Biomarkers of cholestasis

Abstract

Cholestasis is a major pathological manifestation, often resulting in detrimental liver conditions, which occurs in a variety of indications collectively termed cholestatic liver diseases. The frequent asymptomatic character and complexity of cholestasis, together with the lack of a straightforward biomarker, hampers early detection and treatment of the condition. The ‘omics’ era, however, has resulted in a plethora of cholestatic indicators, yet a single, clinically applicable biomarker for a given cholestatic disease remains missing. The criteria to fulfil as ideal biomarker as well as the challenging molecular pathways in cholestatic liver diseases advocate for a scenario in which multiple biomarkers, originating from different domains, will be assessed concomitantly. This review gives an overview of classical clinical and novel molecular biomarkers in cholestasis, focusing on their benefits and drawbacks.

Key words: biomarker, cholestasis, liver, omics.
1. Introduction

Cholestasis is a general term that denotes any pathological status in which bile formation, secretion or flow is chronically or acutely hampered [1–3]. Depending on the anatomical site of this disturbance, cholestasis can be further categorized as intrahepatic or extrahepatic. Hepatobiliary anomalies primarily featuring cholestasis are collectively termed ‘cholestatic liver diseases’. They encompass a variety of disorders with different etiologies, including primary biliary cholangitis (PBC), primary sclerosing cholangitis (PSC), autoimmune hepatitis (AIH) overlap syndrome with PBC or PSC, immunoglobulin G4-related sclerosing cholangitis (IgG4-SC), cystic fibrosis-associated liver disease (CFALD), progressive familial intrahepatic cholestasis (PFIC), benign recurrent intrahepatic cholestasis (BRIC) type 1 and 2, Alagille syndrome, biliary atresia, intrahepatic cholestasis of pregnancy (ICP) and drug-induced cholestasis (DIC) (Table 1) [4,5]. Other diseases can also present with cholestatic comorbidity, comprising hepatobiliary diseases, such as non-alcoholic and alcoholic hepatitis and end-stage liver disease cirrhosis, but also diseases that do not primarily affect the liver, such as hematologic diseases, paraneoplastic syndromes and vascular disorders [4,6]. However, the reported prevalence of cholestatic insults in these pathologies are lower than in the typical cholestatic liver diseases [6].

Prolonged or severe cholestatic conditions are toxic for cholangiocytes and hepatocytes, leading to inflammation, and are likely to progress to liver fibrosis, cirrhosis, and in some cases hepatocellular or cholangiocarcinoma, all of which can have fatal consequences [1]. Unfortunately, current therapies lack cholestatic specificity and rely on symptomatic treatment rather than addressing the underlying pathological cause(s) [7]. Furthermore, cholestatic liver diseases are frequently only detected and diagnosed when clinical manifestations become apparent at an advanced stage of the disease, in part due to suboptimal diagnostic tools [4,7]. This justifies the current need for more sensitive and specific biomarkers that enable both early detection and accurate prognosis for cholestatic liver diseases [8,9].

Over the years, the definition of a biomarker (the shortening of ‘biological marker’) has been specified and extended by several groups. The definition of a biomarker is a quantifiable characteristic of measurable biological processes that correlates with the clinical outcome. It follows then, that an optimal biomarker must be both highly sensitive and specific for a biological, and consequently...
pathological, process, implying a clear relationship between the biomarker and the clinical endpoint [8,10,11]. Accordingly, an ideal biomarker should also display predictive power for clinical outcomes, disease progression and prognosis [9,10]. The measurement of the biomarker must be executable in an objective, accurate and reproducible manner [10], and represent the actual patient status instead of capturing an incomplete or time-depending snapshot [11]. Ideally, clinical assessments and analyses should be low cost and non-invasive in character.

Clearly, fulfilling all these criteria is challenging if not impossible. Even though classical clinical biomarkers are useful in diagnosis and possibly prognosis of cholestasis, they do not allow to unravel the etiology of the cholestatic insult [4,11]. However, with the technological advances in the last two decades, the field of ‘omics’ entered in the search for novel biomarkers in cholestasis and generated an enormous amount of data thanks to high-throughput methods and powerful software. This review paper gives an overview of the current and possible future biomarkers for cholestatic liver diseases, placing a key focus on their advantages and disadvantages. In doing so, this review aims to expand the reader’s insight into the complexity of the search for appropriate cholestatic biomarkers.

