THE METABOLOMIC WINDOW INTO HEPATOBILIARY DISEASE

Diren Beyoğlu and **Jeffrey R. Idle**

Hepatology Research Group, Department of Clinical Research, University of Bern, Bern, Switzerland

Summary

The emergent discipline of metabolomics has attracted considerable research effort in hepatology. Here we review the metabolomic data for nonalcoholic fatty liver disease (NAFLD), nonalcoholic steatohepatitis (NASH), cirrhosis, hepatocellular carcinoma (HCC), cholangiocarcinoma (CCA), alcoholic liver disease (ALD), hepatitis B and C, cholecystitis, cholestasis, liver transplantation and acute hepatotoxicity in animal models. A metabolomic window has permitted a view into the changing biochemistry occurring in the transitional phases between a healthy liver and hepatocellular carcinoma or cholangiocarcinoma. Whether provoked by obesity and diabetes, alcohol use or oncogenic viruses, the liver develops a core metabolomic phenotype (CMP) that involves dysregulation of bile acid and phospholipid homeostasis. The CMP commences at the transition between the healthy liver (Phase 0) and NAFLD/NASH, ALD or viral hepatitis (Phase 1). This CMP is maintained in the presence or absence of cirrhosis (Phase 2) and whether or not either HCC or CCA (Phase 3) develop. Inflammatory signalling in the liver triggers the appearance of the CMP. Many other metabolomic markers distinguish between Phases 0, 1, 2 and 3. A metabolic remodelling in HCC has been described but metabolomic data from all four Phases demonstrate that the Warburg shift from mitochondrial respiration to cytosolic glycolysis foreshadows HCC and may occur as early as Phase 1. The metabolic remodelling also involves an upregulation of fatty acid β-oxidation, also beginning in Phase 1. The storage of triglycerides in fatty liver provides high energy-yielding substrates for Phases 2 and 3 of liver pathology. The metabolomic window into hepatobiliary disease sheds new light on the systems pathology of the liver.

Metabolomics and the liver in brief

Over the past decade or more, many authors have defined the terms metabolomics and metabonomics. It is unproductive and unnecessary to add further to these definitions here. All the reader needs to know from the point of view of hepatobiliary disease, is that metabolomics is a window that offers a view distinct from the lenses of genomics, transcriptomics and proteomics. There can be no other organ where such a plethora of both

Author for correspondence: Professor JR Idle, Hepatology Research Group, Department of Clinical Research, University of Bern, Floor F, Murtenstrasse 35, 3010 Bern, Switzerland, jeff.idle@ikp.unibe.ch, Phone: +41 79 446 24 24, Fax: +41 31 632 49 97.

lipids and water-soluble metabolites are metabolically interchanged. No other organ exceeds the rates of metabolism and energy production and consumption as found in the liver. Not only is the liver the source of myriad endogenous metabolites and precursors used by other organs, but also houses a vast array of detoxication enzymes that are crucial for rendering less toxic, more water-soluble and readily excretable the 1–3 million xenobiotics that we are exposed to in our lifetimes [1]. The hepatic metabolome is therefore a highly complex and dynamic flux of small metabolites (say, <1.5 kDa, to include the larger phospholipid species, such as cardiolipins). Metabolomics in its practice combines high-throughput analytical chemistry, typically, methodologies based upon mass spectrometry or nuclear magnetic resonance spectroscopy, with multivariate data analysis. These technologies permit comparison of "global" metabolite profiles in an "unbiased" fashion for two or more groups of samples. Of course, no metabolomic investigation has ever delivered a global metabolite profile for a sample set, as this would require employment of multiple analytical platforms and several sample preparation protocols that performed from millimolar down to sub-picomolar concentrations. Moreover, different analytical platforms combined with specific sample preparation procedures each provide a different metabolomic window in the metabolic life of the liver. Accordingly, metabolomic findings reported are always biased by the laboratory analytical procedures employed, often highly so.

This notwithstanding, many metabolomic investigators in recent years have entered the field of hepatobiliary disease and a considerable volume of publications has appeared. This review is therefore timely and we will attempt to make sense of a large and heterogeneous set of published studies concerning the varied hepatobiliary elements of pathophysiology where metabolomics has had something to say. This metabolomic window on hepatobiliary disease has furnished an overabundance of potential disease biomarkers. More importantly, in our view, the metabolomic lens has begun to provide new insights into liver disease mechanisms, new understandings that may unmask potential therapeutic targets and, one day, new treatment modalities.

The metabolomic window into nonalcoholic diseases of the liver

Overview

In this review and as depicted in Fig. 1, we will describe the extent to which metabolomics has informed on the progression from the healthy liver to hepatocellular carcinoma (HCC) through the various phases of nonalcoholic fatty liver disease (NAFLD), nonalcoholic steatohepatitis (NASH) and liver cirrhosis. We will also examine what metabolomics has taught about the various influencing factors and putative risk factors for these diseases, such as obesity, diabetes, alcohol, hepatitis B and C virus (HBV, HCV) infection. In addition, we will also review what metabolomics has contributed to the understanding of the change in hepatic function after liver transplantation.

Nonalcoholic fatty liver disease (NAFLD)

Nonalcoholic fatty liver disease (NAFLD) is a highly prevalent condition that affects 15% to 45% persons in developed nations [2] and in both children and adults from all ethnic groups [3]. A diagnosis of NAFLD implies an increased risk of such diseases as cardiovascular

disease, diabetes, colonic adenomas, hypothyroidism and polycystic ovary syndrome [3]. NAFLD is generally considered to be the hepatic manifestation of metabolic syndrome [4]. The reference standard for diagnosing hepatic steatosis remains liver biopsy [3]. Investigators have employed metabolomic protocols in an attempt to define biomarkers that might replace this invasive procedure for a disease of such high prevalence. Table 1 shows a summary of 11 studies with metabolomic components that inform regarding the formation of hepatic steatosis. Animal models and studies in living human subjects and human tissues have been employed. One common finding is that of increased lipid species in the liver and serum/plasma, including cholesterol esters [5, 6], triacylglycerols [4–7], diacylglycerols [4], sphingomyelins [4], various bile salts [8–10], together with lactate [9, 11, 12] and glutamate [11, 13]. In addition, cysteine-glutathione disulfide and both oxidized and reduced glutathione were all reported to be depressed in the liver and serum/plasma [8, 9]. Finally, where diets that instigate fatty liver had been used, depressed concentrations of glucose were reported both in rat liver [14] and mouse serum [11], but in one study, elevated plasma glucose was reported [12]. Taken together with elevated mouse serum/plasma lactate [11, 12], pyruvate and alanine [12], and human plasma lactate [9], these results would suggest that NAFLD engages in cytosolic glycolysis. NAFLD is frequently associated with insulin resistance and insulin has been reported in mice to activate pyruvate kinase M2 [15], the enzyme switch to glycolysis involved in the Warburg effect and thus the production of lactate and alanine from glucose via pyruvate. Furthermore, the reduction in glutathione derivatives in human liver [8] and plasma [9] in NAFLD are clear signs of active oxidative stress in the liver.

The lipidomic component of the observations summarized in Table 1 is of interest. Firstly, it has been reported that phosphocholine, choline, betaine and trimethylamine *N*-oxide (TMAO) were up-regulated metabolites in both liver and plasma of rodents fed diets that provoked fatty liver [11, 12]. This is a clear indication of an increased turnover of phosphatidylcholine and phosphatidylethanolamine species in the liver, thus releasing free fatty acids through the action of phospholipases A1 and A2. These fatty acids, if not catabolized by β-oxidation, will be stored in the liver as triacylglycerols. This is what was observed in the metabolomic studies of animals with fatty liver [4–6]. Therefore, fatty liver is not just a deposition of fat in the liver but rather a rearrangement and repartitioning of lipid stores as has been proposed [5]. Using a mouse 24h starvation protocol, it was observed that the triacylglycerols TG(44:2) and TG(48:3) massively increased in the liver by 2427% and 1198%, respectively. These are the most abundant triacylglycerols in adipose tissue and these findings suggest that adipose may be a source of triacylglycerols deposited in the liver in NAFLD [5]. Secondly, elevated hepatic concentrations of various lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE) and phosphatidylcholine (PC) species have been reported for human steatotic *vs*. non-steatotic livers [8]. These molecules are obvious candidates for the elevated choline and choline metabolites discussed above. Finally, three studies in humans reported elevated bile salts in the liver [8] that spilled over to elevated bile acids in serum/plasma [9, 10]. Bile acids act as signaling molecules in the liver that regulate lipid and glucose homeostasis [3, 16]. Certain bile acids, in particular, chenodeoxycholic acid (CDCA) and deoxycholic acid (DCA) are endogenous ligands that activate the farnesoid X receptor (FXR) [17]. The nuclear receptor

FXR modulates conversion of cholesterol to bile acids by the regulation of the expression of CYP7A1 [3]. Moreover, FXR reduces lipogenesis by downregulating expression of SREBP-1, activates the nuclear receptor PPARα causing an increase in β-oxidation of free fatty acids (FFA), both of which processes reduce hepatic FFA levels [3, 16]. There is a single report of elevated hepatic levels of the bile salts glycochenodeoxycholate 3-sulfate (GCDCA-3S) and taurochenodeoxycholate (TCDCA) in human fatty liver [8]. TCDCA is a relatively weak activator of FXR [17] and GCDCA-3S appears not to have been studied in this regard. It is curious that NAFLD existed in the presence of increased serum/plasma concentrations of glycocholate, taurocholate, glycochenodeoxycholate [9] and deoxycholate [10], which may not reflect hepatic concentrations of the FXR activators CDCA and DCA. This theme will be returned to in the next section.

