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Research Article

Towards the clinical use of peripheral bile acids: Recommendations to limit their preanalytical and analytical sources of variability

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ABSTRACT

Despite more than three decades of research, the use of peripheral bile acids as biomarkers for human liver injury remains inconclusive due to inconsistent findings. Identifying the factors contributing to the variability in published peripheral bile acid data in humans is needed to propose experimental recommendations that could enhance the robustness and reproducibility of future studies. Besides the peripheral blood bile acid data, the metadata on subject demographics (number of subjects, average age, sex distribution, health status, fasted/fed status), the blood matrix analyzed, the matrix volume analyzed, the bile acid extraction process, and analytical technique were extracted from 65 studies involving 215 patient cohorts. Bile acid concentrations in normal cohorts were found to exhibit large inter-study variability. The analytical technique used to measure bile acid concentrations, the fasted/fed status of patients at the time of sampling, the choice of blood collection matrix, the starting volume of this matrix, and the choice of protein precipitation solvent were found to be determinants of this variability. To address these determinants, procedural recommendations are proposed: liquid chromatography-tandem mass spectrometry should be used to measure absolute bile acid concentrations in 50 µl of plasma obtained from a fasted subject, with methanol as the protein precipitation solvent. A lack of studies meeting the recommended criteria makes it difficult to conclude whether variability is reduced. These recommendations must be standardized across future studies to enhance the clinical use of peripheral bile acids as biomarkers of liver injury in humans.

1. Introduction

1.1. Enterohepatic cycle of bile acids

Bile acids (BAs) comprise several molecules built on an identical sterol backbone. Cholic acid (CA) and chenodeoxycholic acid (CDCA) are primary BAs. They are metabolic products of cholesterol in liver hepatocytes (Fig. 1). In humans, most BAs are conjugated

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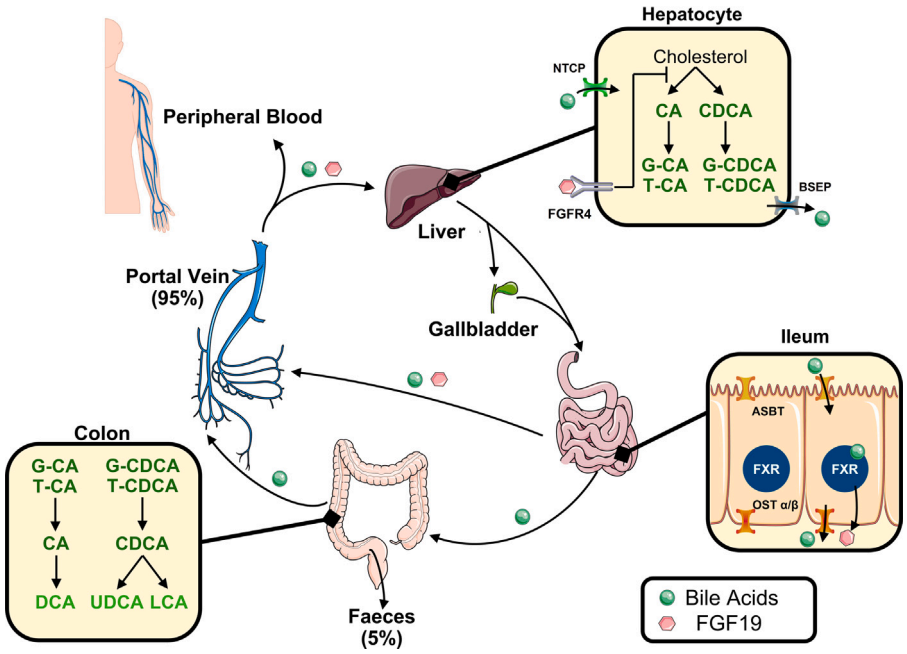

Fig. 1. Schematic of the human enterohepatic circulation. *Abbreviations: ASBT, Apical Sodium–Bile acid Transporter; BSEP, Bile Salt Export Pump; CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; FGF19, Fibroblast Growth Factor 19; FGFR4, Fibroblast Growth Factor Receptor 4; FXR, Farnesoid X Receptor; G-, glycine-conjugated; LCA, lithocholic acid; NTCP, Na^+-Taurocholate Cotransporting Polypeptide; OST α/β, Organic Solute Transporter alpha/beta; T-, taurine-conjugated; UDCA, ursodeoxycholic acid.*

to glycine (G-), with a minority conjugated to taurine (T-), in a ratio of 3:1 (G-:T-) on average [1]. BA conjugation in hepatocytes is highly efficient and increases both their secretion into bile and their water solubility at acidic pH levels preventing precipitation in the duodenum [2], where BAs aid in the absorption of dietary fats. From the liver, conjugated BAs (cBAs) are secreted through the bile salt export pump (BSEP; ABCB11) into the bile duct, from where they are stored in the gallbladder during periods of fasting, or flow directly into the duodenum during prandial periods. From the duodenum, BAs flow through the jejunum and ileum. Throughout the small intestine, cBAs can be passively absorbed through the intestinal wall into the portal vein. However, the majority of cBA absorption occurs in the terminal ileum [3], where the apical sodium–bile acid transporter (ASBT; SLC10A2) mediates the active transport of BAs from the lumen of the ileum into enterocytes. In enterocytes, BAs bind to FXR, a nuclear receptor, with varying affinities according to BA species [4,5]. Activation of FXR stimulates the production and secretion of fibroblast growth factor 19 (FGF19). The latter is brought to the liver with the portal flow where it binds to Fibroblast Growth Factor Receptor 4 (FGFR4) on hepatocytes to inhibit the synthesis of BAs, functioning as an autoregulatory negative feedback mechanism. BAs are exported from enterocytes to the portal vein by the basolateral organic solute transporter (OST-*α*/*β*; SLC51A/SLC51B). BAs not actively transported into enterocytes of the terminal ileum continue towards the colonic lumen where they are rapidly deconjugated by the gut microbial enzyme, bile salt hydrolase (BSH). The so formed unconjugated BAs (uBAs) can undergo dehydroxylation by cooperative bacterial enzymes, to form secondary BAs such as deoxycholic acid (DCA), lithocholic acid (LCA) and ursodeoxycholic acid (UDCA). Lipophilic secondary uBAs are passively absorbed from the colonic lumen through the colonic epithelium into the portal circulation. Remaining colonic BAs are excreted in the feces, accounting for approximately 5% of the BA pool daily. The portal circulation returns BAs to the liver, where they are transported into hepatocytes by basolateral Na^+-taurocholate cotransporting polypeptide (NTCP; SLC10A1) and organic anion transporting polypeptides (OATP; SLCO1B1), completing the enterohepatic circulation (EHC); while a minor fraction of BAs leak into the peripheral circulation.

1.2. Bile acids as biomarkers

Besides their key role in lipid digestion, BAs are important signaling molecules. BAs bind to multiple receptors, including FXR, Takeda-G-protein-receptor 5 (TGR5), pregnane X receptor (PXR), vitamin D receptor (VDR) and liver X receptor-*α* [6]. The activation of these receptors contributes to lipid homeostasis, glucose homeostasis, and to both the regulation of energy metabolism and inflammation [7]. Each individual BA species bind to any of the receptors with a specific affinity. Hence, the concentration these individual BA species have at a given time and location along the EHC creates a unique effect which varies dynamically with time (for example in relation to meal induced gallbladder emptying into the duodenum) and position along the EHC. Many disease processes, ranging from cholestasis to digestive disorders and dysmetabolic disorders, are associated with perturbed BA metabolism. Hence alterations of the size and composition of the BA pool could be promising biomarkers of disease, and particularly of liver

injury. Several studies have investigated the potential use of peripheral BA concentrations as biomarkers in various diseases such as obesity and insulin resistance [8–11], Type 2 Diabetes Mellitus [8,10,12–15], Metabolic Dysfunction-Associated Steatotic Liver Disease (MASLD) [9,16–29], cancers — such as hepatocellular carcinoma [30–33], cholangiocarcinoma [34] and colon cancer [35] - and Alzheimer's disease [36–38]. However, most studies were conducted with small sample sizes, making it difficult to extrapolate results to a large population. In addition, the conclusions of the studies are inconsistent or contradictory, highlighting the need to better understand the potential causes of inter-study variability.