2. Biomarkers of cholestasis

2.1 Clinical biomarkers

2.1.1 Physical biomarkers

The first-line read-out of a possible pathological condition is the patient’s clinical presentation. Although in strict definition not considered a true clinical physical biomarker, patient symptoms deserve high attention since they define the focus of further disease-specific research. At an early stage of cholestasis, patients often remain asymptomatic or present with non-specific clinical symptoms such as fatigue, anorexia, abdominal pain and nausea [2,4,7]. Clinical physical manifestations typical for cholestasis include jaundice, scleral ictus, pruritus and skin excoriations due to scratching, xanthoma, skin pigmentation, dark urine and pale feces. Since bile is necessary for the absorption of fat-soluble vitamins, i.e. vitamin A, D, E and K, symptoms related to deficiencies in these vitamins could be indicative for cholestasis as well [12,13].
When the patient’s clinical presentation raises the suspicion of cholestasis, a first set of measurements aims at differentiating an intrahepatic from extrahepatic cause by visualizing disease-specific morphological features in the hepatobiliary tract using imaging techniques [4]. The preferred technique for detection of intrahepatic and extrahepatic bile duct obstructions or dilatations involves abdominal ultrasonography or ultrasound, since it is non-invasive, portable, relatively inexpensive and allows for sufficient sensitivity and specificity [3,4,14]. However, the obtained findings are operator-dependent and certain abnormalities of bile ducts can be missed in some cases [15]. Computed tomography can overcome these problems, but includes important disadvantages, such as use of radiation and decreased performance in delineating the biliary tree [3,4]. Magnetic resonance cholangiopancreatography is a safe and accurate option to further explore the biliary tree, together with endoscopic ultrasound [3,4]. Although endoscopic retrograde cholangiopancreatography is considered the gold standard for examination of the biliary tract and extrahepatic obstructions, the use of this technique is reserved for highly specific cases due to technical difficulties and risk for associated morbidity and mortality [3,4]. Chronic cholestatic conditions can burgeon into liver fibrosis and even cirrhosis and liver failure. Therefore, early detection of liver fibrosis, characterized by liver stiffness, is essential to determine the severity and prognosis of the cholestatic disease [16]. Elastography techniques are state-of-the-art methods for liver elasticity measurements, with vibration-controlled transient elastography being the most widely used [16,17]. This validated ultrasound-based technique displays high reproducibility and accuracy, especially in PBC and PSC, and has several other advantages, such as rapid measurements, quick result obtention, ease in use and bed-sided application [16–18]. Other less practiced ultrasound-based techniques include static elastography, shear wave elastography, acoustic radiation force impulse imaging and magnetic resonance elastography [17,19].

Assessing clinical physical biomarkers is essential for identifying the nature of the cholestatic condition. When combined with other biomarkers, essentially clinical chemistry biomarkers, they have proven useful for diagnostic purposes [1,4].

2.1.2 Clinical chemistry biomarkers
The most frequently used biomarkers in cholestasis detection are clinical chemistry biomarkers, which can be easily assessed by blood sampling.

Two enzymes whose serum concentrations are most frequently analyzed are alanine aminotransferase (ALT) and alkaline phosphatase (ALP). ALP is considered the most relevant biomarker for cholestasis, given its earlier and more prominent increased serum levels upon cholestatic injury compared with AST. However, the distribution of these enzymes is not limited to the liver [1,4,20]. ALT is detected as two isoenzymes, namely ALT1, present in liver, kidney, fat and heart, and ALT2, detectable in brain, skeletal muscle, kidney and fat [21,22]. In the case of ALP, at least four different isoenzymes are identified, classified by their site of expression, namely placenta, liver/bone/kidney, intestine and germ cells [23]. In addition, whereas ALT is a cytosolic enzyme, ALP is present at the cell plasma membrane. Consequently, the detergent mode of action inherent to the increased bile acid concentrations during cholestasis causes ALP release from the hepatocellular membrane into the systemic circulation [1,3,24–26]. Therefore, increased serum ALP concentrations are more indicative for cholestatic injury whereas increased ALT levels point towards hepatocellular damage as such [1,27]. It must be noted that ALP has a half-life of seven days [28], making detection of elevated ALP serum concentrations possible even after disappearance of the cause of cholestatic injury, such as obstruction [1]. Importantly, bone activity, which is altered in bone diseases and during childhood, is a significant contributor to serum ALP levels [3,29]. Furthermore, external factors, such as pregnancy, smoking and exercise, are frequently associated with increased serum ALP levels [23,29]. Other rare diseases, including Celiac disease and hyperparathyroidism, contribute as well, albeit to a lesser extent [23,29]. Therefore, ALP as such is not considered a highly specific biomarker of cholestasis.

In addition to ALT and ALP, aspartate aminotransferase (AST) levels can be assessed to calculate the AST/ALT ratio. Values higher than 1.5 are suggestive for intrahepatic cholestasis, whereas lower levels are indicative for extrahepatic obstruction [30]. However, in clinical settings, AST measurements are less popular due to an unclear advantage over ALP measurements. This is mainly due to the wide tissue distribution of AST. Whereas the mitochondrial isoform is abundantly present in hepatocytes [31], the cytosolic isoform can be found in skeletal and heart muscle, kidneys and red blood cells [32]. Release of mitochondrial AST occurs in a similar manner as ALT, hence indicating hepatocellular damage.
Cytosolic AST however, can enter the bloodstream under many pathological circumstances, including muscle injury and hemolysis [20,33]. Therefore, increased AST serum levels are not very specific for cholestasis.