Nonalcoholic steatohepatitis (NASH)

NASH is a more advanced stage of NAFLD with a major inflammatory component [2]. NAFLD may progress to NASH, but >80% of cases remain as isolated fatty liver (IFL) with no or minimal progression to cirrhosis and no increased risk of death relative to the general population [3]. It has been estimated that ~11% NASH develop cirrhosis over 15 years and ~7% progress to hepatocellular carcinoma (HCC) over 6.5 years, either via cirrhosis or sometimes directly [3] (see Fig. 1). The origins of the hepatic inflammation in NASH continues to involve a major research effort and one theory posits that the hepatitis originates in visceral adipose, which is intrinsically pro-inflammatory [2]. A study in mice fed a high-fat diet supports this theory [18].

There have been relatively few metabolomic studies addressing the pathobiology of NASH and its progression from simple NAFLD and all these have examined serum/plasma only. Five studies are summarized in Table 2. As with NAFLD, triacylglycerols and several fatty acids were elevated in plasma [7] and like NAFLD, several other fatty acids and LPCs were attenuated in plasma [9]. When a small series of NASH was compared with NAFLD, significant changes in serum concentrations of only three phospholipids were reported [10]. A study using NMR, which, unlike mass-spectrometry-based platforms, does not have the power of detecting a large range of molecules [19], contributed raised serum concentrations of glucose, glutamate and taurine [11]. The greatest metabolomic insights into NASH pathogenesis come from a recent study that combined high-end analytics and targeted gene expression by qPCR [20]. These workers generated NASH in mice fed a methionine- and choline-deficient (MCD) diet. UPLC-ESI-TOFMS metabolomics revealed a statistically significant depression of LPC(16:0), LPC(18:0) and LPC(18:1) in serum with a significant rise in tauro-β-muricholate, taurocholate and 12-HETE for MCD fed mice compared with mice on a normal diet. As a positive control, genetically obese *ob/ob* mice with severe steatosis were administered galactosamine (GalN), which provoked severe inflammation and hepatocyte injury with marked upregulation of hepatic mRNAs coding for TNFα and TGFβ1. Serum of GalN-injected *ob/ob* steatotic mice compared with saline-injected *ob/ob* steatotic mice displayed the same changes in LPCs and bile acids as the MCD fed mice. Thus, the decline in serum LPC and the rise in serum bile acids is a signature of the inflammatory component of NASH, rather than the steatotic component. To investigate further the mechanisms involved in these perturbations of LPC and bile acid homeostasis in

the NASH model, hepatic mRNA levels were determined by qPCR for genes involved in the metabolism and transport of LPC, bile acids and 12-HETE. Lysophosphatidylcholine acyltransferases (LPCAT) that convert LPC to PC [21] were all upregulated with two- to four-fold elevations in hepatic *Lpcat1, Lpcat2* and *Lpcat3* mRNAs in the NASH model. Additionally, the transporters SLC10A1 and SLCO1A1 that uptake bile salts into hepatocytes and the transporters ABCC1 and ABCC4 that export bile acids from the liver were highly downregulated and upregulated, respectively [20]. Taken together, these observations explain how the inflammatory phenotype of NASH in a mouse model results in the changes in serum metabolites described in Table 2 and this is shown in Fig. 2. Importantly, similar perturbations have been observed in NASH patients [9], suggesting that similar mechanisms may operate in humans. Finally, it should be stated that biomarkers for NASH are limited and therapeutic options are poorly developed, which serves to emphasize the need for further metabolomic research in this area.

Fibrosis and cirrhosis

Liver fibrosis is a scarring process involving the deposition of excess connective tissue in response to injury. Cirrhosis may be considered as the end-stage of this reaction, comprising formation of fibrous septa and hepatocyte nodules. Oxidative stress provokes the inflammatory reactions and apoptosis involved in the generation of cirrhosis [22]. It is now clear that NAFLD/NASH may develop into cirrhosis, although the histological features of precursor NASH in the cirrhotic liver may be challenging to diagnose [23]. Cirrhosis may arise due to a large number of causes, principal among which are not only NAFLD/NASH but also alcoholic fatty liver disease and viral hepatitis B or C (see Fig. 1). There are no cut-off values for laboratory analyses that give a diagnosis of cirrhosis [24] and so the generation of novel metabolomic biomarkers to detect early cirrhosis has become a justifiable aim. Table 3 summarizes such studies.

Three studies have been conducted in rats administered hepatotoxins to provoke fibrosis and cirrhosis. Histopathology confirmed that rats exposed to thioacetamide in their drinking water developed hepatic fibrosis after one month and cirrhosis after three months. Liver extracts examined by NMR had higher levels of lactate [22], suggesting a degree of anaerobic metabolism within the fibrotic liver. Two studies treated rats with carbon tetrachloride (CCl_4), which induced fibrosis [25, 26] and the authors evaluated treatment with the Chinese medicine Xia Yu Xue Decoction [25] or scoparone, a drug isolated from a medicinal plant [26]. Many metabolomic signals were reported after CCl_4 administration, including decreases in the urinary excretion of certain amino acids and gut flora metabolites (which were mostly reversed by Xia Yu Xue Decoction) [25] and an increased urinary excretion of glycocholate [26]. Neither serum nor liver tissue was examined in these studies. Thus, hepatic fibrosis provoked in a normal, rather than fatty, rat liver, is associated with somewhat minor changes in the urinary metabolome.

Eight metabolomic investigations of hepatic cirrhosis have all been performed on human materials, six on serum [27–32], one on liver biopsies [33], and one on faeces [34]. No clear picture emerges from these studies. An increased serum concentration of non-essential amino acids [27] and certain D-amino acids [28], a decreased serum concentration of

essential amino acids [27, 28, 31], suggests that the cirrhotic liver has an impaired ability to metabolize both protein and D-amino acids. Other notable observations include the decrease in several LPCs in serum of cirrhotics versus healthy volunteers, whether the cirrhosis was due to alcohol or hepatitis B [29]. This pattern is similar to that observed for NASH (Table 2), although the cirrhotic patients studied had a background of alcohol abuse or hepatitis B. Moreover, glycochenodeoxycholic acid and glycocholic acid concentrations were also elevated in serum [29]. Clearly, the mechanism proposed by Gonzalez and colleagues [20] shown in Fig. 2 may apply not only to NASH but other inflammatory liver diseases.

Selective impairment of hepatic β-oxidation was apparent from a reduced serum carnitine and increased serum palmitoleoylcarnitine (16:1) and oleoylcarnitine (18:1) concentrations [32]. Impaired ammonium detoxication in cirrhosis is implied from a reported shift from hepatic levels of glutamine and glucose to glutamate [33]. Finally, a very interesting report catalogued changes in the faecal metabolome between 24 healthy volunteers and 17 cirrhotics [34]. In faeces from cirrhotic patients, there was an increased concentration of the major LPCs (16:0, 18:0, 18:1, 18:2) and a decreased faecal excretion of chenodeoxycholic acid and 7-ketolithocholic acid, the latter reported as a gut flora metabolite of the former by *Bacteroides intestinalis* [35]. The data on the faecal excretion of LPCs and bile acids further supports and enhances the mechanism outlined in Fig. 2.

Hepatocellular carcinoma (HCC)

More than half a million people are diagnosed each year with hepatocellular carcinoma (HCC). The disease has a poor prognosis, generally because of its late presentation and its incidence is growing in developed countries. There has been considerable research effort to try to define biomarkers that would aid earlier detection and thus improve patient outcomes. Many researchers, particularly in China, have employed metabolomic protocols towards this end. Table 4 contains details of 24 metabolomic investigations of human HCC [27, 32, 36–57], three of chemically-induced rat HCC [42, 46, 58] and two of hepatocellular adenomas in the flatfish *Limanda limanda* [59, 60]. Many investigators of human HCC employed healthy volunteers as a control group, especially for the collection of serum/plasma or urine [27, 32, 36, 37, 39–41, 43–45, 47–50, 53, 57], others used cirrhotics as a comparator group [36, 37, 39, 45–48, 50–52, 54], while others included acute hepatitis [36, 37], chronic hepatitis [36, 37, 46, 48, 50], benign liver tumours [43] and acute myeloid leukemia [45] as comparator groups. These metabolomic comparisons have permitted insights into the biochemical transitions to HCC from various precursor states, at least as viewed through serum/plasma or urine. A relatively few studies have addressed the hepatic metabolome directly by interrogating tumour tissue and paired uninvolved liver, for human HCC [38, 55, 56], chemically-induced rat HCC [42] and fish hepatocellular adenoma [59, 60]. Two recent reports also combined transcriptomic and metabolomic analyses of human HCC [55, 56]. As will be demonstrated below, comparison of the outputs of metabolomic investigations of NAFLD/NASH, cirrhosis and HCC will permit a new understanding of the chain of biochemical events that lead from a healthy liver to HCC.