A recent systematic review found that total BA concentrations as well as circulating UDCA, T-CA, CDCA, T-CDCA, G-CA, G-UDCA, G-CDCA, T-UDCA and CA were significantly elevated (in order of most-elevated to least-elevated) in MASLD patients compared to healthy controls [39]. Though the average concentrations of these BAs were increased, the intrinsic high inter-individual variability in BA concentrations meant that there was an overlap between MASLD and healthy cohorts. But individual studies draw contradictory results. For example, Zhang et al. observed a decrease in peripheral G-DCA concentrations in MASLD patients compared to healthy controls [27]; conversely Ferslew et al. observed an increase in peripheral G-DCA concentrations [29], while concentrations reported by Wu et al. exhibited no change [28]. Another review exploring the use of systemic BAs as biomarkers for diverse liver diseases showed that the studies did not agree on which individual BAs or BA ratios are relevant as biomarkers of specific liver dysfunction, including MASLD [40]. This study highlighted inconsistencies in the reported results related, in part, to differences in the methods used in BA assays. Except for the prognostic value of systemic BAs in intrahepatic cholestasis of pregnancy, studies have failed to provide evidence for using systemic BAs as a liver disease biomarker.

To address this, we propose a comprehensive investigation into the influence of preanalytical determinants (such as health status, fasted/fed status, the material analyzed), as well as the influence of analytical determinants (such as the interface, the choice of columns and standards), on BA data reporting. Thus, we recommend that providing and harmonizing the data on peripheral BA concentrations is critical to increase their comparability between studies in order to increase their potential use as promising biomarkers.

2. Methods

2.1. Literature search

A literature search was performed in PubMed as of May 2024 with the following search: *(((bile acid) AND (human)) AND (((peripheral) OR (circulating)) OR (systemic))) AND ((concentration) OR (level))*. The search was complemented by a manual screening of the list of references for relevant articles based on predetermined inclusion and exclusion criteria.

2.2. Inclusion and exclusion criteria

Studies that reported absolute peripheral concentrations of individual BAs or absolute peripheral concentrations of total BAs were included. Only studies written in English and conducted on humans were included. Editorials, commentaries, letters to the editor, conference proceedings, abstracts, and monographs were not included.

2.3. Data and metadata extraction

Data for the most abundant primary and secondary conjugated and unconjugated BAs (CA, CDCA, DCA, UDCA, LCA, G-CA, G-CDCA, G-DCA, G-UDCA, G-LCA, T-CA, T-CDCA, T-DCA, T-UDCA, T-LCA) were extracted directly from the main publication or supplementary material of the studies. The arithmetic mean concentrations in a study cohort of each of these BAs was extracted, or when unavailable, the geometric mean or median was extracted and presented as the arithmetic mean in this study. In cases where data were only presented in graphical form and not in tabular form, graphs were digitized using WebPlotDigitizer (automeris.io) when possible. When concentrations for these 15 BA species were not available, total BA concentrations (totalCA, totalCDCA, totalDCA, totalUDCA, totalLCA) were extracted when available. When concentrations for these 15 BA species were presented, but the total BA concentrations were not, concentrations of the conjugated and unconjugated forms of BAs were summed (e.g. totalCA = CA + G-CA + T-CA). If BA concentrations of cohorts at multiple timepoints were available, these were also included as separate datasets — one dataset per available time point. The units of concentration were standardized to nM for comparison, using molecular weights reported in PubChem (https://pubchem.ncbi.nlm.nih.gov) where necessary. Total BA concentrations in a study cohort were either taken directly from the study when available or calculated (e.g Total BA = totalCA + totalCDCA + totalDCA + total UDCA + total LCA). When concentrations of the 15 BAs for a study cohort were available, the mean ratio of cBAs to uBAs was calculated in Eq. (1), and the mean ratio of secondary to primary BAs was calculated in Eq. (2).

$$\frac{cBA}{uBA} = \frac{(G{-}CA{+}T{-}CA{+}G{-}CDCA{+}T{-}CDCA{+}G{-}DCA{+}T{-}DCA{+}G{-}UDCA{+}T{-}UDCA{+}G{-}LCA{+}T{-}LCA)}{(CA{+}CDCA{+}DCA{+}UDCA{+}LCA)} \quad (1)$$

$$\frac{SecondaryBAs}{PrimaryBAs} = \frac{(DCA{+}G{-}DCA{+}T{-}DCA{+}UDCA{+}G{-}UDCA{+}T{-}UDCA{+}LCA{+}G{-}LCA{+}T{-}LCA)}{(CA{+}G{-}CA{+}T{-}CA{+}CDCA{+}G{-}CDCA{+}T{-}CDCA)} \quad (2)$$

The preanalytical information we retrieved includes the health status of the subjects, whether the subjects were in a fasted or fed state, the average age of the cohorts and the size and sex distribution of the cohorts when available. A cohort of patients was considered *unhealthy* if they had a condition that could directly impact the enterohepatic cycle of bile acids: cirrhosis, obstructive

jaundice, (mild and severe) cholestasis, cholelithiasis, adenomyoma, ileal resection, primary sclerosing cholangitis, non-alcoholic fatty liver (NAFL), non-alcoholic steatohepatitis (NASH), hepatocellular carcinoma (HCC), cholangiocarcinoma, gallbladder cancer, primary colon cancer, viral hepatitis B and C, alcoholic liver disease, and various combinations thereof. By contract, we defined *normal* individuals here as *'having no known diseases directly affecting the organs involved in the EHC'*.

Then, the material used for analyses was recorded (serum or plasma). Additionally, the method by which BAs were extracted from this material and the analytical technique used to quantify the BA concentrations was extracted. For analytical techniques, there are many forms of Liquid Chromatography–Mass Spectrometry (LC–MS). Studies using any of these forms were clumped together and identified as using LC–MS in this study. In the studies utilizing LC–MS, information about whether the sample was directly injected or whether steps were taken to extract BAs was obtained. This includes information concerning which internal standards (ISs) were used, the reagent used for protein precipitation, the use of solid phase extraction (SPE), the use of evaporation under vacuum or nitrogen, and in which reagent the residue was reconstituted before being injected into the LC–MS equipment. In addition, the type of LC and the interface between the liquid chromatograph and the mass spectrometer was identified, as appropriate.

2.4. Visual plotting and statistics

The data for each cohort were plotted using Python and graphed as individual points and presented as the mean±standard deviation. Statistical analyses were performed using a combination of GraphPad Prism 8 and the SciPy Statistical functions (scipy.stats).

3. Results

3.1. Large inter-cohort and inter-individual variability in peripheral bile acid concentrations in humans

A total of 65 studies encompassing 215 patient cohorts and 10,629 individuals were utilized in this study. Of the 215 patient cohorts, 169 reported the concentration of individual BA species (Table 1), while the remaining 46 reported the total concentration of CA, CDCA, DCA, LCA and UDCA (Table 2).

First, we observed that 'unhealthy' cohorts (cohorts of patients with a condition that could directly impact the EHC of BAs) exhibit increased mean individual BA concentrations across most BAs compared to 'normal' cohorts (Fig. 2A). Concentrations of half of the main individual BAs (UDCA, G-CA, G-CDCA, G-DCA, T-CA, T-CDCA, T-DCA, T-UDCA) are significantly elevated in unhealthy cohorts, while concentrations of the other half (mainly unconjugated) are similar in both groups of cohorts on average. The concentrations of the sum of each BA (= unconjugated-BA + T-BA + G-BA) are significantly elevated in unhealthy cohorts, except for totalLCA (Fig. 2B).

However, despite these significant differences, there is a massive overlap between BA concentrations in the normal and unhealthy cohorts, meaning that the use of peripheral BA concentrations to distinguish between normal and unhealthy individuals is not possible.

3.1.1. Liver disorder cohorts

To explore whether this significant overlap of peripheral BA concentrations between unhealthy and normal cohorts could be reduced, datasets from cohorts with liver diseases were stratified into three groups: cholestasis, HCC and metabolic-dysfunction associated liver diseases.

First, as expected, there is a significant difference in the total BA concentrations between cholestatic cohorts and normal cohorts (Fig. 3A). But there are no differences between cholestatic cohorts and cohorts with metabolic-dysfunction associated liver diseases or HCC. The secondary-to-primary BA ratios in cholestatic cohorts are significantly lower than in normal cohorts.

For HCC, there is a significant difference between the cBA-to-uBA ratios compared to normal cohorts (Fig. 3B) and the secondary-to-primary BA ratios in HCC cohorts are significantly lower than in both the normal and metabolic disorders cohorts (Fig. 3C).

For the metabolic disorders cohorts, categorizing them by the stage of liver disease progression into NAFL (non-alcoholic fatty liver), NASH (non-alcoholic steatohepatitis) or cirrhosis shows that NASH cohorts have significantly larger total BA concentrations (Fig. 3D) and cBA-to-uBA ratios (Fig. 3E) than cohorts with NAFL. In some studies, stages of the liver disease progression were not separated, and are presented here as NAFLD. Meanwhile, there are no differences in the secondary-to-primary BA ratios between cohorts with different stages of NAFLD progression (Fig. 3F). There is insufficient data regarding cirrhotic cohorts, though they may have elevated total BA concentrations, but more evidence is needed.

