify reliable end points for clinical trials, to be consistently and universally used
- Criteria for UDCA partial response must be clearly established
- Identify novel biomarkers discriminating high-risk patients at the time of diagnosis
- Carry out trials with UDCA + biologics as first-line treatment in high-risk patients
- Consider investigating the efficacy of anti-IL-17 agents
- Obtain an orphan-disease status for PBC
- Generate data on long-term safety and efficacy of FXR- and PPAR-agonists in PBC, including impact on fibrosis and oncogenicity

- Evaluate combination therapies, such as the combination of FXR- and PPAR-agonists
- Investigate the PXR pathway
- Investigate the secretin/secretin-receptor axis as a novel, stage-specific therapeutic approach
- Investigate the immunological features of UDCA-responder versus UDCA-incomplete responder PBC patients

6 Conclusions

The meeting held in Lugano, Switzerland, last September provided a unique opportunity for scientists with different backgrounds to share the latest advances in our understanding of PBC. Due to the complexity of the field, collaborative efforts are essential in order to promote research directed at developing a more effective treatment, and possibly cure, for this unrelenting disease.

Table 1. Histologic staging and grading system of primary biliary cholangitis. Adapted from (32,34).

STAGING		GRADING	
FIBROSIS		CHOLANGITIS ACTIVITY	
Absence of portal fibrosis or fibrosis involving only portal tracts	0	Absence of cholangitis or mild damage of the bile duct epithelium	0
Portal and periportal fibrosis or fibrosis with incomplete septa	1	Chronic cholangitis in < 1/3 of the portal tracts	1
Fibrosis with complete septa	2	Chronic cholangitis in 1/3-2/3 of the portal tracts	2
Cirrhosis	3	Chronic cholangitis in > 2/3 of the portal tracts	3
BILE DUCT LOSS		HEPATITIS ACTIVITY	
Absence of bile duct loss	0	Interface hepatitis: absent Lobular hepatitis: absent or minimal	0
Bile duct loss involving < 1/3 of the portal tracts	1	Interface hepatitis: focal in a few portal tracts Lobular hepatitis: focal parenchymal necrosis	1
Bile duct loss involving 1/3-2/3 of the portal tracts	2	Interface hepatitis: moderate in several portal tracts Lobular hepatitis: mild to moderate	2
Bile duct loss involving > 2/3 of the portal tracts	3	Interface hepatitis: moderate-severe in the majority of portal tracts Lobular hepatitis: moderate or confluent necrosis	3

Presence of orcein-positive granules	
Absence of granules	0
Granules in several periportal hepatocytes in < 1/3 of the portal tracts	1
Granules in a variable number of periportal hepatocytes in 1/3-2/3 of the portal tracts	2
Granules in the majority of periportal hepatocytes in > 2/3 of the portal tracts	3

Total = 0	Stage 1, no progression
Total = 1-3	Stage 2, mild progression
Total = 4-6	Stage 3, moderate progression
Total = 7-9	Stage 4, advanced stage

Table 2. Main features of animal models of primary biliary cholangitis.

Spontaneous models							Xenobiotic-induced models	
	NOD.c3c4 (200)	dnTGFbRII (201)	IL-2Rα -/- (219)	Scurfy mouse (226)	Ae2_{a,b} -/- (123)	ARE -Del -/- (166)	Guinea pig (228)	2-OA-BSA immunized (229)
Female dominance	yes	no	no	no	no	yes	no	no
Anti-mitochondrial antibody	50-60%	100%	100%	100%	30%	100%	100%	100%
Cholestasis	no	yes	no	no	yes	yes	no	yes
Portal lymphocytic infiltration	+++	+++	+++	++++	++	++	+++	+++
Small bile duct destruction	++	+++	+++	+++	++	+++	+++	+++
Granulomas	+	-	-	-	-	+	++	+
Eosinophils	++	-	-	+	+	-	-	-
Fibrosis	30%	+	-	-	-	+	-	+
Other features	Biliary cysts	Moderate colitis	Severe anemia, IBD	Short life span	Variable histology, difficult to breed		Late onset	Severe peritonitis

Table 3. Clinical trials with biologics in primary biliary cholangitis.