Disease progression from fatty liver to hepatocellular carcinoma

The metabolomic observations encompassed in Tables 1–4 have been combined into a visual format (Fig. 3) which permits a biochemical view of the changes occurring from fatty liver through cirrhosis to HCC. Only observations reported in at least two independent human studies have been entered into this Figure. The paramount conclusion is that elevated bile acids and lowered LPCs are common across all three groups of pathology. The bile acids affected include GCA, TCA, GDCA and GCDCA. A whole range of LPCs, comprising saturated, monounsaturated and polyunsaturated long-chain and very long-chain fatty acids are affected. The probable mechanisms by which these metabolic perturbations have occurred were discussed above and are shown, in part, in Fig. 2. Increased biliary excretion of phospholipids is an additional factor already discussed above. Of importance is that these alterations in hepatic metabolism would appear to occur very early in the chain of events leading from the normal liver to HCC (Fig. 1) and therefore must be maintained throughout the progression to HCC.

As shown in Fig. 3, NAFLD/NASH (see Tables 1, 2) is characterized by upregulation of lactate, glucose, glutamate and tyrosine, together with the downregulation of cortisone. This would suggest that, in the fatty liver states, hepatic glucose is mobilized from glycogen almost certainly due to insulin resistance [61]. The rise in lactate may be a sign of a degree of metabolic remodelling to aerobic glycolysis in response to elevated glucose, although there was little evidence of the other glycolytic metabolites, pyruvate and alanine [56], being elevated in NAFLD/NASH. The rise in glutamate is a sign of reduced cytosolic glutamine synthesis and thus an impairment of ammonium detoxication [62]. This is usually associated with cirrhosis and liver failure, but may also be manifest in NASH [63]. The upregulation of tyrosine in NAFLD/NASH is at odds with a single report in which plasma tyrosine was lower in NAFLD than controls [64]. Elevated tyrosine is more likely to be correct as the metabolomic report [9] employed a more specific analysis. Finally, the metabolomic investigations of NAFLD and NASH [9] have reported that plasma cortisone is downregulated. This is consistent with the upregulation of 11β-hydroxysteroid dehydrogenase type 1 (HSD11B1) in visceral obesity, metabolic syndrome and type 2 diabetes [65, 66] and its role in NAFLD [67]. HSD11B1 activates cortisone to cortisol in liver and adipose tissue. Thus, the aforementioned metabolomic signals are consistent with known biochemical characteristics of fatty liver. In particular, elevated serum bile acids and reduced LPCs are in accord with known changes in gene expression in NASH (Fig. 2).

As shown in Table 3, a relatively small number of metabolomic studies have addressed the conversion of either normal or fatty human liver states to cirrhosis. Only two metabolomic markers specific to cirrhosis could therefore be defined, downregulation of the branched-chain amino acids (BCAAs) valine and isoleucine. Lowered plasma BCAAs in cirrhosis was first observed almost six decades ago [68] and is due to hepatic metabolism of BCAAs to provide carbon skeletons for the TCA cycle [69]. Noteworthy is the carry forward from NAFLD/NASH into cirrhosis of elevated bile acids and reduced LPCs (Fig. 3).

The greatest number of human metabolomic studies was conducted in HCC and, not surprisingly, there occur a large number of metabolomic changes in HCC relative to cirrhosis or to control subjects (Table 4). As shown in Fig. 3, there are many signs of a

metabolic reprogramming in the livers of HCC patients, detected by metabolomics. For example, the decrease in glucose, citrate and glycerol 3-phosphate coupled with an increase in pyruvate are all signs of the Warburg effect [70], a switch from mitochondrial respiration to cytosolic aerobic glycolysis [71, 72]. By a metabolomic comparison of paired HCC biopsies and uninvolved liver tissues, we have calculated that the switch to the aerobic glycolysis in HCC is no more than four-fold [56]. Although tumours are generally considered to synthesize fatty acids *de novo* from citrate via acetyl-CoA [72], the accumulated metabolomic data in HCC (Fig.3) tend to point to increased fatty acid β-oxidation, with elevated acetate and 2-oxoglutarate (immediate precursor of carnitine) and reduced free fatty acids, carnitine and carnitine esters. Furthermore, some transcriptomic types of HCC, in particular G1 and G3, displayed markedly reduced 1-palmitoylglycerol, 1-stearoylglycerol and palmitate compared with surrounding uninvolved liver tissue [56]. Thus, metabolic reprogramming in HCC appears to comprise a modest Warburg shift to glycolysis and a major upregulation of fatty acid catabolism in some tumour types.

The metabolomic window into other hepatobiliary diseases

Alcoholic liver disease

The consumption of alcoholic beverages leads to exposure of the liver to ethanol. While many consider the pharmacological effects of ethanol consumption enjoyable, ethanol is nevertheless a solvent that can exhibit potent toxicological effects, in particular, on the liver. Alcohol exposure to laboratory animals can provoke a range of pathologies that parallels nonalcoholic liver disease. For example, 20 to 40 kg micropigs voluntarily consume an ethanol-supplemented diet (40% daily energy needs), developing peak blood ethanol levels >200 mg/dl and, within 6 months, hepatic steatosis, inflammation and fibrosis. Alcoholic micropigs displayed increased hepatic TG levels relative to controls with elevated fatty acid ratios of 16:1n7/16:0 and 18:1n9/18:0, due to increased stearoyl-CoA desaturase activity. The authors concluded that increased *de novo* lipogenesis and reduced LPC synthesis and export were responsible for the accumulation of TG during alcoholic steatohepatitis (ASH) [73]. Athymic nude mice gavaged with ethanol solutions from 5% gradually to 40%, developed mild hepatic hemorrhage, with elevated serum PC, decreased saturated and monounsaturated LPC, and elevated polyunsaturated LPC levels [74]. Similarly, rats fed 5% ethanol developed fatty infiltration after 2 months with mild inflammation and oxidative stress after 3 months. NMR metabolomics suggested that hepatic fatty acids and TG increased and plasma fatty acids and PC decreased [75]. These contradictions may reflect a species difference but more likely underscore the relative weakness of NMR as a lipidomic tool.

Another approach to study alcohol-induced liver disease (ALD) has been to employ the *Ppara*-null mouse, since the nuclear receptor PPARα is a master regulator of hepatic lipid metabolism whose biochemical effects can be detected through metabolomics, both in humans [76] and in the mouse [77]. *Ppara*-null and control mice were fed a 4% ethanol-containing liquid diet and an isocaloric control diet, respectively. After one month, steatosis with elevated hepatic TGs was observed for the *Ppara*-null mice only. Metabolomic analysis revealed elevated indole-3-lactic acid associated with the development of ALD in ethanol-

treated *Ppara*-null mice [78]. In an enlarged study, these authors reported that indole-3-lactic acid and phenyllactic acid were potential biomarkers for early ALD [79]. CYP2E1 is the principal ethanol-inducible hepatic enzyme responsible for ethanol metabolism and hepatotoxicity [80]. A metabolomic study in *Cyp2e1*-null and control mice reported that the ethanol metabolite acetate can acetylate taurine in the liver, leading to ethanol-dose-dependent production of *N*-acetyltaurine [81], a potential biomarker of ethanol hepatotoxicity. This reaction was found only in wild-type animals with hepatic CYP2E1.

Viral hepatitis B and C

Evaluation of liver disease in patients with hepatitis B or C is essential to identify patients who require antiviral therapy and to determine prognosis. Staging of liver fibrosis and the occurrence of cirrhosis associated with HBV or HCV infection is traditionally done by biopsy, but now there has been a move towards the use of noninvasive biomarkers [82]. None of the serum biomarkers that were originally developed for hepatitis C involves small molecules. Metabolomic studies in hepatitis B and C patients are very timely. The first study of its kind to evaluate deteriorating liver function in chronic hepatitis B using metabolomics was conducted in China, where HBV infection occurs in 80–90% of HCC cases [39]. Using LCMS, they established a decline in serum LPC(16:0), LPC(18:0), LPC(18:1) and LPC(18:2), together with an elevation of GCDCA (or its isomer GDCA) [83]. Another Chinese study reported similar results when examining the progression of chronic hepatitis B to cirrhosis [84]. This, of course, is the same fingerprint as seen in NALFD/NASH, cirrhosis and HCC (Figs. 2, 3). It was also reported that serum GCA, GCDCA and TCA were elevated in hepatitis B-induced cirrhosis [39]. There do not appear to be metabolomic studies comparing HBV-positive and HBV-negative subjects. It should also be pointed out that HBV may cause HCC in the absence of cirrhosis. Currently, there are no biomarkers for predicting HCC development in HBV-positive patients without cirrhosis and this should be a priority for metabolomic research.

HCV infection accounts for 70% of chronic hepatitis and 30% of liver transplants in developed countries [85–87]. Regarding HCV, atomic emission spectroscopy on scalp hair has been performed in 73 HCV-positive and 82 HCV-negative subjects, the hair concentrations of Ca, Cu, Fe, Mg, Mn and Zn determined and the data analyzed by multivariate data analysis [88]. This metallomics [89] study showed that Mg, Ca and Zn were most closely associated with HCV infection. No biological discussion of the findings was made. There has been a claim that NMR metabolomics on urine can distinguish HCV-infected from uninfected persons [90], although little data were provided. A metabolomic comparison of HCV-infected and mock-infected hepatocytes revealed small but significant increases in alanine, tyrosine and adenosine with HCV infection [91]. Interestingly, similar elevations have been recorded for NAFLD/NASH (tyrosine) and HCC (adenosine) (see Fig. 3). Preliminary findings in HCV-infected tree shrews (*Tupaia belangeri chinensis*) suggested that HCV affects many pathways in the liver, with alterations in LPCs and bile acids (as for other liver diseases, Fig.3), carnitine esters, fatty acids and LPEs [92]. It is clear, therefore, that both HBV and HCV infection, together with NASH, trigger similar molecular events represented by the mechanisms shown in Fig. 2. Moreover, both alcohol- and HBV-induced cirrhosis displayed higher bile acids and lower LPCs than healthy

controls in an almost identical manner [29]. It would appear that the depressed LPCs and elevated bile acids in serum represent a phenotype of hepatitis and cirrhosis independent of etiological origin, and that this phenotype is carried forward into any resultant HCC.