In all cases where there are significant differences, the overlap between the two groups being compared, is so important that successfully classifying a liver disease based on peripheral BA profiles has a high probability of being inaccurate.

Table 1
Biological and experimental characteristics of the individual bile acid concentration datasets obtained from the included studies.*The number in rounded brackets indicated the number of datasets*.

	Health status	Fed status	Material analyzed	Analytical technique	Reference(s)
Individual Bile Acids (169)	Normal (76)	Fasted (53)	Serum (38)	LC–MS (35)	[8,11,13,20,22–29,41–47]
				GC–MS (1)	[48]
				GLC (1)	[49]
				HPLC (1)	[50]
			Plasma (15)	LC–MS (14)	[9,10,12,15,18,19,21,51–54]
				GC–MS (1)	[55]
		Fed (19)	Serum (11)	LC–MS (11)	[13,16,17,29,33,56]
			Plasma (8)	LC–MS (5)	[52,56]
				GC–MS (3)	[55]
		Unknown (4)	Serum (2)	LC–MS (2)	[32,57]
			Plasma (2)	LC–MS (2)	[34,58]
	Unhealthy (78)	Fasted (51)	Serum (38)	LC–MS (31)	[20,22–29,41,43,45]
				GC–MS (2)	[48]
				GLC (1)	[49]
				HPLC (1)	[50]
			Plasma (13)	LC–MS (12)	[9,15,18,19,21,51,53]
				GC–MS (1)	[55]
		Fed (15)	Serum (12)	LC–MS (12)	[16,17,20,29,33,59–61]
			Plasma (3)	GC–MS (3)	[55]
		Both (2)	Plasma (2)	LC–MS (2)	[30,35]
		Unknown (10)	Serum (5)	LC–MS (5)	[31,32]
			Plasma (5)	LC–MS (5)	[34,58]
	Both (2)	Fasted (1)	Plasma (1)	LC–MS (1)	[62]
		Both (1)	Plasma (1)	LC–MS (1)	[30]
	Unknown (13)	Fasted (7)	Serum (7)	LC–MS (7)	[37,38,63]
		Both (1)	Plasma (1)	LC–MS (1)	[35]
		Unknown (5)	Plasma (5)	LC–MS (5)	[36,64]

Table 2
Biological and experimental characteristics of the total bile acid concentration datasets obtained from included studies.*The number in rounded brackets indicated the number of datasets*.

	Health status	Fed status	Material analyzed	Analytical technique	Reference(s)
Total Bile Acids (46)	Normal (25)	Fasted (7)	Serum (7)	GC–MS (4)	[61,65–67]
				GLC (2)	[59,68]
				Capillary Electrophoresis (1)	[17,69]
		Fed (18)	Serum (18)	LC–MS (1)	[17]
				GC–MS (16)	[61,65,66]
				GLC (1)	[59]
	Unhealthy (20)	Fasted (13)	Serum (13)	GC–MS (7)	[61,67,70–73]
				GLC (2)	[59,68]
				HPLC (1)	[74]
				Capillary Electrophoresis (3)	[69]
		Fed (7)	Serum (7)	LC–MS (1)	[17]
				GC–MS (5)	[61]
				GLC (1)	[59]
	Both (1)	Fasted (1)	Plasma (1)	LC–MS (1)	[14]

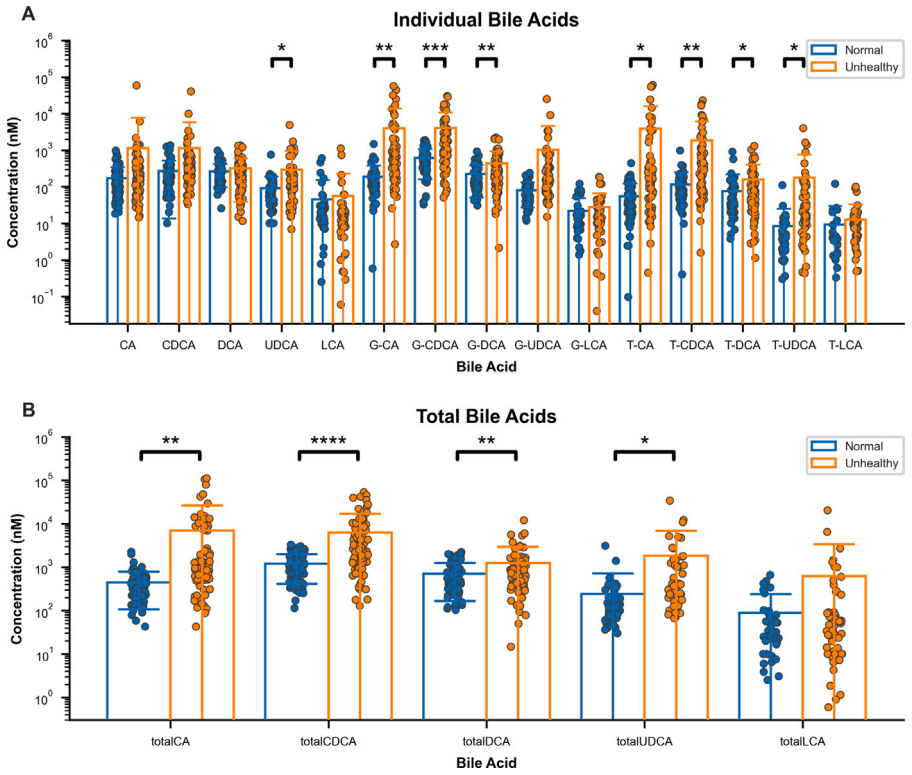

Fig. 2. Inter-cohort variability in bile acid concentrations is large. Mean individual bile acid (A) and total bile acid (B) concentrations in normal (without enterohepatic disorders) and unhealthy cohorts (with enterohepatic disorders) were plotted as their mean±standard deviation. Statistics performed by two-tailed unpaired Welch's t-test, blank $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

3.1.2. Normal cohorts

We then more deeply analyzed individual BA species in normal cohorts. This reveals substantial inter-individual variability for a given BA. Even in a healthy context (Fig. 2A), a 2 log fold difference in range is observed for the 15 BAs. In the majority of cohorts, G-CDCA is the most abundant BA, with concentrations ranging between 30 nM and 2000 nM. This is followed by CDCA and DCA, which both have similar mean concentrations. In contrast, T-LCA and T-UDCA are the least abundant BAs, with mean concentrations of ~10 nM. The mean concentrations of G-conjugated BAs are between two- and ten-fold higher than their T-conjugated counterparts.

Beyond individual BAs, total BA concentrations also varied considerably between cohorts and individuals (Fig. 2B), with totalCDCA being the most abundant. This is followed by totalDCA, totalCA, totalUDCA and totalLCA in descending order. totalCDCA has the widest range from 100 nM to 1900 nM, while totalLCA has the smallest, ranging from 0 nM to 700 nM.

Having established that overlap exists between the peripheral BA concentrations in diseased and normal cohorts and that normal cohorts exhibit considerable variability, we wanted to identify potential analytical and preanalytical sources of this variability in BA concentration measurements.

3.2. Analytical sources of variability in peripheral bile acid concentrations

In the past, HPLC and GLC were mostly used to quantify BA concentrations. However, owing to the higher sensitivity, specificity and throughput of LC–MS and GC–MS, HPLC and GLC are now obsolete. Consequently, the vast majority of studies have used either LC–MS or GC–MS (Fig. 4A). Of the 65 studies included here for normal cohorts, 49 studies used LC–MS and 10 studies used GC–MS. The fact that GC–MS requires a complex, time-consuming derivatization step to convert the BAs to a volatile form, and that LC–MS has a higher sensitivity and throughput, has led to LC–MS becoming the primary analytical technique for measuring individual BAs (Table 1), while studies in which GC–MS was used reported total BAs (Table 2).

Focusing on studies solely using LC–MS to measure BA concentrations revealed that individual BA (Fig. 4B) and total BA (Fig. 4C) concentrations in normal cohorts also exhibit large inter-cohort variability, reinforcing the need to identify sources of this variability. The LC–MS workflow involves many steps that could impact the measured BA concentration. On the LC part, the choice of column, interface, internal standards, mobile phase, autosampler injection method and chromatographic separation method could all be determinants of the variability of BA concentration measurements.