Another serum biomarker that is typically increased during cholestasis is the microsomal enzyme γ-glutamyltransferase (GGT). GGT is a membrane-bound enzyme and shows, like ALP, a wide distribution not only in many tissues, including kidney, liver, pancreas and intestine [34], but also in circulating cells, such as leucocytes and lymphocytes [35,36]. In contrast, whereas isolated increased serum ALP concentrations can be found in some cholestatic diseases, isolated augmentation in GGT levels is less cholestasis-specific [4]. Indeed, even though GGT is the most sensitive liver enzyme [37], it lacks specificity, since serum levels are elevated in a variety of other diseases and conditions, such as renal insufficiency, hyperthyroidism, diabetes, pancreatitis, myocardial infarct, obesity and alcohol intake [29,38]. Hence, the main clinical relevance of serum GGT measurements lies in the possibility of excluding bone diseases as cause for raised ALP levels, and to better comprehend and categorize increased ALP levels in growing children [29].

5′-nucleotidase (5′-NT) is another enzyme whose serum levels increase upon cholestatic injury. Present in the liver as well as in other organs, it is mainly found at the surface of the cell plasma membrane [29]. As for GGT, serum concentrations of 5′-NT are independent from bone diseases, and, in contrast to ALP levels, pregnancy and childhood are no significant confounders [39,40]. However, human clinical laboratory use of this biomarker is seldom due to unclear advantages over combined ALP and GGT evaluations in hepatobiliary diseases [41].

A non-enzymatic biomarker that can be easily assessed in blood is bilirubin. Unconjugated or indirect bilirubin is a pigmented degradation product from hemoglobin, derived from the lysis of aged red blood cells. This water-insoluble form circulates in the blood bound to albumin to be transported to the liver. Once arrived in hepatocytes, bilirubin is released from albumin and conjugated with glucuronic acid to obtain water-soluble conjugated (direct) bilirubin, which can then be excreted at the canalicular membrane into the bile duct and stored in the gallbladder. Cholestatic conditions, such as bile duct obstruction, can cause increased amounts of conjugated bilirubin in hepatocytes, followed by regurgitation of the latter into the blood, a process taking place in prolonged or severe cholestatic
circumstances [20,42]. Consequently, serum conjugated hyperbilirubinemia is an indicator of cholestasis, albeit in a more advanced disease stage [1,4]. In clinical settings, total and (un)conjugated bilirubin concentrations can be obtained, allowing to distinguish hepatic from extrahepatic causes of hyperbilirubinemia [20].

The quantitative limits indicative for cholestasis are fixed at concentrations 1.5 times above the upper limit of normal (ULN) for serum ALP and 3 times above the ULN for serum GGT [3,4]. However, these values are still subject for debate and are considered more as guidelines than rigid cut-offs, since distinct values have been established for specific cholestatic diseases [3,4,40]. In addition, despite ALP and GGT being early biomarkers of cholestasis, often even elevated in asymptomatic patients, absence of altered levels in symptomatic patients is not unusual [4]. In these cases, clinical judgement should be prioritized in diagnostic investigations. Furthermore, depending on the type of cholestatic liver disease, other clinical chemistry biomarkers can or must be included for diagnosis as well. In this regard, ALT can be of diagnostic help in detection of PBC and IgG4 positivity is in the vast majority of cases required for confirmation of IgG4-SC [3,4,43]. In some conditions, like DIC, a combination of several clinical parameters proves advantageous to distinguish between other forms of liver injury [4,44].

None of the clinical chemistry biomarkers discussed above are truly liver-specific and each one bears its own pitfalls, most importantly the alterations in levels in a variety of pathologies and conditions. Nevertheless, the combination of multiple parameters can, at least in part, overcome this problem. Table 2 gives an overview of clinical chemistry biomarkers used in clinical settings for differential diagnosis of the most common cholestatic liver diseases.

2.1.3 Histopathological biomarkers

Histopathological assessment following liver biopsy is sometimes considered when cholestasis is suspected, but when the clinical physical and chemistry biomarkers do not allow for clear diagnosis or represent atypical features [4,45]. The collected liver tissue is analyzed by a pathologist, who can identify certain morphological alterations and patterns typically found in cholestasis and thus representing histopathological biomarkers.
The main goal is to differentiate between intrahepatic and extrahepatic cholestasis, with specific attention to mechanic obstructions, since therapeutic treatment and prognosis highly differ for both types of cholestasis [46]. Histologically, cholestasis of any etiology mainly consists of bilirubin granules deposition in the cytoplasm of centrilobular hepatocytes, bile plugs in dilated canaliculi and a variable degree of bile regurgitation into the perisinusoidal space with phagocytosis by Kupffer cells [46,47]. In extrahepatic cholestasis, bile accumulation becomes more prominent and extends to periportal areas with time, which is associated with biliary infarcts and portal tract changes, including dilated bile ducts and ductular reaction [46–48]. In line with this, some morphological patterns portray general features observed in cholestasis, whereas others can be indicative for a precise type and stage of cholestatic liver disease. Thus, cholestasis-induced alterations predominate in centrilobular hepatocytes independent from the type of cholestatic disease [46], whereas bile duct epithelial cell damage is a typical feature observed in early stage PBC [45].