Cholangiocarcinoma

Cholangiocarcinoma (CCA) is an aggressive cancer originating from the biliary tract. It would appear that obesity, diabetes, hepatitis B and C, alcohol use and cirrhosis are all major risk factors for CCA, suggesting a common pathogenesis with HCC [93]. It has also been proposed that genetically impaired biliary excretion of phospholipids underlies CCA [94, 95]. Metabolomic investigations support this view, with lower phosphatidylcholine and elevated glycine- and taurine-conjugated bile acids reported in the bile of CCA patients [96, 97].

Cholestasis and cholecystitis

Interruption of bile flow may have an extrahepatic and obstructive or an intrahepatic and biochemical basis. An NMR metabolomic study has been performed in rats in an attempt to use urinary biomarkers to distinguish the two mechanisms [98]. Metabolomics revealed that cholestasis induced in *Fxr*-null mice by a cholic acid diet resulted in increased urinary excretion of bile salt tetrols, predominantly $3\alpha,6,7\alpha,12\alpha$-tetrahydroxy-$5\beta$-cholestan-26-oyltaurine, due to an adaptive upregulation of the steroid-hydroxylating cytochrome P450 CYP3A11 in these mice [99]. An adaptive response was also characterized in a rat cholestasis model, with a shift from cytotoxic to cytoprotective bile acids in plasma and urine [100].

Injection of *Escherichia coli* into the rabbit gallbladder produces a model for acalculous cholecystitis (AAC). Compared to saline-injected controls, AAC animals displayed increased serum LDL and VLDL, with decreased serum phospholipids, lactate, 3-hydroxybutyrate, citrate, lysine, asparagine, histidine and glucose as demonstrated by NMR metabolomics [101]. These observations need to be refined with the use of LCMS-based metabolomics.

Liver transplantation

As shown in Fig. 1, several end-stage liver diseases require transplantation. A metabolomic study of single patient with hepatitis B and HCC who underwent two consecutive liver transplants showed that the first failed graft was associated with elevated blood lactate, uric acid, citrate, glutamine and methionine, diagnostic of dysfunctional hepatic metabolic fluxes [102]. A series of 15 HCC patients displayed increased valine, alanine, acetone, succinate, glutamine, choline, lactate and glucose one day after transplantation. After 7 days, lipids and choline increased while glucose and amino acids decreased [31]. The metabolomic window appears to offer new insights into specific hepatic metabolic changes in the transplantation perioperative period.

Miscellaneous other hepatobiliary diseases

Metabolomic studies have been reported that are of relevance to Wilson's disease [103, 104], primary biliary cirrhosis [105], primary sclerosing cholangitis [105], the hepatic stage of malaria [106–108], as well as various aspects of hepatic encephalopathy [109–112].

The metabolomic window into acute liver toxicity in animal models

High-throughput metabolomic screening of hepatotoxins in laboratory animals first used NMR and pattern recognition algorithms [113–115] but, in early studies, also employed Fourier-transform infrared spectroscopy [116]. Metabolomic profiles of numerous hepatotoxins in laboratory animals have been described, and include hydrazine [117], bromobenzene [118, 119], methapyrilene [120], methylenedianiline [121], D-galactosamine [121–123], clofibrate [121], allyl formate [124], the anti-HBV compound Bay41-4109 [125], paracetamol [126–133], isoniazid [134, 135], carbon tetrachloride [131, 136–138], α-naphthylisothiocyanate [137], perfluorododecanoic acid [139], valproate [140], Huang-yao-zi [141], dimethylnitrosamine [142], polychlorinated biphenyls [143, 144], 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [143], methamphetamine [145], (+)-usnic acid [146], pentamethychromanol [147] and methotrexate [131]. Detailed analysis of these drug-induced liver injury (DILI) studies falls beyond the scope of this review. However, the reader is directed to The Liver Toxicity Biomarker Study on DILI and closely related topics that have been reviewed [148–153].

A proposed metabolomics-based model for major liver disease

Based upon a review of the available literature, we propose a three-stage progression from hepatic insult of the healthy liver to carcinoma (Fig. 4). A core metabolomic phenotype (CMP) arises early in this progression and comprises readily discernible changes in bile acids and phospholipids (Tables 1–4, Fig. 3). The CMP is maintained whether or not cirrhosis arises and/or HCC or CCA develop (Stages 2 and 3, respectively). This CMP is common to all etiologies in Stage 1, including NAFLD/NASH, ALD and viral hepatitis. Other metabolomic perturbations distinguish the different stages (Fig. 3). We also propose that the metabolic remodelling described for HCC [56] begins at the transition from Phase 0 to Phase 1 as a consequence of the presence of inflammatory signalling in the liver, as outlined in Fig. 2. Thus, this body of accumulated metabolomic data may begin to cast further light on hepatobiliary diseases.