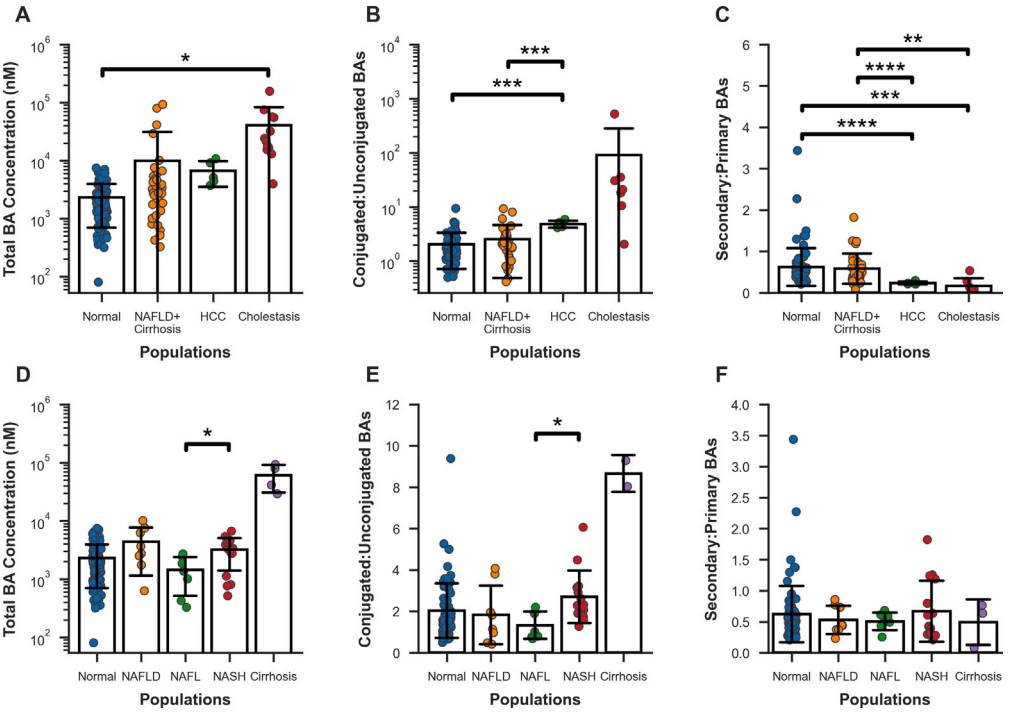

Fig. 3. Circulating bile acids as a marker of liver diseases. The mean of the total bile acid concentrations (A), conjugated-to-unconjugated bile acid ratios (B) and secondary-to-primary bile acid ratios (C) in normal cohorts (those without enterohepatic disorders), cohorts with metabolic-dysfunction associated liver disorders (NAFLD + cirrhotic), HCC, and cholestatic cohorts were plotted as their mean±standard deviation. The mean of the total bile acid concentrations (D), conjugated-to-unconjugated bile acid ratios (E) and secondary-to-primary bile acid ratios (F) in normal, NAFLD, NAFL, NASH and cirrhotic populations were plotted as their mean±standard deviation. Statistics performed by Games–Howell Multiple Comparison Test, blank $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

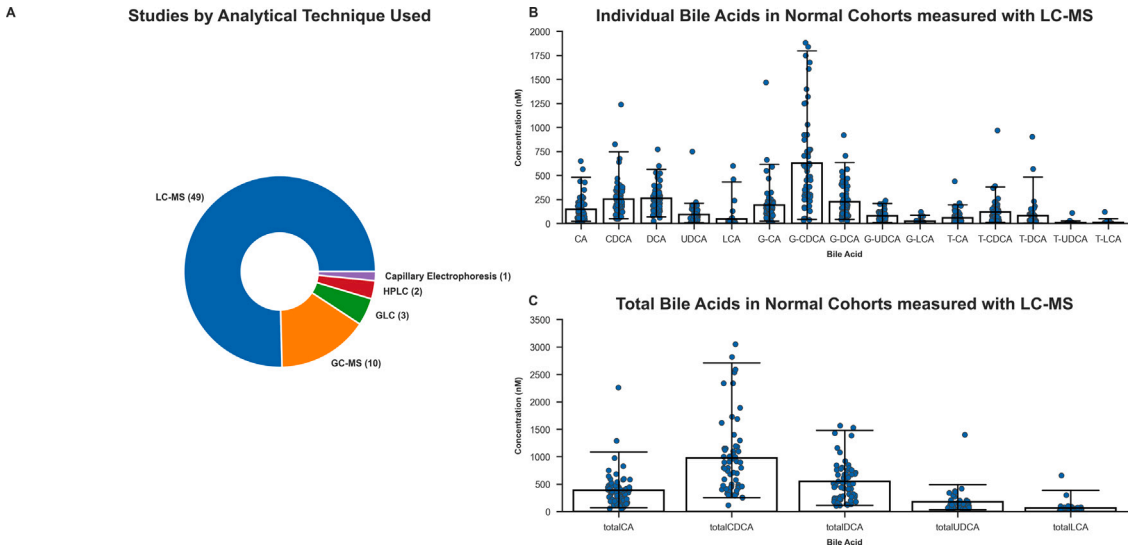

Fig. 4. LC–MS is the predominant analytical technique to measure bile acid concentrations. The analytical technique used in the 65 included studies were tallied and presented as a pie chart (A). The number in brackets indicates the number of studies using the respective technique. Mean individual bile acid concentrations (B) and total bile acid concentrations (C) in normal cohorts (those without enterohepatic disorders) when LC–MS was the analytical technique used were collected from literature and plotted as their mean±standard deviation. *Abbreviations: GC–MS, gas Chromatography–Mass spectrometry; GLC, gas liquid chromatography; HPLC, high performance liquid chromatography*.

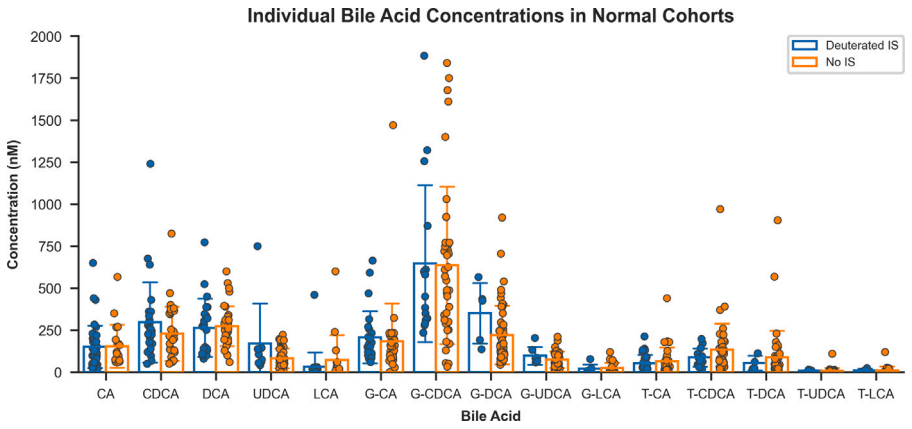

Fig. 5. The use of a specific deuterated analogue internal standard for a target bile acid does not affect the measure bile acid concentrations. Mean individual bile acid concentrations from studies that used LC–MS in normal cohorts (those without enterohepatic disorders) were stratified for whether a specific deuterated analogue internal standard (Deuterated IS) or a non-analogous internal standard (No IS) was used for a target bile acid and plotted as their mean±standard deviation. Statistics performed by two-tailed unpaired Welch's t-test, blank $p > 0.05$. *Abbreviations: IS, internal standard*.

3.2.1. Column

Various parameters related to the column could impact its ability to separate BAs, including the particle size inside the column, the column length, the inner diameter of the column, the column temperature and whether the column has degraded over time. But in all the studies utilizing LC–MS, Reversed-Phase Liquid-Chromatography (RPLC) columns were used. RPLC columns separate analytes based on their hydrophobicity. Unconjugated BAs are more hydrophobic than their G- and T- conjugates.

3.2.2. Interface

The ionization interface is critical for converting the separated analytes from the liquid phase into the gas-phase ions that can be detected by the mass spectrometer. All studies utilizing LC–MS for the measurement of BA concentrations use electrospray ionization (ESI).

3.2.3. Internal standards

Accurate and reproducible quantification of BAs by LC–MS/MS necessitates the use of stable isotope-labeled internal standards (SIL-IS), preferably deuterated analogues of the target analytes. These standards compensate for variable extraction efficiency, matrix effects, and ionization variability, which are particularly pronounced in complex biofluids like plasma or serum.