Although proven highly informative, especially in cases of uncertainty, liver biopsies are not considered the preferred method for standard evaluation and confirmation of cholestatic diseases due to several limitations. The most common route for obtaining a liver specimen is percutaneously, which is not always possible, like in obese patients [49]. Alternative methods include laparoscopic, transvenous or plugged biopsy [49]. Regardless of the route used, performing a liver biopsy is an invasive procedure, frequently associated with complications and in rare cases death [49,50]. Furthermore, the obtained sample is evaluated by a pathologist, which includes some subjectivity, and inter-operator and intra-operator differences have been reported [51]. Additionally, sampling variability is a major limitation of liver biopsies. Cholestasis-induced alterations are not necessarily uniformly distributed throughout the entire liver and different liver zones can present with varying disease stages as well. Consequently, the spatiotemporal information regarding the cholestatic disease is dependent on the liver specimen analyzed [52]. Therefore, the collected biopsy should measure sufficient dimensions, and an inclusion of at least 10 portal fields has been suggested [4,49,50]. Despite these disadvantages, histopathological assessment of the liver remains necessary for diagnosis of some specific cholestatic diseases, such as small-duct PSC and antimitochondrial antibody-negative PBC, and to confirm malignancy [50].
2.2 Molecular biomarkers

2.2.1 Genetic biomarkers

Genomics, broadly the study of the structure, function and interaction of genes, has contributed substantially to identifying genetic causes and risk factors in cholestasis. In this regard, genome-wide association studies (GWAS), *i.e.* large-scale hypothesis-free studies in which whole genomes of cases versus controls are analyzed in order to associate altered genes with the clinical phenotype, together with next-generation sequencing (NGS), have provided a tremendous amount of genes involved in a variety of cholestatic diseases. In this light, 6 types of PFIC, each one characterized by a distinct clinical presentation, are currently known, with classification into the respective subtypes depending on the mutated gene [53]. These monogenic diseases are typified by a clear genotype-to-phenotype association; hence the affected gene can serve as a genetic biomarker. Practically, the patient’s DNA is tested against a gene panel, which includes several genes known to be mutated in specific cholestatic diseases, allowing for diagnosis and adequate treatment. Table 3 displays the current existing genetic cholestasis testing panel, together with the associated disease upon gene mutation.

Monogenic liver diseases represent only a small fraction of liver diseases and, given the multifaceted functions of the liver, it may not be surprising that the vast majority of cholestatic diseases encompass multiple affected genes [53,54]. These polygenic diseases are presumed to be of multifactorial cause, in the sense that disease onset is dependent on higher-order interactions of multiple susceptibility genes and environmental influences [54–56]. The proportional contribution of genetic and environmental factors to the pathogenesis is overly complex to unravel and appears to vary across diseases. Thus, the D19H variant in the \( ABCG8 \) gene, which encodes the hepatocanalicular cholesterol hemitransporter, is the major risk factor for gallstone development [57]. In a large-scale Swedish study, 21% of twins with gallstones were carrier of at least one D19H allele [58]. This indicates a major contribution of one gene variant, but also highlights the importance of other environmental factors, underlined by the fact that the D19H variant was present in 9% of non-affected individuals [58,59].

In the majority of cholestatic liver diseases, unravelling the specific genetic contribution is even more challenging. A plethora of genetic polymorphisms has been associated with the autoimmune diseases PBC [60] and PSC [61], with a significant number occurring in the genetic region coding for the human
leucocyte antigen (HLA) complex, which is essential in immunity regulation. However, the HLA region is highly polymorphic and many of the identified HLA risk loci in PBC and PSC have been associated with other autoimmune and immune-mediated disorders [62–64]. Accordingly, the presence of these genetic risk factors rather implies a global susceptibility for (auto)immune diseases than for the specific disease per se [62,65]. Furthermore, only 10% of PBC [66] and PSC [67] patients carry identified susceptibility genes, indicating an important contribution of environmental factors in pathogenesis and possibly other yet unidentified rare variants [53,60].

Clearly, altered genes can serve as valuable biomarkers in monogenic liver diseases, whereas diagnosis based on solely genetic biomarkers is less straightforward in polygenic complex diseases [65,68]. Furthermore, regarding the latter, the presence of a genetic risk factor does not necessarily imply ultimate development of the disease, but must be considered as an indicator of predisposition [53,68,69]. In addition, identifying genetic risk factors eventually leading to cholestatic insult is extremely difficult due to the complexity and multifactorial causes of cholestasis. Extended knowledge regarding causes and pathways in cholestatic disease development is necessary to validate the use of a specific altered gene as a genetic biomarker for a given cholestatic disease [68,69].