Biologic	Trial design	Number of patients	Endpoint	Results
Rituximab (307)	Single-center open label	13	Normalization and/or 25% improvement of ALP levels	23% biochemical response at week 24, not sustained at week 72 Improved pruritus in 60% of patients
Rituximab (308)	Single center open-label	6	Safety and changes in B-cell function	2 patients discontinued for infections Decrease of memory B- and T-cells; increase of regulatory T-cells
Anti-CXCL10 antibody (309)	Multicenter open-label phase IIa	26	Safety, pharmacodynamic and pharmacokinetic parameters, liver function tests	Early termination after interim futility analysis
Ustekinumab (167)	Multicenter, open-label, proof of concept	20	ALP decrease > 40% from baseline or normalization or < 1.67xULN	No patient achieved ALP endpoint

ALP, alkaline phosphatase; ULN, upper limit of normal.

Figure legends

Figure 1. Primary biliary cholangitis (PBC) timeline. AMA, anti-mitochondrial antibody; IIF, indirect immunofluorescence; PDC-E2, pyruvate dehydrogenase E2 subunit; UDCA, ursodeoxycholic acid.

Figure 2. The interlobular bile duct is surrounded by a heavy lymphoplasmacytic infiltrate. The lining epithelium is attenuated with occasional intraepithelial inflammatory cells. The pinkish stroma in the right lower area represents a macrophage-rich, granulomatous inflammation. Magnification x200. Courtesy of Professor Yoh Zen, Institute of Liver Studies, King's College Hospital, London, UK.

Figure 3. Immunofluorescence pattern of anti-mitochondrial antibody on rodent kidney tissue (left) showing strong staining of mitochondria rich distal tubules, and rodent stomach (right) showing staining of the gastric parietal cells.

Figure 4. Immunofluorescence pattern on HEp2 cells of anti-nuclear antibodies typical of primary biliary cholangitis: multiple-nuclear-dots (MND) pattern (left) and rim-like/membranous (RML) pattern (right).

Figure 5. Schematic representation of cholangiocyte bicarbonate secretion regulated by secretin and acetylcholine. Bicarbonate-rich bile leads to production of deprotonated bile acids, which cannot invade cells in the absence of transporters. In contrast, bicarbonate-poor bile leads to

impaired deprotonation of the bile acids, which can invade cholangiocytes promoting apoptosis. Adapted from (133). SR, secretin receptor; AR, acetylcholine receptor; cAMP, cyclic adenosine 3', 5'-monophosphate; PKA, protein kinase; AE2, anion exchanger 2; CFTR, cystic fibrosis transmembrane conductance; AQP1, aquaporin 1; TMEM16A, transmembrane member 16A; BAH, protonated bile acids; BA, deprotonated bile acids; InsP₃R₃, type III inositol 1,4,5-triphosphate receptor.

Figure 6. Schematic representation of primary biliary cholangitis pathogenesis: red arrows indicate the pathway leading to cholangiocyte injury triggered by altered bile acid homeostasis, whereas blue arrows indicate the pathway leading to cholangiocyte injury triggered by immunological damage.

Figure 7. Triangular relationship between cholestasis, bile acids and microbiota. Adapted from (195).

Figure 8. Nuclear receptors are ligand-activated transcription factors. Their pharmacological activation leads, on the one hand, to altered bile composition via reduced bile acid synthesis and enhanced bicarbonate, water and phospholipid biliary secretion, and, on the other hand, to modulation of immunological manifestations. As a consequence, drugs targeting nuclear receptors provide cholangiocyte protection from inside the bile ducts, i.e. from bile toxicity, as well as from outside the bile ducts, i.e. from the immunological attack. These drugs are referred

to as immunometabolic drugs since they impact both on bile metabolism and on immunological phenomena. Adapted from (280). Courtesy of Professor Michael Trauner.

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Figure 1

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