When individual BA concentrations of the peripheral blood of normal cohorts, measured by LC–MS were stratified by whether a specific deuterated analogue of a target BA was used as the internal standard (e.g. d4-CA for CA, d4-G-CA for G-CA, d4-T-CA for T-CA), no significant differences in BA concentrations were observed when a different, non-analogous but appropriate SIL-IS was employed (Fig. 5).

3.3. Pre-analytical sources of variability

As LC–MS is the most widely used analytical technique to measure BA concentrations, we restricted further analysis to studies using LC–MS when investigating the preanalytical sources of measurement variability in normal cohorts.

3.3.1. Fed/fasted status

First, we investigated the impact of the fasted/fed status at the time of sampling on the concentration of individual BAs in normal cohorts. Normal cohorts fed at the time of sampling tend to have higher mean concentrations of G-BAs than fasted cohorts, with the exception of G-CA (Fig. 6A). Of the uBAs, mean DCA concentrations are significantly higher in fed normal cohorts, while mean concentrations of CA, CDCA, UDCA and LCA are similar in both conditions. T-CDCA is the only T-BA that is significantly elevated in fed normal cohorts, while the other T-BAs remain similar in both conditions. The totalDCA mean concentrations are increased in fed normal cohorts compared to fasted, while the mean concentrations of the other totalBAs remain similar in both conditions, though data for totalUDCA in fed normal cohorts are limited (n = 3) (Fig. 6B).

To reduce the variability linked to feeding, samples should always be obtained in the fasted state, unless specifically investigating postprandial effects.

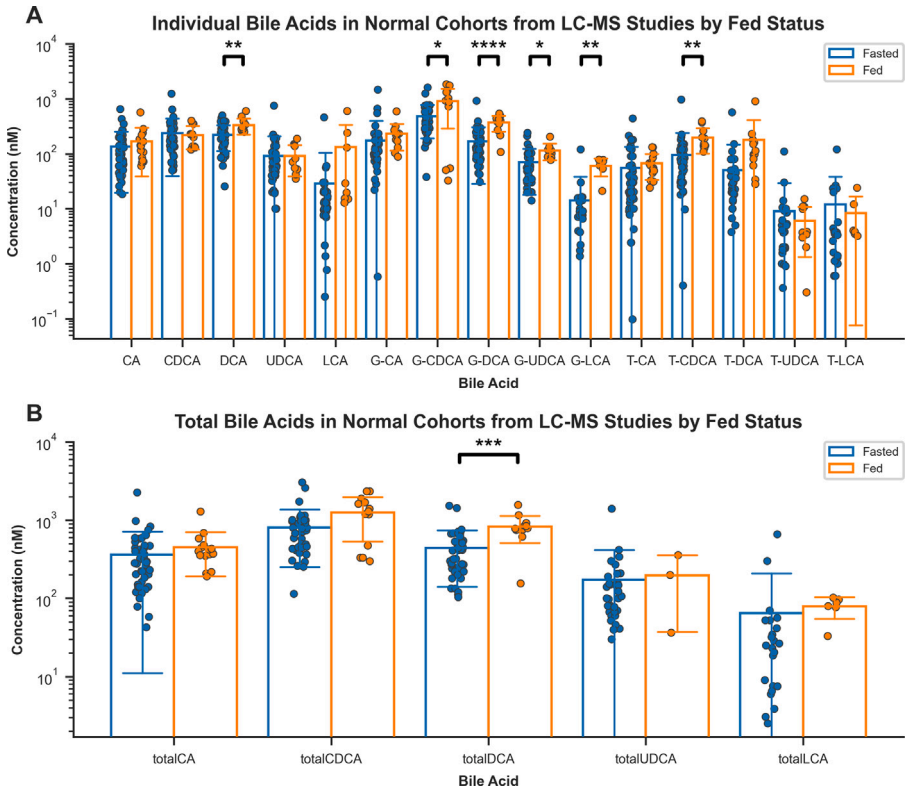

Fig. 6. Normal cohorts fed at the time of sampling tend to have elevated mean bile acid concentrations than fasted normal cohorts. Mean individual bile acid (A) and total bile acid (B) concentrations from studies that used LC–MS in normal cohorts (those without enterohepatic disorders) were stratified for whether the cohorts were fasted or fed at the time of sampling and plotted as their mean±standard deviation. Statistics performed by two-tailed unpaired Welch's t-test, blank $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

3.3.2. Analyzed material

Secondly, peripheral blood can be collected as either serum or plasma. To collect serum, blood is allowed to clot naturally and then centrifuged, enabling the removal of coagulation factors, blood cells and fibrin clots. On the other hand, plasma is collected by first adding an anticoagulant such as heparin, EDTA or citrate, and then immediately centrifuged to separate blood cells and liquid plasma.

Serum samples obtained from normal cohorts fasted at the time of sampling tend to have higher individual BA concentrations than plasma samples on average (Fig. 7A). The mean concentrations of CA, CDCA, UDCA, G-CA and T-CA in serum samples are significantly higher than those in plasma. The mean concentrations of totalBAs are also similar in both serum and plasma samples, with only totalCA significantly higher in serum samples (Fig. 7B). Though serum samples exhibit higher BA concentrations on average, across both individual BAs and total BAs, plasma samples seem to exhibit lower variation compared to serum samples. However, a comparison of variance via a Brown–Forsythe test reveals no statistically significant difference between the variance of two groups across all BAs (Suppl. Tab. 1).

3.3.3. Starting volume

Of the 13 datasets of BA concentrations measured using LC–MS in plasma from normal cohorts fasted at the time of sampling, 7 of them used 50 µl of plasma (Fig. 8A). The next most used volume of plasma was 100 µl. Similar ranges of total BA concentrations can be measured when both 50 µl and 100 µl of plasma are originally used. This is also true for G-CDCA, the most abundant BA of the 15 BAs focused on in this study, while there are no measurements of T-UDCA, the least abundant BA of those focused on here, from 100 µl of plasma, though T-UDCA concentrations as low as 0.3608 nM can be measured from 50 µl, suggesting that 50 µl is sufficient to measure low abundant BAs (Suppl. Fig. 1). No correlation between the starting volume of plasma and the concentration of BAs measured is observed. Thus, 50 µl of plasma appears to be a suitable starting volume to ensure accurate absolute BA concentration measurements whilst potentially reducing the volume of blood required to be drawn from a subject.

Similarly, in datasets of BA concentrations measured using LC–MS in serum from normal cohorts at the time of sampling, 50 µl is the most commonly used starting volume (Fig. 8B). While the average total BA concentration across cohorts when the starting volume is 50 µl is less than when it is 100 µl, the fact that a concentration as high as 6500 nM can be measured when the starting volume is 25 µl, and that total BA concentrations as low as 30 nM can be measured with a starting volume of 50 µl, suggests 50 µl can cover a sufficient range of BA concentrations.

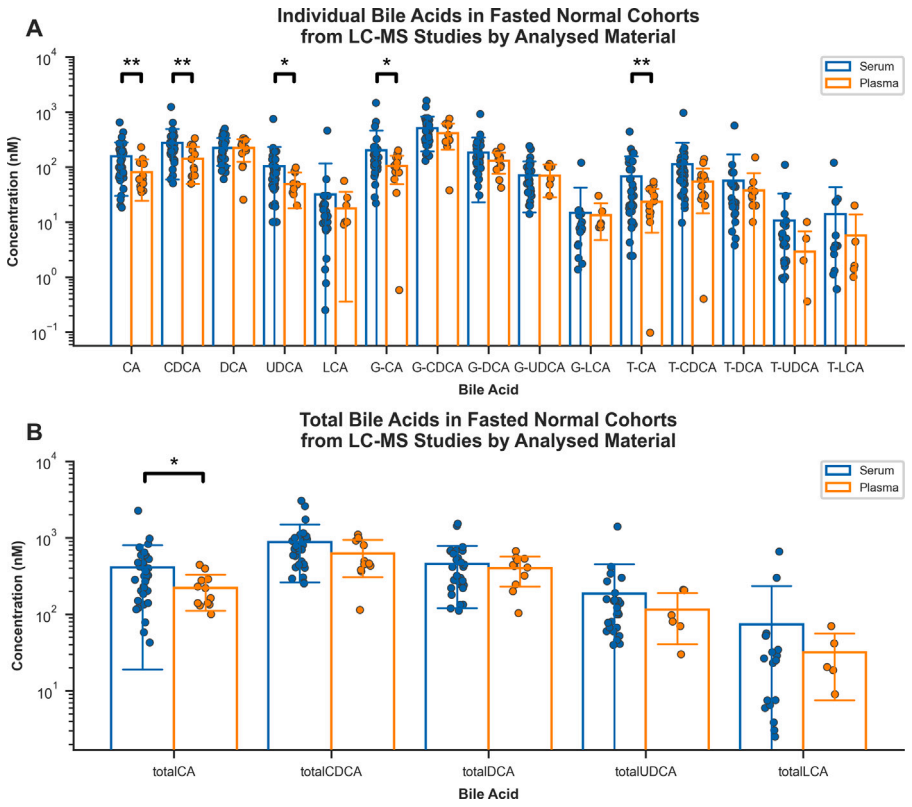

Fig. 7. Bile acid concentrations in peripheral serum tend to be higher than those in peripheral plasma. Mean individual bile acid (A) and total bile acid (B) concentrations from studies that used LC–MS in normal cohorts (those without enterohepatic disorders) which were fasted at the time of sampling were stratified for whether the sample analyzed was serum or plasma, and plotted as their mean±standard deviation. Statistics performed by two-tailed unpaired Welch's t-test, blank $p > 0.05$, $*p \leq 0.05$, $**$ $p \leq 0.01$.