2.2.2 Epigenetic biomarkers

Epigenetics refers to the study of heritable alterations in the genome without changes in the primary DNA sequence [70]. Indeed, the ultimate transcriptional output of the cell DNA depends on a variety of non-genetic molecular pathways with as major mechanisms DNA methylation, histone modifications and subsequent chromatin remodeling, and regulation by non-coding RNAs [9,71,72]. Non-coding RNAs can be divided in long non-coding RNAs and short non-coding RNAs, with microRNAs (miRNAs) the most extensively studied and characterized [72,73]. MiRNAs are approximately 22 nucleotide long RNA molecules that bind messenger RNA (mRNA) through complementary base-pairing. It is noteworthy that they play an important role in physiological liver development and as an expected consequence a variety of miRNAs has been associated with different stages of many chronic liver diseases [73].
The epigenome contributes considerably to the ultimate phenotype of a cell. As such, the study of epigenetic patterns and their alterations is gaining interest as a source of useful biomarkers in disease [74]. The major advantage of epigenetic biomarkers is that the contribution of non-genetic factors, such as age, diet, microbiome and exercise, is incorporated, since epigenetic regulations are strongly responsive to these environmental triggers [9,74]. This is reflected in the bulk of the recently discovered potential epigenetic biomarkers for cholestatic conditions, of which a vast majority is linked with bile acid metabolism, the key player in developing cholestatic conditions and highly dependent on environmental factors [75]. In this regard, trimethylation of histone H3 lysine 4 (H3K4) is critical for the activation of several bile acid transporter genes by nuclear receptors and is downregulated in cholestasis, rendering the trimethylated H3K4 a possible epigenetic biomarker for cholestasis [76]. MicroRNA-210 (miR-210) levels are increased in cholestatic mice and binding to their target causes decreased bile salt export pump (BSEP) levels and consequently a disturbed bile acid metabolism [77]. Interestingly, elevated hepatic miR-210 levels are also found in PBC patients, introducing possibilities for this small non-coding RNA as biomarker and potential therapeutic target in cholestatic diseases [77]. Another non-coding RNA that gained interest during the last decade is the long non-coding RNA H19 [78]. Liver H19 levels are elevated in cholestatic mice and humans and although bile acid accumulation is suggested causal for this observation, the exact mechanism remains to be elucidated [78–80].

This highlights a significant complication of epigenetic biomarkers, namely that a profound understanding of the underlying epigenetic regulatory mechanisms, which are highly dynamic and complex, in healthy and cholestatic conditions is required to fully exploit the potential of these new biomarkers [74]. At the same time, the dynamicity and adaptational capacity to environmental cues of these regulation mechanisms encompass the main advantage of epigenetic biomarkers, namely that they allow to explain interindividual variability and pave the way to precision medicine [9,74].

### 2.2.3 Transcriptomic biomarkers

The transcriptome is the complete collection of all RNA molecules in a cell or cell population. This includes coding mRNA, which is translated into proteins, but also non-coding RNA, such as transfer
RNA (tRNA), ribosomal RNA (rRNA) and miRNA. In its wide sense, transcriptomics involves the study of both categories of RNA, nevertheless generally it is mostly applied to mRNA [81,82]. The earliest technique developed to study (whole) transcriptomes is the microarray, which uses multiple known nucleic acid sequences attached to solid spots to act as detecting probes upon hybridization with a complementary sequence [83]. This method is quick in generating and processing data, and allows for quantification and detection of known transcripts, however, it is not applicable for discovering novel sequences [84]. The more recent RNA sequencing method, an application of NGS, overcomes this drawback and moreover is more sensitive and resolutive [84–86]. The characteristics inherent to each technique have defined their applicability in specific research domains. Microarrays have proven a valuable tool to study alterations in gene expression profiles in the field of drug development and toxicity testing of known compounds [84,87]. Regarding cholestasis, a certain number of studies have assessed transcription profiles in DIC in vitro, in vivo and ex vivo, and allowed for identification of altered transcribed genes or gene sets upon application of cholestatic drugs [88–92]. Interestingly, cluster analysis of unique gene expression profiles generated by several hepatotoxicants demonstrated that drugs known to cause cholestatic injury were categorizable in the same cluster, proposing a future role for these transcripts as biomarkers in toxicological studies [88]. The RNA sequencing technology on the other hand is highly appreciated as high-throughput screening technique for identification of novel altered transcripts induced by drugs [84,93]. Transcriptomics have been proven useful in other domains than solely DIC, and a plethora of altered transcription profiles has been associated with other cholestatic conditions, such as PBC [94,95], PSC [94,96] and gallstones [97,98]. However, these discovered altered gene signatures encourage to unravel the underlying pathological mechanism instead of being usable biomarkers, since many of them lack clearly described functions and rather support or provide hypotheses regarding disease pathways [81,94,99]. Nevertheless, after future research will have shed more light on these compelling questions, transcriptomic biomarkers may be of great value as clinical biomarkers, since they allow for information of a precise tissue at a specific time point of the disease [84]. Furthermore, alterations in transcripts likely occur early in disease, before clinical phenotypical manifestations or detectable changes in
classical chemistry biomarkers. This renders transcriptomic biomarkers a promising tool for early detection or even prediction of cholestatic liver diseases [87].