Acknowledgments

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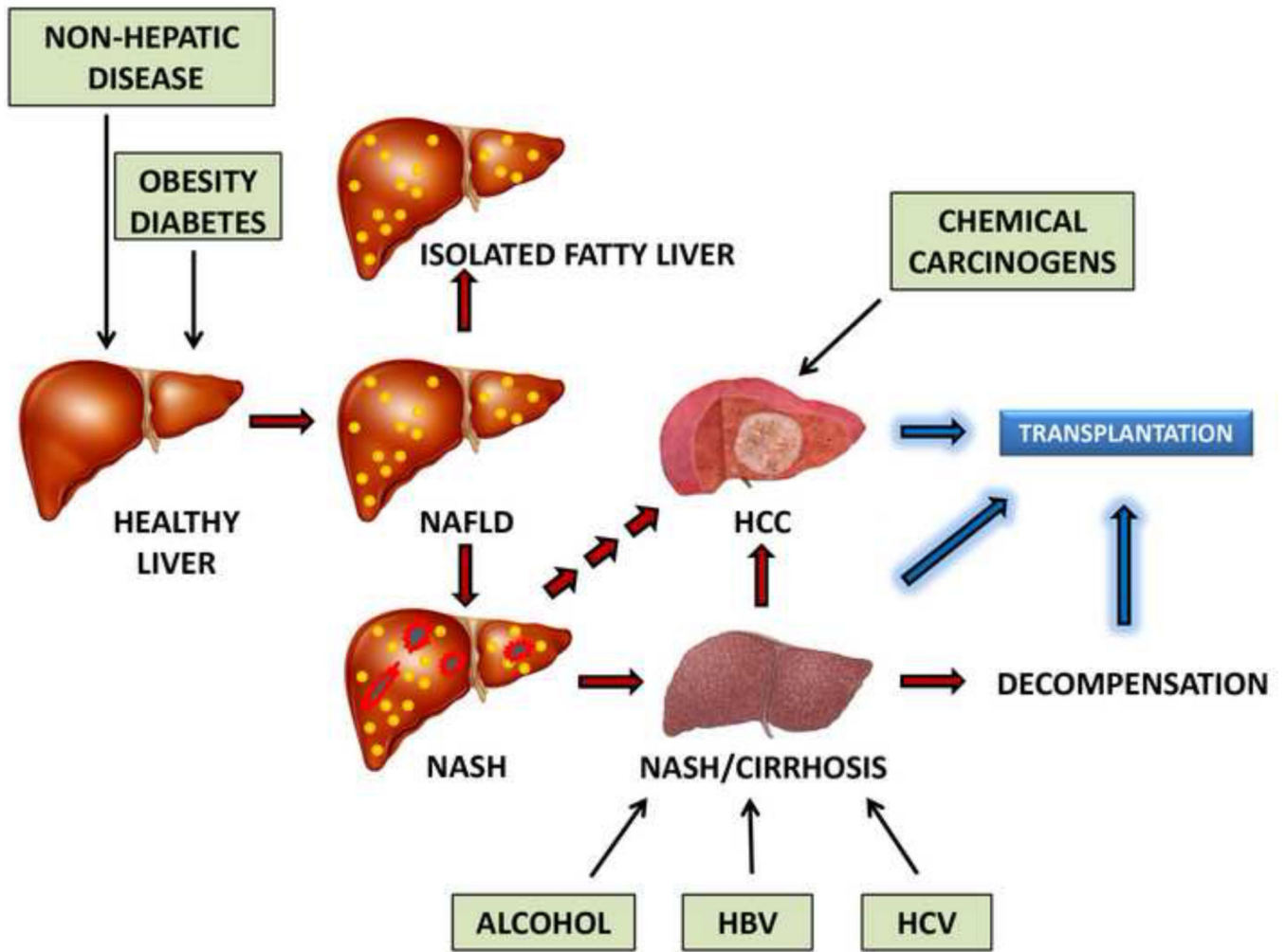
Fig. 1. Major liver diseases and potential influencing factors
This schematic shows the development of NAFLD from a healthy liver various influencing factors (green boxes). Steatosis is shown in yellow. NAFLD mostly becomes isolated fatty liver, but some cases progress to NASH, showing both steatosis and inflammatory necrosis (shown in red and black). NASH may progress to cirrhosis and then to HCC or to HCC directly. HCC, cirrhosis and decompensated cirrhosis may all be treated by liver transplantation. Chemical carcinogens, such as aflatoxin B_1, together with alcohol and HBV and HCV infection, are all potential influencing factors (green boxes).
Abbreviations used: NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus.
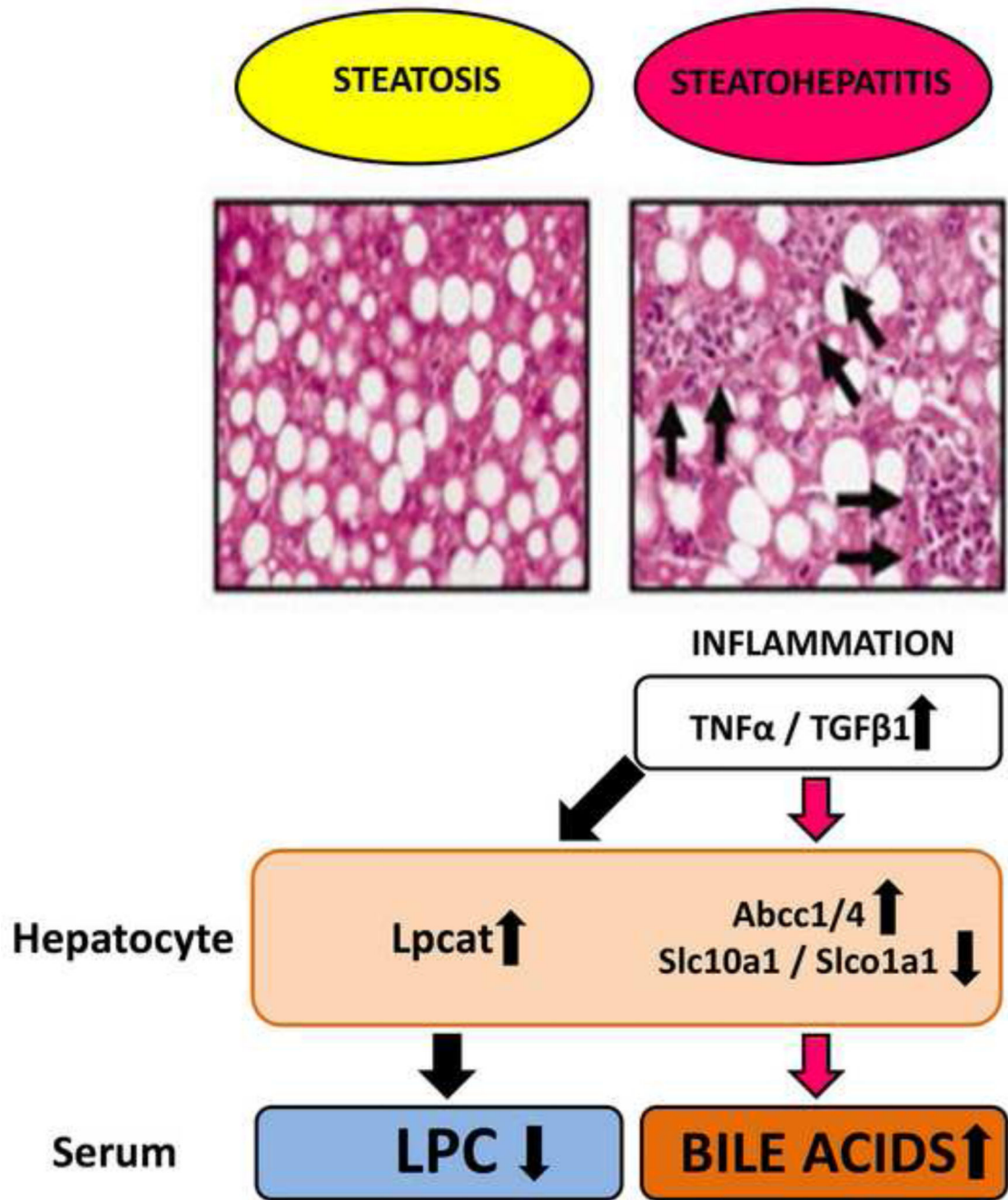
Fig. 2. Mechanisms leading to lowered LPCs and elevated bile acids in serum in NASH
Reproduced with permission from Tanaka *et al.* Disruption of phospholipid and bile acid
homeostasis in mice with nonalcoholic steatohepatitis. *Hepatology* 2012;56:118–129.
Abbreviations used: LPC, lysophosphatidylcholine.
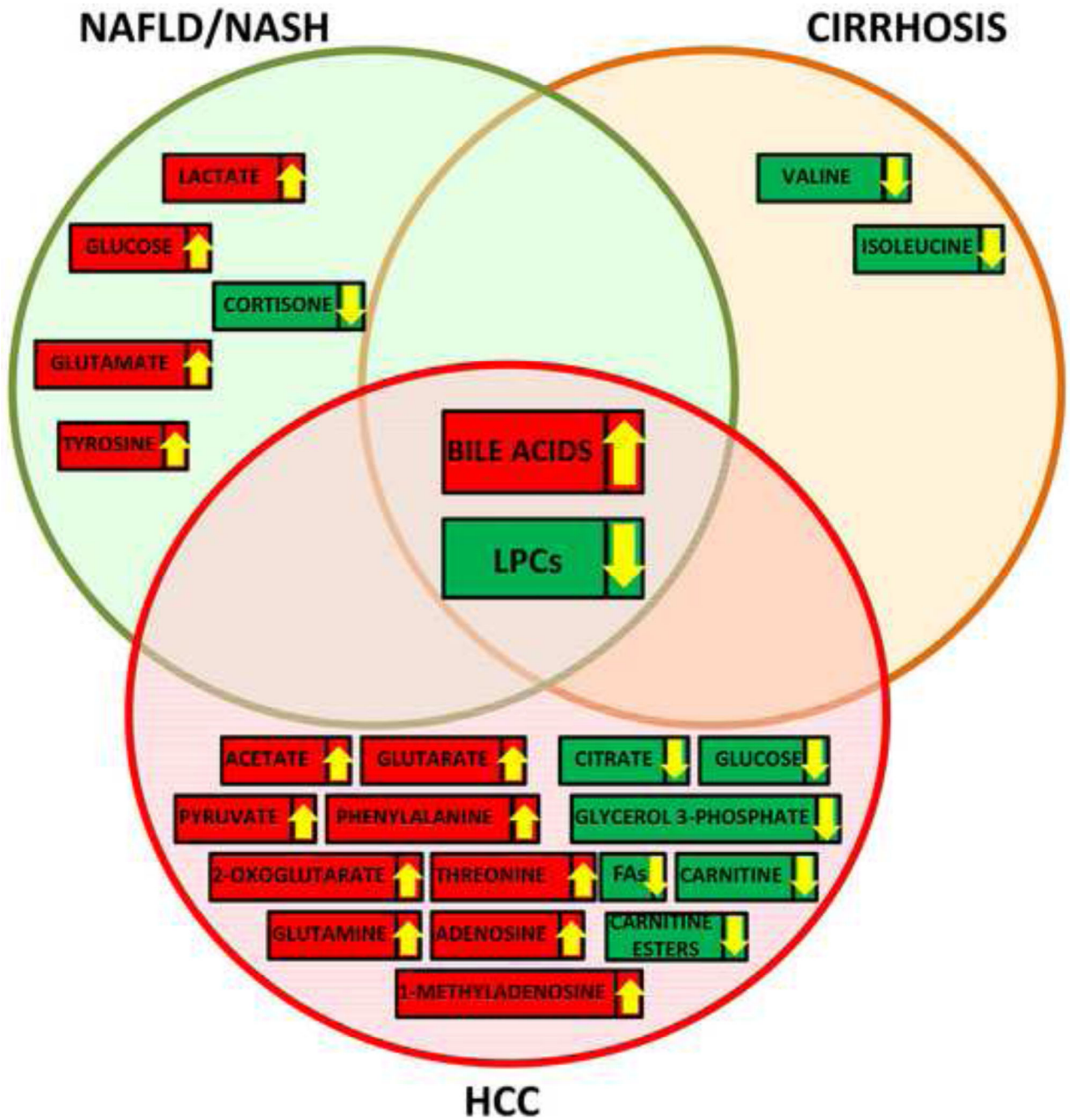
Fig. 3. Venn diagram showing the up- and down-regulated metabolites in NAFLD/NASH, cirrhosis and HCC