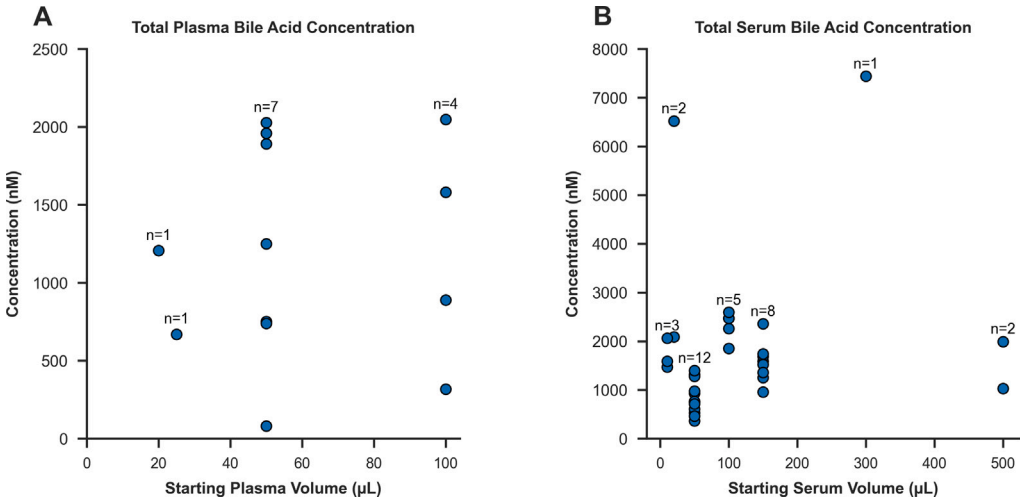

Fig. 8. A wide range of bile acids concentrations are detected in a 50 µl starting volume. The mean total bile acid concentrations measured using LC–MS in plasma (A) and serum (B) of normal fasted cohorts were plotted against the starting volume used.

3.3.4. Protein precipitation solvent

A critical step in the upstream processing of a sample to be analyzed by LC–MS is the precipitation of proteins, during which proteins in serum and plasma are removed to minimize the matrix effect. These proteins would otherwise interfere with BA analysis by binding to BAs or by clogging the chromatography column. The two most common methods involve using either an

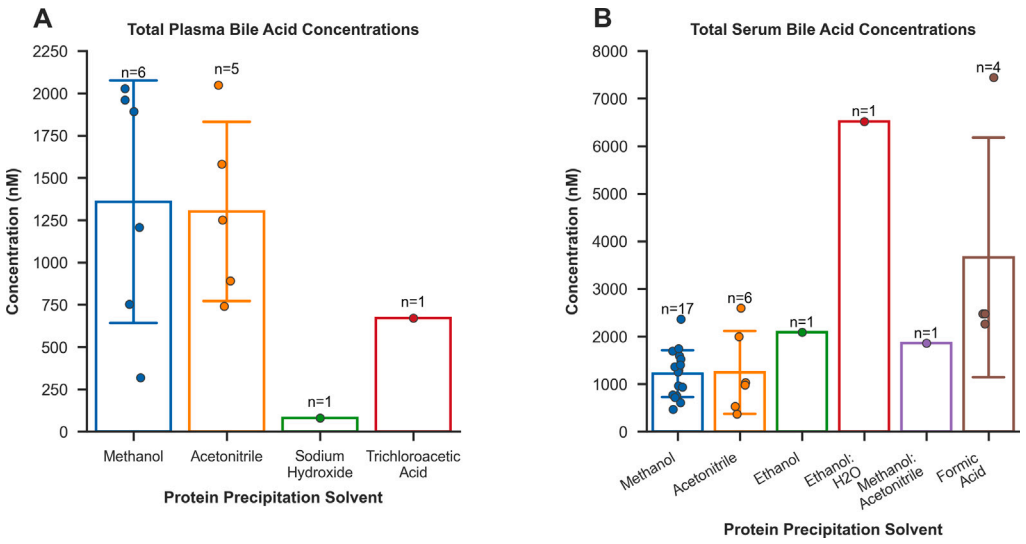

Fig. 9. Methanol and acetonitrile are the most commonly used protein precipitation solvents. The mean total bile acid concentrations measured using LC–MS in plasma (A) and serum (B) of normal fasted cohorts were stratified for the solvent used for protein precipitation.

organic solvent for precipitation, such as methanol, ethanol or acetonitrile, or an acid for precipitation, such as formic acid or trichloroacetic acid. Protein precipitation using ice-cold methanol or acetonitrile (3:1 solvent-to-plasma ratio) remains the most common approach for preparing samples. However, co-precipitation of phospholipids can lead to ion suppression, particularly in positive ion mode. Thus, additional cleanup via SPE may improve method robustness, especially in clinical applications where throughput and reproducibility are highly important. Another method is alkaline hydrolysis which involves the addition of an alkaline such as sodium hydroxide, followed by heating to facilitate hydrolysis and then centrifugation to remove the precipitated proteins. However, exposure to strong alkaline conditions may lead to the degradation of BAs.

Since no difference was observed in the total BA concentrations measured when 50 μl and other starting volumes of serum or plasma were used, we compared the protein precipitation solvent used in studies considering both serum and plasma and all starting volumes (Fig. 9). Methanol and acetonitrile were the two most commonly used protein precipitation solvents in both plasma (Fig. 9A) and serum (Fig. 9B) from normal fasted cohorts in which LC–MS was used as the analytical technique. In plasma, the average total BA concentrations when methanol and acetonitrile were used as the protein precipitation solvent are roughly similar, while the impact of using sodium hydroxide or trichloroacetic acid instead cannot be ascertained because these were only used in one cohort each. In serum, methanol is the most commonly used solvent (n=17), followed by acetonitrile (n=6). The average total BA concentrations are similar when both solvents are used, while the impact of using ethanol, diluted ethanol or a mixture of methanol and acetonitrile is unclear due to their use being limited to one cohort each. Meanwhile, the average total BA concentrations in serum when formic acid was used as the protein precipitation solvent are higher than when both methanol and acetonitrile were used. However, since the number of cohorts is relatively low (n=4) and because there is no data regarding the use of formic acid as the protein precipitation solvent in plasma samples, it is difficult to conclude if BA concentrations are higher on average when formic acid is used. Low abundance BAs such as G-LCA and T-LCA were only detected in the plasma and serum of fasted normal cohorts when methanol was used as the protein precipitation solvent (Suppl. Fig 2).

3.4. Recommendations to limit variability

The main sources of variability in BA concentration determination are the choice of analytical technique, the fasted/fed status of the subjects at the time of sampling, the starting material analyzed, the starting volume of this material, and the solvent used to precipitate proteins that would interfere in the analysis. To address and standardize these sources of variability we propose recommendations (Table 3). If we filter the studies according to the suggested recommendations, a clear reduction in the observed inter-cohort variability in BA concentrations of normal cohorts is not evident since studies meeting the recommended criteria are few (Fig. 10). Testing for a change in variance via a Brown–Forsythe test between all the LC–MS normal cohorts and the filtered cohorts found G-UDCA to be the only BA whose variance is significantly lower when the data is filtered (Suppl. Tab. 2).