2.2.4 Proteomic biomarkers

Even though transcriptomics has great potential in providing novel clinical applicable biomarkers for cholestatic diseases, it also suffers from some drawbacks. mRNA levels do not always correspond well with their respective protein concentrations [100]. This renders mRNA data difficult to interpret, since only the transcribed protein is biologically active and thus representative for disease status. Moreover, this biologically active state of the protein is orchestrated by post-translational modifications, such as acetylation, glycosylation and phosphorylation, all processes which are undetectable in mRNA levels or sequences [101]. In addition, one mRNA sequence can give rise to a variety of proteins due to protein polymorphisms or alternative splicing [102]. Proteomics, the analysis of all translated proteins of a cell or tissue, including their functions and interactions, overcomes these inconveniences.

The procedure of proteomic biomarker discovery starts with depletion of abundant proteins in the sample, usually plasma or serum, in order to detect and/or quantify low abundant proteins [103]. After depletion, proteins are separated by two-dimensional gel electrophoresis and visualized by staining techniques [104]. Significant protein spots are excised out of the gel, digested and identified by mass spectrometry (MS) techniques [103,105]. The most frequently applied strategy in proteomics is the bottom-up approach, which aims at identifying the primary structure of the protein [106,107]. Here, proteins are digested into peptides prior to MS coupled with other techniques like gel electrophoresis or liquid chromatography [106]. The top-down approach on the other hand relies on intact proteins, which are fractionated by their molecular weight or immunoprecipitation [107]. This approach is less sensitive compared with bottom-up MS and is generally used for larger proteins in order to determine sequence variants [107]. MS techniques allow for high-throughput and are more specific and sensitive compared with other analytical methods. However, covering all proteins in a sample is challenging and minor or weak signals are frequently missed [108]. Hence, in some cases protein-arrays are used instead of MS. Sadly, the lack of well-established arrays limits their utility in biomarker discovery [105,109].
Although promising, currently the amount of studies relying on proteomics to assess (novel) biomarkers in liver disease is sparse, and the majority of them has been focused on hepatocellular carcinoma [109,110]. The few studies investigating cholestasis associated diseases by proteomic techniques mainly concentrated on the bile proteome, given the evident role of bile in cholestatic conditions [111].

The advantage of the use of this biological fluid lies in the fact that pathology associated protein concentrations are higher in fluids surrounding the lesions than in plasma or serum, where they will become highly diluted and probably even undetectable [112]. On the other hand, collecting bile samples is an invasive procedure and bile acids and other molecules present in the sample may interfere with bile proteins during the analytical procedure [112]. Nevertheless, studies relying on human bile samples for proteomic purposes have been proven promising. In this regard, it has been demonstrated that cholangiocarcinoma can be distinguished from PSC patients based on bile protein patterns [113]. Interestingly, in an independent prospective study, some of these bile proteins were found to be significantly elevated in cholangiocarcinoma compared with PSC patients [114]. This result was later expanded by analyzing blood and urine as sample matrix. Whereas urine seemed to be a valid diagnostic tool to separate cholangiocarcinoma from PSC conditions, blood was unworkable, due to the marked range between low and high abundancy proteins [115]. However, it was concluded that a combination of bile and urine sample analysis would be necessary to achieve increased specificity [115].

Despite the limited yet encouraging results, potential proteomic biomarkers are not entirely validated for use in clinical settings to diagnose or prognose cholestatic pathologies [109]. The technological advances in the last decades made high-throughput identification of novel proteins altered in liver disease possible, but the lack of knowledge of their functions and interactions in normal conditions hinders their utility as biomarkers [109,110,116]. In addition, the complete human proteome remains to be elucidated and standardized protocols for sample preparation or data analysis to be developed. The Human Liver Proteome Project, as part of the Human Proteome Organization, aims at resolving the aforementioned issues and plays an essential role in exploiting the full potential of proteomic biomarkers in cholestatic liver diseases [117,118].

2.2.5 Metabolomic biomarkers
Of all ‘omics’ technologies, metabolomics is one of the latest to have gained research interest. It can best be described as the analysis of all metabolites or low molecular weight intermediates, which are context-dependent, in a biological sample [119,120]. The term ‘metabonomics’ was first defined by Nicholson and colleagues as ‘the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification’ [121]. Both terms are frequently used interchangeably, since the difference in definition is somewhat ambiguous [122,123]. Currently their differentiation is proposed to rely on differences in analytical techniques [122,123]. For the sake of simplicity, the term metabolomics will be used hereafter.