Elevated bile acids and lowered lysophosphatidylcholines are common across the pathological evolution in humans and comprise a core metabolomic phenotype. For abbreviations, see Fig. 1.
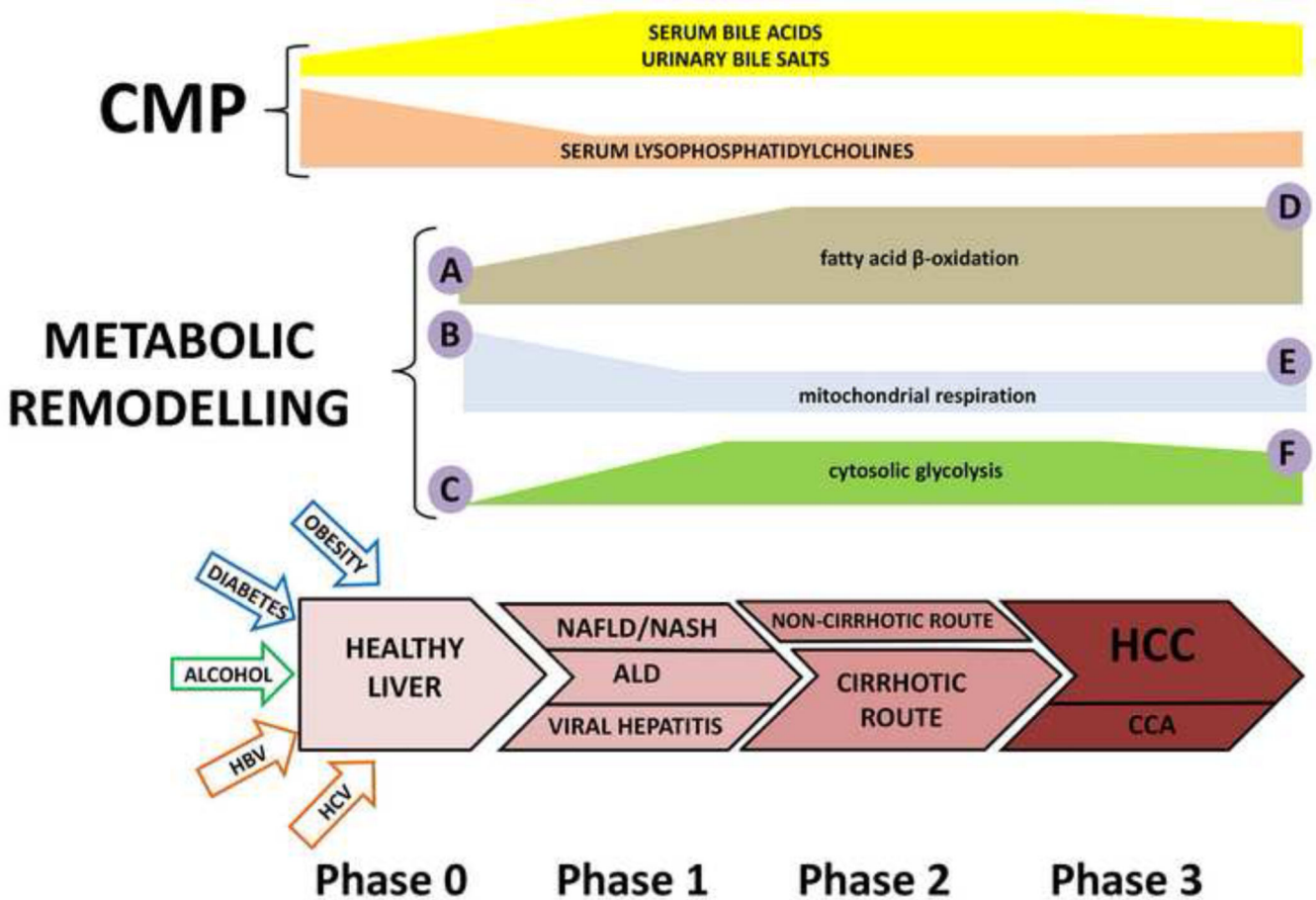
Fig. 4. Diverse hepatic insults leading to a core metabolomic phenotype en route from the healthy liver to HCC and CCA

Elevated serum bile acids and urinary bile salts together with decreased serum lysophosphatidylcholines represent the core metabolomic phenotype (CMP). A metabolic remodelling begins in the transition from the healthy liver (Phase 0) to NAFLD/NASH, ALD or viral hepatitis (Phase 1). During Stage 1 there occurs a Warburg shift from mitochondrial respiration to cytosolic glycolysis, together with an increase in fatty acid β-oxidation. This metabolic remodelling persists through cirrhosis (Phase 2) and into carcinoma (Phase 3). Note that the sum of the carcinoma energy production D+E+F is greater than the summed energy production A+B+C in the healthy liver.

Abbreviations used: CCA, cholangiocarcinoma; CMP, core metabolomic phenotype; ALD, alcoholic liver disease. For other abbreviations, see Fig. 1.

Table 1

Summary of metabolomic studies examining the development of NAFLD

species [ref]	tissue	platform	up-regulated	down-regulated	conclusions
Human **[9 obese with normal liver; 24 NAFLD]** **GNMT−/− mice** [10]	serum	UPLC-ESI-QTOFMS	Common to NAFLD & GNMT−/− mice: DCA	Common to NAFLD & GNMT−/− mice: FAs 20:2n6, 18:3n3/18:3n6, LPC(20:2), LPC(20:1), SM(36:3), SM(d18:2/16:0), SM(18:2/14:0), SM(d18:1/12:0), LPC(20:2), LPC(20:1), TDCA, TCDCA, DG(18:0/22:6)	lipogenesis↑
Human **[50 HV; 25 NAFLD]** [7]	plasma	HPTLC GCFID LCMS	Triacylglycerols, FAs: 16:0, 14:1n5, 16:1n7, 18:1n9, 18:1n7, 18:3n6, 18:4n3, 20:3n6, 22:5n3, 15-HETE	-	
Human **[25 HV; 11 NAFLD]** [9]	plasma	UPLC-ESI-TQMS GCMS	GCA, TCA, GCDCA, 4:0-carnitine, glutamate, tyrosine, lactate	cysteine-glutathione disulfide, LPC(18:1), cortisone, uridine	oxidative stress↑ Bile salts↑
Human **[23 non-steatotic; 23 steatotic]** [8]	liver	UPLC-ESI-QTOFMS	Dextrin, GCDCA-3-sulfate, TCDCA, glycerophosphocholine, LPC(16:0), LPC(18:3), LPE(16:0), LPE(18:0), LPE(18:3), PC(36:5), PC(36:2), PC(36:4)	GSH, GSSG, L-glutamyl-L-lysine, L-leucyl-L-proline, glutamate	Oxidative stress↑ Bile salts↑ Phospholipid synthesis↑ Hepatic glucose catabolism↑
Mouse fed methionine and choline deficient diet vs. control mice **Human** **[28 HV; 15 NAFLD; 11 NAFLD with necro-inflammation]** [12]	serum	NMR	Mice: lactate NAFLD: lactate, glutamate	Mice: glucose, choline, TMAO, betaine, VLDL	Glycolysis↑
Mouse [fed high-fat diet] [12]	plasma	NMR	lactate, pyruvate, glucose, fucose, phosphatidylcholine, TMAO, alanine	albumin	glucose uptake/mobilization↑ glycolysis↑ phospholipid synthesis↑
Mouse [fed high-fat diet] [12]	plasma	NMR	lactate, pyruvate, glucose, fucose, phosphatidylcholine, TMAO, alanine	albumin	glucose uptake/mobilization↑ glycolysis↑ phospholipid synthesis↑
Mouse [24h starvation] [5]	liver	FPLC HPTLC LCMS	Cholesterol esters, triacylglycerols TG(49:4), TG(44:2), TG(48:3)	phosphatidylcholine	mobilization of TGs from adipose to liver↑
Mouse (LDLr−/−) fed high-fat diet ± cholesterol [4]	liver	NMR	Triacylglycerols, diacylglycerols, sphingomyelins, G-6-P, G-1-P, glycerol	PUFA/MUFA, fumaric acid	cholesterol is influencing factor SCD1↓
Rat H4IIEC3 hepatoma cells treated with PA (apoptosis) ± OA(steatosis) [14]	cells	GCMS	Associated with PA + OA (steatosis): fructose, gluconate, glutamate, desmosterol	Associated with PA alone (apoptosis): adenosine, malate, serine, citrate, aspartate, C16 ceramide, diacylglycerol	None for NAFLD, but several for NASH (lipoapoptosis phenotype)
Piglet [Caesarean section] [6]	liver	NMR	Total lipid (>5 mg/g liver), cholesterol esters, triacylglycerols, glycerol	glycerol phosphate	gluconeogenesis from glycerol↓

Abbreviations used: HV, healthy volunteers; PA, palmitic acid; OA, oleic acid; NMR, nuclear magnetic resonance spectroscopy; FPLC, fast performance liquid chromatography; HPTLC, high performance thin-layer chromatography; LCMS, liquid chromatography-mass spectrometry; GCFID, gas chromatography with flame ionization detection; UPLC, ultraperformance liquid chromatography;

ESI, electrospray ionization; TQMS, triple quadrupole mass spectrometry; QTOFMS quadrupole time-of-flight mass spectrometry; TMAO, trimethylamine *N*-oxide; TG, triacylglycerol (triglyceride); FA, fatty acid; 15-HETE, (±)-15-hydroxy-5*Z*,8*Z*,11*Z*,13*E*-eicosatetraenoic acid (non-enzymic oxidation product of arachidonic acid); DCA, deoxycholic acid; GCA, glycocholic acid; TCA, taurocholic acid; GCDCA, glycochenodeoxycholic acid; TCDCA, taurochenodeoxycholic acid; G-6-P, glucose-6-phosphate; G-1-P, glucose-1-phosphate; GNMT, glycine *N*-methyltransferase; PUFA, polyunsaturated fatty acids; MUFA, monounsaturated fatty acids; LPC, lysophosphocholine; SCD1, stearoyl-CoA desaturase-1