3.5. An alternative to BA concentrations — peripheral BA proportions are significantly altered in enterohepatic disorders

Besides concentrations, we investigated if peripheral BA proportions could be a more reliable biomarker of liver status. 47 out of the 169 datasets (Table 1) provide concentrations for all the 15 individuals BAs focused on in this study. 15 of these 47 datasets are from normal cohorts, while the remaining 32 are from unhealthy cohorts with enterohepatic disorders. 14 of these 32 are from

Table 3
The identified sources of variability in bile acid concentration measurements and the recommendations to reduce this variability. *Abbreviations: LC–MS, liquid-Chromatography–Mass spectrometry.*

Source of variability	Recommendation
Analytical Technique	LC–MS
Fasted/Fed Status	Fasted
Analyzed Material	Plasma
Starting Volume	50 µl
Protein Precipitation Solvent	Methanol

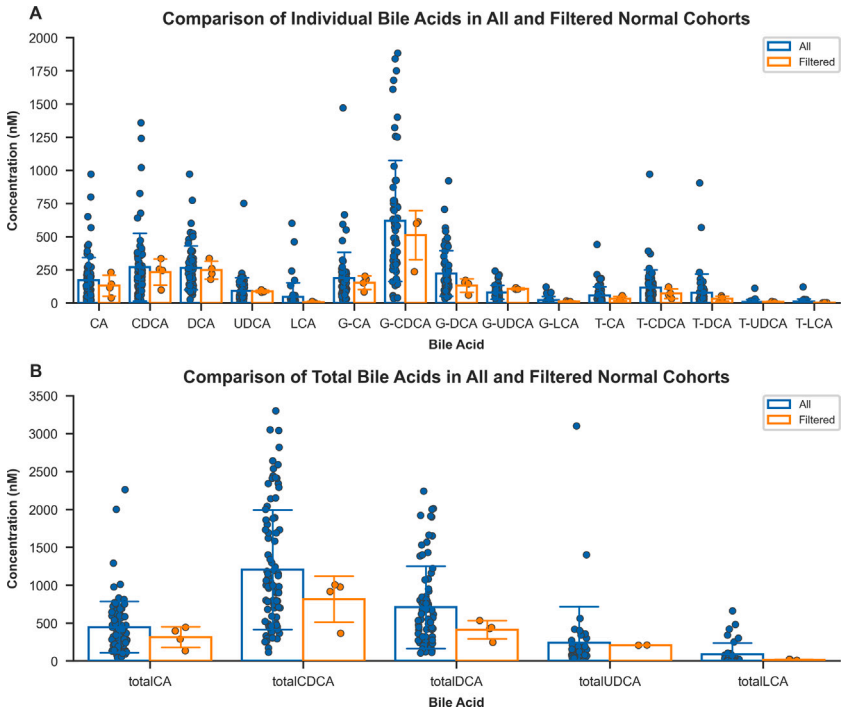

Fig. 10. Data collected in accordance with the suggested recommendations are limited. Mean individual bile acid (A) and total bile acid (B) concentrations from all studies were plotted against those from studies that met the preanalytical and analytical criteria specified in Table 3. Statistics performed by Brown–Forsythe test, blank $p > 0.05$, $^{*}p \leq 0.05$.

cohorts with metabolic-dysfunction associated liver diseases (NAFL, NASH & Cirrhosis), with the other 18 from cohorts with other liver diseases such as viral hepatitis, ALD, HCC (3 cohorts) and cholestasis (1 cohort). For each dataset, the concentration of each of the 15 BA species was summed for the total BA concentration, and the proportion of each individual BA of this total was determined.

In these 47 cohorts, the proportions of 11 out of the 15 BAs were altered. The proportions of CA, CDCA, DCA, LCA, G-DCA, and G-LCA are decreased in unhealthy cohorts (Fig. 11). Of these, the proportions of CDCA, DCA and UDCA are significantly lower in cohorts with other enterohepatic disorders compared to metabolic-dysfunction associated enterohepatic disorders (Suppl. Fig. 3). Proportions of DCA, LCA, G-DCA and G-LCA are significantly decreased in normal cohorts compared to cohorts with metabolic-dysfunction associated disorders (Suppl. Fig. 4). Meanwhile, the proportions of G-CA, G-UDCA, T-CA, T-CDCA and T-UDCA of total BAs are increased in unhealthy cohorts. Of these, T-CA and T-CDCA proportions are significantly elevated in cohorts with metabolic-dysfunction associated enterohepatic disorders compared to other disorders (Suppl. Fig. 3) and are significantly higher in cohorts with metabolic-dysfunction associated enterohepatic disorders than in normal cohorts (Suppl. Fig. 4). Looking into the preanalytical and analytical sources of variability previously identified here revealed that 14 out of the 15 normal cohorts were fasted at the time of sampling, and BA concentrations were measured in plasma in only 3 of these 14 cohorts.

4. Discussion

This study aimed to, first, assess the range of variability in peripheral BA concentrations reported in literature and, then, identify the preanalytical and analytical sources influencing this variability. A total of 65 studies, encompassing 10,629 individuals, were utilized in the analysis.

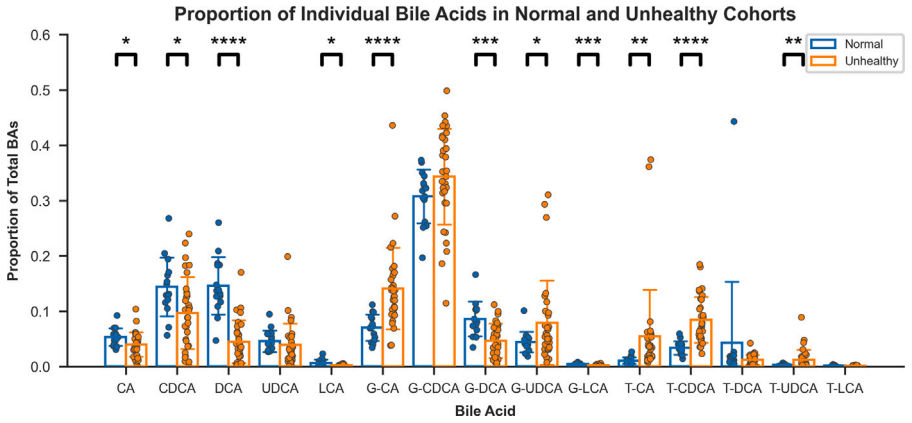

Fig. 11. Circulating individual bile acids as a proportion of total bile acid concentrations are significantly altered in populations with enterohepatic disorders. Mean concentrations of peripheral bile acids from 47 cohorts in which concentrations of the 15 bile acids focused on in this study were reported in normal cohorts (without enterohepatic disorders) and unhealthy cohorts (with enterohepatic disorders) were plotted as their mean±standard deviation. Statistics performed by two-tailed unpaired Welch's t-test, blank $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

4.1. Analytical sources

Instrument

LC–MS is the predominant analytical technique for measuring individual BA concentrations. While direct comparison with GC–MS data for individual BAs was inconclusive due to limited GC–MS data (Suppl. Fig 5), total BA concentrations measured by GC–MS were, on average, higher than those from LC–MS. This difference likely stems from GC–MS requiring cleavage of all conjugates, thus including all structural subclasses like sulfated BAs, unlike LC–MS. For total BA concentrations, we only summed the concentrations of the unconjugated and G-/T-conjugated forms. Thus, GC–MS results for total BAs are expected to be more accurate and higher, while LC–MS remains better suited for individual BA analysis.

Column

In all studies using LC–MS, RPLC with C18 columns is the standard for BA separation. However, achieving baseline resolution of isobaric species (e.g., CA vs. DCA or CDCA vs. UDCA) and conjugated forms can be challenging, often requiring lengthy gradient programs (20–30 min for full BA panels). The potential for interference of isobaric compounds has to be carefully considered during LC–MS analysis of BAs. The number of isomeric species is vast, with many sharing identical MS/MS fragmentation patterns without unique diagnostic fragments, making them difficult to distinguish solely by mass spectrometry. This highlights the importance of optimized column selectivity and gradient conditions to minimize interference in LC–MS/MS BA assays. Modified C18 stationary phases like phenyl-hexyl [75,76], pentafluorophenyl (PFP) [77,78], and biphenyl columns [77,79] offer improved selectivity for isomers and conjugates through $\pi - \pi$ interactions, dipole moments, and steric effects. The specific composition of stationary phases and other RPLC column parameters, such as the particle size inside the column, the column length, the inner diameter of the column, the column temperature and whether the column has degraded over time, which can all significantly influence variability, were not analyzed in this study. Future studies should investigate these factors to better understand their impact on BA quantification.

Interface

All included LC–MS studies used Electrospray Ionization (ESI) as the interface. While crucial for converting analytes to gas-phase ions, a more granular analysis of the ESI parameters used in the different studies, such as positive/negative ion mode, flow rates and interface design, was not performed, due to a lack of detail in the majority of studies. These parameters could impact ESI performance [80] and hence contribute to variability.