The main techniques used in metabolomics are nuclear magnetic resonance (NMR) spectroscopy and MS. The major advantage of NMR spectroscopy is that biological samples do not require preanalytical treatment [123,124]. This renders this technique highly appreciated for in vivo and in situ studies [125]. Even though NMR spectroscopy knows a relatively simple workflow and provides highly reproducible results, it lacks sensitivity [119,124]. MS largely overcomes this issue, and is furthermore more suitable for high-throughput profiling of metabolites [124]. The downside of this technique however is the necessity for separation of biological fluids prior to analysis [122,123]. The most frequently used separation techniques are liquid and gas chromatography, rendering metabolites soluble and volatile respectively [123,124].

As metabolites represent the end product of biological pathways, they are considered a recapitulation of all foregoing upstream information, including genetic, epigenetic, transcriptomic and proteomic data [119,126]. This renders measurement of metabolite concentrations highly useful in biological pathway identification, since they designate the net result of all preceding steps in the studied biological system, in contrast to protein or mRNA concentrations [119]. Furthermore, changes in metabolite patterns occur rapidly and provide information regarding disturbed known pathways, allowing for early detection of disease [119,123]. Not surprisingly the liver, as principal metabolically active tissue, and the hepatic metabolome have gained much interest in the field of metabolomics. As for proteomics, many investigations focused on discovering biomarkers in hepatocellular carcinoma. In this regard, the search for metabolomic biomarkers in cholestatic conditions, as possible premalignant stage of hepatocellular carcinoma or cholangiocarcinoma, has started to grow during this last decade [122].
Up until now, the majority of studies relied on cholestatic murine models to detect associated altered metabolite levels in liver, urine, serum or plasma, with a main focus on bile acids [127–133]. Each study provided novel discovered metabolites whose concentrations were altered in cholestatic conditions. However, the limited number of studies and the use of different experimental models renders conclusions regarding definite valid metabolomic cholestatic biomarkers premature.

Efforts have also been made to allow for metabolomic differentiation between several (cholestatic) liver diseases, such as between autoimmune hepatitis and PBC [134], PBC and PSC [135,136], PBC and Celiac disease [137] and ICP and asymptomatic hypercholanemia of pregnancy [138]. In this regard, evidence is growing that PBC and PSC could be distinguished based on metabolic primary and secondary bile acid patterns and concentrations [134–136,139].

Although metabolomic biomarkers are highly valued in cholestasis and in liver pathology in general, the field of metabolomics is still in its infancy. Due to the novelty of this research area, the current available techniques and analyses are not entirely optimized for their purpose neither globally standardized, which complicates data processing and comparison [125,126]. In addition, the complete human metabolome comprises a tremendous amount of molecules, which are far from being all identified [140]. So far, discovered metabolites in cholestatic diseases are to be regarded as potential biomarkers, but should foremost be used as a tool to shed more light on disease mechanisms [123,141].

3. Conclusions and future perspectives

Cholestasis is a relative mild pathology that, if left untreated, can develop into life-threatening conditions. The clinical work-up normally starts after suspicion of cholestasis based on the patient’s clinical presentation. However, due to the initial asymptomatic character, cholestatic conditions do not unfrequently pass undetected until a more advanced disease stage [3,4]. Traditional clinical serum biomarkers are currently the gold standard in the detection of cholestasis, being frequently combined with physical and histopathological biomarkers [3,4]. However, even when combining several traditional biomarkers, the required specificity, sensitivity and accuracy is not always achieved, and false negative results persist [7,142]. In addition, given the possible severe outcomes of cholestasis and
the subsequent financial losses from a societal and individual patient point of view, there is an urgent need for biomarkers capable of determining disease prognosis and severity [27,143,144]. With the era of ‘omics’, a tremendous amount of novel possible molecular biomarkers has been discovered during the search of fulfilling the demanding criteria of biomarkers. However, the vast majority of these newly discovered potential biomarkers have ambiguous clinical relevance due to a lack of adapted software and standardized protocols for data analysis, as well as insufficient knowledge of existing molecular pathways [9,145,146]. In addition, unraveling their precise role in cholestatic conditions, hence estimating a utility as biomarker, is extremely puzzling. Each of these molecules intervenes at a specific point in the pathological pathway, influencing and being influenced by upstream as well as downstream events and molecules. The utility of these novel ‘omics’ technologies and discoveries therefore lies in filling the gaps in our present understanding of biological and pathological molecular pathways by integrating and combining multiple ‘omics’ studies [84,145,146].