Table 2

Summary of metabolomic studies examining the development of NASH

species [ref]	tissue	platform	up-regulated	down-regulated	conclusions
Human [9 obese with normal liver; 24 NAFLD: 9 NASH] [10]	serum	UPLC-ESI-QTOFMS	NASH vs. NAFLD: PC(14:0/20:4), LPC(18:1)	NASH vs. NAFLD: LPC(24:0)	A few lipid changes between NAFLD and NASH of uncertain origin.
Human 28 HV; 6 NASH	serum	NMR	Glucose, glutamate, taurine	-	Increased glucose mobilization.
Humans [HV 50; NASH 50] [7]	plasma	HPTLC GCFID LCMS	Triacylglycerols, FAs: 14:1n5, 16:1n7, 18:1n9, 18:1n7, 18:3n6, 20:3n6, 22:6n3, 5-HETE, 8-HETE, 15-HETE, 11-HETE		lipogenesis↑
Human [25 HV; 24 NASH] [9]	plasma	UPLC-ESI-TQMS GCMS	N-Acetylthreonine, aspartate, glutamate, phenylalanine, tyrosine, 3-(4-hydroxyphenyl)-lactate, kynurenine, isoleucine, leucine, valine, ornithine, glutamylvaline, γ-glutamyl-leucine, γ-glutamyl-phenylalanine, γ-glutamyl-tyrosine, erythronate, mannose, glucose, pyruvate, lactate,2-oxoglutarate, carnitine, propionylcarnitine, butyrylcarnitine, 2-methylbutyroylcarnitine, GCA, TCA, GCDCA, xanthine, urate, pseudouridine, erithritol	N-Acetylglycine, betaine, histidine, phenylacetate, indolepropionate, 2-aminobutyrate, cysteine-glutathione disulfide, glycerate, 20:5n3, 22:6n3, 11:1n1, 20:4n6, 2-hydroxypalmitate, 3-carboxy-4-methyl-5-propyl-2-furanpropanoate, glycerophosphocholine, LPC(18:1), LPC(18:2), LPC(20:4), cortisone, threonate, hippurate, catechol sulfate, indoleacrylate, 3-phenylpropionate	Elevated bile acids GCA, TCA and GCDCA a sign of liver injury or insulin resistance. High rate of GSH turnover reflective of oxidative stress. Origin of certain depressed PUFAs and LPCs uncertain. Increased glucose mobilization and formation of pyruvate and lactate suggests cytosolic glycolysis. Elevation of essential amino acids suggests increased protein turnover.
Mouse fed methionine- and choline-deficient diet [11]	serum	UPLC-ESI-QTOFMS	Tauro-β-muricholate, TCA, 12-HETE	LPC(16:0), LPC(18:0), LPC(18:1)	Disruption of bile acid and phospholipid homeostasis due to hepatic inflammatory signaling.

For abbreviations, see Table 1 footnotes.

Table 3

Summary of metabolomic studies examining the development of hepatic fibrosis and cirrhosis

species [ref]	tissue	platform	up-regulated	down-regulated	conclusions
Human [63 HV; 36 LC] [27]	serum	NMR	Acetate, pyruvate, glutamine, "N-acetylglycoproteins", 2-oxoglutarate, taurine, glycerol, tyrosine, 1-methylhistidine, phenylalanine	LDL, VLDL, leucine, isoleucine, valine, acetoacetate, choline, unsaturated lipid	Downregulation of essential amino acids suggests depressed protein turnover.
Human [16 HV; 25 LC] [28]	serum	GCxGC-TOFMS	D-Alanine, D-proline	L-Alanine, L-valine, L-isoleucine, L-leucine, L-serine, L-asparagine	Targeted amino acid analysis reveals loss of ability by the cirrhotic liver to metabolize D-amino acids
Human [22 HV; 18 LC (alcohol); 19 LC (HBV)] [29]	serum	UPLC-ESI-QTOFMS	GCDCA, GCA, L-acetylcarnitine, myristamide, oleamide (only in alcohol cirrhosis)	LPC(16:0), LPC(18:2), LPC(18:0), LPC(20:3), LPC(20:5) myristamide, oleamide (only in HBV cirrhosis)	Pattern very similar to NASH and so NASH signature dominates serum picture in cirrhosis, irrespective of origin as alcohol or HBV.
Human [HV and LC (HBV)] [30]	serum	LCMS	GCDCA	LPCs	Confirmatory of bile acid and phospholipid perturbations.
Human [30 HV; 30 LC (compensated); 30 LC (decompensated)] [31]	serum	NMR	-	In both compensated and decompensated LC: isoleucine, valine	Many other changes recorded, but with OPLS-DA correlations <0.85
Human [30 HV; 30 CHB; 30 LC] [32]	serum	LCMS	Relative to HV: 16:1-carnitine, 18:1-carnitine	Relative to HV: carnitine, pimeloylcarnitine Also relative to CHB: PE(22:6/16:0), PE(20:4/18:0)	Elevation of two MUFA-carnitines suggests reduced β-oxidation of these two fatty acids.
Human [24 HV; 17 LC] [34]	faeces	UPLC-ESI-QTOFMS	LPC(16:0), LPC(18:0), LPC(18:1), LPC(18:2)	CDCA, 7-ketolithocholic acid, urobilin, urobilinogen	Increased LPCs in faeces consistent with lower serum LPCs in LC. Biliary excretion of bile acids known to be reduced in LC
Human [57 non-LC; 11 LC] [33]	liver	NMR	UFA, phosphocholine, glutamate, phosphoethanolamine	Choline, TMAO, α-glucose, glutamine, aspartate, β-glucose	Shift from glutamine and glucose to glutamate suggests a net release of ammonium and impaired ammonium detoxication.
Rat treated with CCl4, then with Xia Yu Xue Decoction [25]	urine	GCMS	Apart from propionate and leucine, all changes due to CCl4 were reversed by Xia Yu Xue Decoction.	Effect of CCl4: Propionate, benzoate, leucine, octanoate, phenol, glycine, indole, oleic acid, lysine	Some metabolic changes of uncertain origin that may be associated with fibrosis
Rat treated with CCl4 [26]	urine	UPLC-ESI-QTOFMS	Glycocholate		Hard to evaluate in this model if GCA is really a marker for fibrosis
Rat treated with thioacetamide [22]	liver	NMR	Lactate, choline, proline, "glutamine/glutamate"	TMA, glycogen, inosine, fumarate	Raw data very poor and these NMR findings have uncertain validity

Abbreviations used: LC, liver cirrhosis; CHB, chronic hepatitis B; TMA, trimethylamine; CCl4, carbon tetrachloride; UFA, unsaturated fatty acid units (-CH=CH-CH2-), GCxGC-TOFMS, Two-dimensional gas chromatography time-of-flight mass spectrometry. OPLS-DA, orthogonal partial least squares projection to latent structures-discriminant analysis, PE, phosphatidylethanolamine. For other abbreviations, see footnotes to Table 1

Table 4

Summary of metabolomic studies examining the development of hepatocellular carcinoma

species [ref]	tissue	platform	up-regulated	down-regulated	conclusions
Human [63 HV; 39 HCC] [27]	serum	NMR	Acetate, "N-acetylglyco-proteins", pyruvate, glutamine, 2-oxoglutarate, glycerol, tyrosine, 1-methylhistidine, phenylalanine	LDL, VLDL, valine, acetoacetate, choline, taurine, "unsaturated lipid"	Increased lipid catabolism because of LDL/VLDL↓ and acetate↑. Many signs that TCA cycle is impaired – pyruvate↑, acetoacetate↓, glutamine/2-oxoglutarate↑ Essential amino acids and metabolites elevated due to increased protein turnover.
Human [25 HV; 25 LC(HBV); 24 HCC] [39]	serum	HPLC-ESI-TOFMS	TCA, GCA, bilirubin, TCDCA, GCDCA, FA(18:1), carnitine, acetylcarnitine	Hypoxanthine, phytosphingosine, dihydrosphingosine, LPC(18:2), LPC(18:3), LPC(16:1), LPC(18:0), taurine, 6-methyl-nicotinic acid	HCC/HV ratios much smaller than LC/HC ratios, suggesting that HCC diminishes the LC metabolomic phenotype. Bile acid and LPC findings consistent with other data. Data suggest reduced β-oxidation of FAs. Reduced sphingosines suggest increased ceramide synthesis and thus, increased death signalling.
Human [38 HV; 41 HCC] [44]	serum	UPLC-ESI-TQMS	1-Methyladenosine	-	A biomarker study that compared 1-methyladenosine ± AFP
Human [90 HV; 48 LC; 82 HCC] [47]	serum	UPLC-ESI-QTOF-MS	Canavaninosuccinate, phenylalanine, GCDCA, oleamide	LPC(16:0), LPC(18:0), PC(16:0/22:6), PC(16:0)/20:4), PC(18:0/18:2)	Canavaninosuccinate synthesized by argininosuccinate synthase, presumably induced in HCC. Decreased PCs as well as LPCs suggests increased biliary excretion of phospholipids, rather than increased synthesis of PCs from LPCs by Lpcat (Fig. 2).
Human [30 HV; 30 CHB; 30 LC; 30 HCC] [48]	serum	UPLC-ESI-QTOF-MS	GCA, GCDCA, 16:1-carnitine,	Tryptophan, LPC(14:0), 10:0-carnitine, 10:1-carnitine, 8:0-carnitine, 6:0-carnitine	Increased β-oxidation of short- to medium-chain FAs.
Human [30 HV; 30 CHB; 30 LC; 30 HCC] [50]	serum	UPLC-ESI-QTOF-MS	16:1-carnitine, 18:1-carnitine, PE(22:6/16:0)	carnitine, pimeloylcarnitine	Reduced β-oxidation of MUFAs.
Human [184 LC; 78 HCC] [51]	serum	UPLC-ESI-QTOF-MS	-	Relative to LC: GCA, GDCA, TCA	Bile acid export to blood mostly a feature of LC not HCC.
Human [49 LC; 40 HCC] [52]	serum	UPLC-ESI-QTOF-MS	Relative to LC: PhePhe	Relative to LC: TCDCA, GDCA, 3β, 6β-dihydroxy-5β-cholan-24-oic acid, 18:1-carnitine, 18:2-carnitine	Bile acid export to blood mostly a feature of LC not HCC. Increased β-oxidation of FA(18:1) AND FA(18:2) in HCC.

species [ref]	tissue	platform	up-regulated	down-regulated	conclusions
Human [93 LC; 28 small HCC; 33 large HCC] [54]	serum	NMR	Glutamate, acetate	Glutamine	Shift from glutamine in LC to glutamate in HCC suggests defect in ammonium detoxication in HCC. Acetate↑ suggests increased β-oxidation of FAs.
Human [6 HV; 22 AML; 7 LC; 20 HCC] [45]	plasma	UPLC-ESI-QTOF-MS	Bilirubin, biliverdin, GDCA, DCA 3-sulfate, 7α-hydroxy-3-oxochol-4-en-24-oic acid, 3-oxachol-4,6-dien-24-oic acid, LPC(24:0)	LPC(14:0), LPC(16:0), LPC(18:0), LPC(18:1), LPC(18:2), LPC(18:3), LPC(20:2), LPC(20:3), LPC(20:4), LPC(20:5), LPC(22:6), FA(24:0), FA(24:1)	Increased bile acid transport into blood, including fetal bile acids. Increased metabolism of LPCs and/or biliary excretion.
Human [30 HV; 28 HCC] [32]	plasma	GCTOF-MS	No significantly elevated molecules in HCC vs. HV	No significantly depressed molecules in HCC vs. HV	Multiple comparisons not allowed for.
Human [71 HV; 24 BLT; 82 HCC] [43]	serum urine	UPLC-ESI-QTOF-MS GCTOF-MS	**Serum:** GCDCA, GCA, cysteine, fumarate, 2-oxoglutarate, lactate, pyruvate, inosine, erythronate, carnitine **Urine:** GCA, dopamine, adenosine, urate, xanthine, phenylalanine, dihydrouracil, hypotaurine, threonine, N-acetylneuraminic acid	**Serum:** AA, EPA, DHA, glycerol, FA(14:0), FA(24:1), glycine, serine, aspartate, citrulline, ornithine, kynurenine, tryptophan, lysine, glucosamine, 5-oxo-proline, phenylalanine, β-alanine, α-tocopherol, glycerate, 3-amino-2-piperidone, D-arabino-hexos-2-ulose, arabinose, creatinine, oleamide, phosphate **Urine:** Cysteine, TMAO, homovanillate, normetanephrine, adenine, cysteic acid, 6-aminohexanoate, creatine	Reduced ammonium detoxication through the urea cycle. Metabolic reprogramming to glycolysis. Increased export of bile acids into blood and then to urine. Reduced serum free carnitine consistent with increased β-oxidation of FAs.
Human [50 HV; 27 LC; 30 acute hepatitis; 20 chronic hepatitis; 48 HCC] [36]	urine	HPLC	Assay specific for cis-diols and so nucleosides detected: pseudouridine, 1-methyladenosine, xanthosine, 1-methylinosine, 1- and 2-methylguanosine, N^4-acetylcytidine, adenosine	-	Probably a sign of increased tRNA turnover and of inflammation rather than HCC.
Human [50 HV; 27 LC; 30 acute hepatitis; 20 chronic hepatitis; 48 HCC] [37]	urine	LCMS	-	-	Reanalysis by LCMS of same samples as [232.] with no further information.
Human [17 HV; 16 HCC (11 with HCV] [57]	urine	NMR	No elevated molecules.	Glycine, TMAO, hippurate, citrate	Decreased glycine may drive decreased urinary hippurate.
Human [20 HV; 19 paired pre- and post-operative HCC patients; patients divided into 7 recurrent; 11 nonrecurrent; one unknown] [49]	urine	GC-TOFMS	Adenine (RPost/NRPost), threonine (RPost/RPre)	-	Not corrected for multiple comparisons. Most findings not statistically significant.

species [ref]	tissue	platform	up-regulated	down-regulated	conclusions
Human [12 HV; 25 HCC] [53]	urine	UPLC-ESI-QTOF-MS	GCA	-	Increased bile acid export into blood in HCC vs. HV.
Human [20 HV; 20 HCC] [40]	urine	GCMS	A total of 18 metabolites listed as different between HV and HCC, but no correction for multiple comparisons was made. Therefore, only xylitol and urea elevated.	-	Xylitol a sign of a switch from the TCA cycle to the pentose phosphate pathway and thus from catabolism to anabolism, e.g. synthesis of nucleic acids
Human [24 HV; 21 HCC] [41]	urine	UPLC-ESI-QTOF-MS	A total of 15 metabolites listed as different between HV and HCC, but no correction for multiple comparisons was made. Therefore, no metabolites elevated.	Three metabolites significantly reduced: carnitines 4:0, 8:1 and 9:0	Authors claim the decline of acylcarnitines in urine is a sign of reduced β-oxidation in HCC. It is surely a sign of increased β-oxidation on HCC.
Human [31 HCC from 17 patients; 14 adjacent nontumour livers] [38]	liver	NMR	Glutamine, glutamate	α-glucose, β-glucose	Increased glycolysis.
Human [30 pairs HCC and nontumour tissue; 356 HCC] [55]	liver	LCMS GCMS	55 Annotated metabolites upregulated in HCC, of which 5-methylthioadenosine, 4:0-carnitine, 6:0-carnitine, 16:0-carnitine, 18:0-carnitine and ophthalmate had greatest fold change	103 Annotated metabolites downregulated in HCC, of which NAD$^+$, glycerol 3-phosphate, LPC(18:2), GCA and xanthosine had greatest fold change	Consistent with shift to glycolysis. Decreased β-oxidation of short- and long-chain FAs.
Human [31 pairs HCC and nontumour tissue; 59 HCC typed by transcriptomics as G1 to G6] [56]	liver	GCMS	-	Downregulated in HCC: glucose, glycerol 2- and 3-phosphate, malate, alanine, myo-inositol, FA(18:2). Downregulated in transcriptomic groups G1 and G3: palmitate, 1-palmitoylglycerol, 1-stearoylglycerol	Consistent with shift to glycolysis in HCC. Consistent with increased β-oxidation of long-chain FAs in groups G1 and G3.
Rat [20 DEN-treated HCC; 28 control rats] Human [262 HCC sera; 76 LC sera; 74 hepatitis sera] [46]	serum	UPLC-ESI-QTOF-MS	Three molecules elevated in HCC rat serum – LPC(22:5), LPE(16:0), TCA Same three molecules used as biomarkers to distinguish HCC, LC and hepatitis	-	Only elevated bile acid consistent with other data.
Rat [5 control; 5 DEN-treated HCC; 5 DEN-treated HCC with lung metastases (HLM)] [58]	serum urine	GCTOF-MS	**Serum:** A total of 47 metabolites listed, of which 18 had P<0.05 by ANOVA for control, HCC and HLM. No correction for multiple comparisons was made. Nine remained significant after Bonferroni correction. Elevated: lactate, tyrosine **Urine:** 13 metabolites had P<0.05 by ANOVA for control, HCC and HLM. Only one remained significant after Bonferroni correction. No metabolites elevated.	**Serum:** Oxalate, glutamate, arabinose, glucose, stearate, adipate, phosphoinositide Urine: hippurate	Decreased hippurate may be a sign of decreased glycine or CoA availability. Glycine appeared to rise >4-fold in rat HCC. Therefore, most likely explanation, other than an effect on the gut flora, is decreased CoA availability due to enhanced β-oxidation of FAs.
Rat [5 control; 5 DEN-treated HCC; 5 DEN-treated HCC	liver	NMR	A total of 15 metabolites listed as different between control and HCC rats, but no correction for multiple comparisons was made. Six remained significant after	Three metabolites significantly reduced: TMAO, glucose, glycogen	Increased glycolysis and β-oxidation of FAs.

species [ref]	tissue	platform	up-regulated	down-regulated	conclusions
with lung metastases (HLM)] [42]			Bonferroni correction. Elevated: Leucine, acetate, glutamine		
Dab (*Limanda limanda*) [9 hepatocellular adenomas; 9 paired nontumour samples] [60]	liver	FTICR-MS	M_R = 521.3500 (probably, LPC(18:1) [3 ppm error])	M_R = 495.3343 (probably, LPC(16:0) [13 ppm error]) M_R = 533.2902 (?) M_R = 591.2066 (?)	Possible changes in LPCs in this liver tumour.
Dab (*Limanda limanda*) [10 hepatocellular adenomas; 10 paired nontumour samples] [59]	liver	NMR	Propionate, succinate, lactate	Lysine, phosphocholine	Switch to glycolysis in the tumour.

Abbreviations used: FTICR, Fourier transform ion cyclotron resonance mass spectrometry; M$_R$, relative molecular mass (molecular weight); GCTOFMS, gas chromatography time-of-flight mass spectrometry; BLT, benign liver tumour; AA, arachidonic acid; EPA, 5Z,8Z,11Z,14Z,17Z-eicosapentaenoic acid; DHA, 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoic acid; TQMS, triple quadrupole mass spectrometry; AFP, α-fetoprotein; LPE, lysophosphoethanolamine; TCA, taurocholic acid; CHB, chronic hepatitis B; RPost, recurrent HCC post-surgery; NRPost, nonrecurrent HCC post-surgery; RPre, recurrent HCC pre-surgery; For other abbreviations, see footnotes to Table 1