An adverse outcome pathway (AOP) has been developed for cholestatic liver injury [147]. An AOP is a conceptual construct that links an initial molecular initiating event with an adverse outcome through intermediate key events, hence being of high value in discerning molecular disease pathways [148]. The AOP for cholestatic liver injury focusses on inhibition of the BSEP as initiating event, resulting in increased liver bile acid accumulation [147]. This triggers two cellular responses, namely an initial deteriorative response, characterized by mitochondrial impairment, inflammation, oxidative stress and cell death, and an adaptive response, aiming at counteracting the increased bile acid concentration by inducing transcriptional changes [147]. Recently, the robustness of the developed AOP has been tested by transcriptional analysis of genes involved in both responses [149]. In compliance with the established AOP, the OATP1B1 and the SLC10A1 genes were significantly upregulated, leading to the conclusion that these genes could be potential early biomarkers of cholestatic disease (Table 4) [149]. This highlights the value of AOPs in unravelling molecular disease mechanisms as well as their important contribution to biomarker discovery.

In the near future, different strategies and optimizations will be essential for translation of obtained data into clinical practice and management of cholestasis. First of all, there is insufficient reproducibility between results, mainly due to a lack of standardization in analytical methods and a concrete definition
of analytical variables in laboratory and clinical set-ups, despite great technical advances [146,150]. Efforts must be put on developing and validating standardized bioinformatic tools as well as open-access databases for cataloging novel discoveries [145,146]. In this regard, several databases have originated the last decade, such as the Human Protein Atlas and the Human Metabolome Database. Second, increased collaboration must occur between key players in biomarker discovery, validation and implementation, namely industry and academia [146]. Whereas in general the former excels in providing tremendous amounts of potential biomarkers generated by use of high-throughput sophisticated technology, the latter furnishes indispensable knowledge by time-consuming research [11]. Both aspects are complementary though and their collaboration will speed up and ameliorate the research for biomarkers in cholestasis and many other pathologies [146]. Third, with the increased interest in precision medicine, interpersonal and intrapersonal variability should be taken into account when assessing potential biomarkers, and this especially during clinical trials [11,144]. On the one hand, monitoring multiple biological parameters and ‘omics’ biomarkers of one single person can balance intrapersonal variability, hence augmenting the chance of establishing representative biomarkers, which is less probable when measuring so-called snapshots in time of clinical parameters [11]. On the other hand, an increased patient data availability by continuous monitoring of multiple parameters would allow to correct for interpersonal variability, subsequently aiding in a better patient stratification [144,146]. This would empower the creation of more homogenous patient groups, hence leading to more confident conclusions [144,146].

The perfect future biomarker for cholestasis ideally should allow for more precise and faster diagnosis, but also prognosis of the disease. Furthermore, assessment should be non-invasive and rapid, and results easily quantifiable. Importantly, in terms of laboratory settings the biomarker should be interspecies extrapolatable and preferably accessible via high-throughput techniques [8,142,147]. Of course, such demanding requirements bear considerable costs in terms of discovery, development, clinical studies and analytical validation [11]. It is however not expected that one single novel molecular biomarker will replace traditional clinical biomarkers, but rather that multiple biomarkers from different fields will be assessed together in order to obtain more qualitative, complete and reliable information [145]. This
holistic approach will bear fruit not only in diagnosis, treatment and prognosis of cholestasis but even in other complex diseases, especially with the upcoming era of personalized medicine [11,69,145].
Executive summary

- Cholestasis can result in detrimental liver conditions when left untreated.
- There is an urging need for more sensitive and specific biomarkers allowing for early detection of cholestatic liver conditions.
- Biomarkers can arise from two domains:

Clinical biomarkers:
- Clinical biomarkers are classical biomarkers and include physical, clinical chemistry and histopathological biomarkers.
- They are frequently assessed together for diagnosis of (the type of) cholestatic liver injury.
- No single specific biomarker for cholestatic liver exists and each clinical biomarker bears its own benefits and drawbacks.

Molecular biomarkers:
- Molecular biomarkers are novel biomarkers and include genetic, epigenetic, transcriptomic, proteomic and metabolomic biomarkers.
- Technological advances, high-throughput techniques and software development have contributed to the generation of a tremendous amount of potential novel biomarkers.
- The lagging behind of the mechanistic understanding of cholestatic liver diseases limits their clinical applicability.
- Expectedly multiple biomarkers originating from different fields will be assessed concomitantly for diagnosis and evaluation of (cholestatic) diseases.
References


   * This paper provides clinical practical guidelines for diagnosis, treatment and prevention of cholestatic liver diseases, established by a panel of experts selected by the EASL governing board.


* This review describes the anatomically ascending disease course of cholestatic liver diseases together with a new pathophysiological view on PSC and PBC.


*This review describes the role of genetic testing in monogenic liver diseases and discusses the complexity of polygenic liver diseases.*


*This is the first large-scale study attributing genetic gallstone disease risk to a genetic variant in the ABCG8 gene.*


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* This topical review gives an overview of the pathophysiology of cholestatic diseases, experimental study models and the role of 'omics'.


** This is the first paper describing an AOP for drug induced cholestatic liver injury.


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Disclosures

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