Internal standards

The use of analogous SIL-ISs in LC–MS studies, while not strictly necessary to reduce individual BA variability, is crucial for compensating for variable extraction efficiency, matrix effects, and ionization variability in complex biofluids. Ideally, each BA species or structural subclass should have a structurally identical or closely analogous SIL-IS for accurate quantitation. However, cost and availability often lead to limited SIL-IS use (e.g., d4-CA alone), potentially introducing bias. SIL-ISs should be selected based on structural similarity, retention time, and ionization mode compatibility, with matrix-matched standard curves for calibration. Additionally, labeled standards would also facilitate inter-laboratory harmonization.

Mass spectrometer

While LC–MS offers high sensitivity and throughput, LC–MS/MS provides enhanced specificity and structural confirmation, critical for distinguishing isobaric or structurally similar BAs. All forms of LC–MS/MS were broadly classified as LC–MS in this study.

Triple quadrupole mass spectrometers, in Multiple Reaction Monitoring (MRM) mode, especially, remain the gold standard for sensitive, reproducible, and accurate quantification of known BAs. MRM offers high sensitivity by monitoring specific precursor-to-product ion transitions but provides limited structural insight and can be compromised by co-eluting isomers without sufficient chromatographic resolution. Expanding MRM panels can also reduce sensitivity due to the increased number of transitions, unless scheduled MRM (sMRM) or dynamic MRM strategies are employed [57,81–83].

Parallel Reaction Monitoring (PRM) on high-resolution platforms like Orbitrap mass spectrometers offers enhanced specificity by capturing full MS/MS spectra of targeted precursor ions. This allows for retrospective data interrogation and better differentiation of co-eluting isomers. However, PRM generally has lower acquisition speed and sensitivity compared to optimized MRM. Thus, PRM is ideal for structurally complex BA panels or when identification certainty is paramount, but less suited for high-throughput, strictly quantitative workflows [84–86].

Emerging technologies like Electron-Activated Dissociation (EAD) on ZenoTOF systems offer richer, orthogonal fragmentation patterns, enabling superior structural elucidation and differentiation of BA isomers. When paired with the Zeno trap, EAD boosts MS/MS sensitivity. While powerful for qualitative confirmation and discovery-mode workflows, EAD currently exhibits lower sensitivity for low-abundance quantification in routine clinical samples [87,88]. In summary, triple quadrupole MRM remains the gold standard for sensitive, high-throughput quantification of known BAs; Orbitrap PRM offers greater specificity and flexibility for complex or isomeric profiles; and ZenoTOF-EAD platforms provide the most structural information for qualitative and discovery-based applications.

4.2. Preanalytical sources

Fasted/fed status

BA concentrations are generally higher in fed cohorts due to postprandial stimulation. To accurately assess inter-cohort variability, samples should ideally be obtained in a 12-hour fasted state. If studying postprandial responses, blood collection should be performed at a defined, consistent time after a standardized meal in otherwise fasting individuals, with experimental validation to account for gallbladder variability.

Blood collection matrix

Serum samples tend to yield higher BA concentrations than plasma, while plasma shows lower variability. Logistically, plasma is easier and faster to prepare, requiring no clotting time, and has a longer shelf-life compared to serum (years vs. months) [89]. Studies have shown significant differences in (non-BA) metabolite concentrations between serum and plasma from the same patients [90,91]. While some studies observed higher metabolite concentrations in serum, speculating about a volume displacement effect [92], this theory is debated as plasma contains coagulation factors like fibrinogens [93]. Although these studies used analytical techniques other than LC–MS (^{1}H-NMR and Flow Injection Analysis-Mass Spectrometry) and did not include BAs [90,91], it is reasonable to assume similar differences could exist for LC–MS BA concentrations. A study on antidepressant quantification by LC–MS also found inconsistencies when using calibration curves from one matrix to quantify analytes in others [94], underscoring the blood collection matrix as a source of variability. Standardizing the matrix is crucial for valid clinical interpretation. Additionally, the choice of anticoagulant impacts metabolite concentrations [90]. Further investigation is needed to better understand how serum or plasma from the same patient affects LC–MS BA concentrations and the reasons for discrepancies. Given its logistical ease and higher reproducibility of metabolite concentrations [90,91], plasma is likely the better choice of matrix.

Starting volume

Since some BAs are low-abundance, it is a possibility that too low volumes of plasma may result in low abundant BAs being below the limit of detection. A starting plasma volume of 50 µl is generally sufficient for detecting a wide range of BA concentrations and is comparable to 100 µl. Data for volumes less than 50 µl is limited. We recommend using 50 µl of plasma for accurately measuring absolute BA concentration while taking a reasonably low blood volume. On-column concentrations, which could influence sensitivity independent of the starting volume of plasma, are rarely considered but could also be an important determinant.

Protein precipitation

Methanol and acetonitrile appear to be the most effective solvents for protein precipitation, leading to higher total BA concentrations in plasma. A comparison of extraction techniques followed by HPLC–MS/MS found acetonitrile precipitation followed by C18-SPE to be most suitable for serum extraction of cBAs and uBAs [41]. Methanol precipitation alone could detect the same BAs but at lower levels. Protein precipitation is a critical first step to minimize matrix effects, particularly ion suppression [95]. Methanol and acetonitrile are widely used for their efficiency in denaturing plasma proteins [96], though performance varies by sample type, BA subclass, and concentration. Methanol is known to improve recovery for hydrophilic BAs, while acetonitrile often yields cleaner extracts beneficial for downstream SPE. SPE is increasingly used as a second-tier cleanup method after protein precipitation [41,81,83,85,96]. By selectively retaining analytes or removing matrix interferences based on polarity or charge, SPE can reduce background noise, improve signal-to-noise, and enhance quantification accuracy. Common sorbents include C18,

hydrophilic–lipophilic balance (HLB), and mixed-mode ion-exchange. Although SPE adds complexity, its utility is especially relevant for low-abundance or conjugated BAs prone to matrix effects, and it lends itself to automation [41]. Despite these advantages, standardized SPE protocols are lacking. Further research is warranted to determine if routine SPE improves inter-laboratory reproducibility and overall analytical robustness. Additional factors influencing extraction include sample volume [81], stability of conjugated BAs [97], and alternatives like phospholipid removal plates [98]. While methanol-based protocols are generally mild, maintaining near-neutral pH is important to prevent deconjugation of sulfated or G-conjugated BAs. Internal standard recovery checks and pooled sample replicates are critical for monitoring extraction efficiency [97].

Overall, while methanol precipitation is the most common extraction strategy due to its simplicity, further refinement of purification steps such as SPE may be necessary for accurate quantification of low-abundance or structurally complex BA species. To reduce variability and improve comparability, standardization of methodologies is essential, including fasting status, sample type, analytical technique, sample volume, and protein precipitation methods.

Peripheral BA proportions appear to be significantly altered in subjects with enterohepatic disorders, even with varying methodologies. While standardization is crucial for accuracy, peripheral BA proportions may still provide valuable information for diagnosis and monitoring. Access to absolute BA concentrations or proportions for individual subjects, rather than just cohort means, is critical for advancing research. Standardized sampling, preparation, and analysis are prerequisites for future studies investigating the diagnostic or prognostic value of circulating BAs.

4.3. Bile acids as promising clinical biomarkers of liver injury — perspectives

The study's main limitation is that variability determinants were identified across studies and cohorts, not within samples from the same patients. Future research requires within-patient studies to confirm these findings. In addition, publishing absolute BA concentrations for individual patients, not just cohort means, is crucial for understanding inter-individual variability.

The gut microbiota significantly influences BA metabolism, transforming primary BAs into secondary BAs, which impacts BA diversity and signaling. Accounting for the microbiota's contribution could improve the diagnostic and prognostic value of peripheral BA measurements. The extent to which microbial BA modifications are captured in peripheral blood is still not fully understood. Portal blood BA concentrations might be more informative for liver status, but it is not routinely accessible.

Further work is needed to determine whether changes in BA concentrations or proportions are more informative biomarkers of disease. Our study suggests that differences in BA proportions between normal and unhealthy cohorts are more prominent than differences in concentrations (Suppl. Fig 6). More data is needed to support this.

The vast majority of studies included in this manuscript measured BAs in academic research laboratories, using unique sample preparation and LC–MS/MS protocols. The other studies either sent their samples to third party commercial metabolomic platforms, such as Metabolon (Metabolon, Inc., USA) for BA quantification or used the AbsoluteIDQ Bile Acid kit (Biocrates Life Sciences AG, Austria) for sample preparation before using their own LC–MS/MS equipment to quantify BAs. Metabolon offers a service to perform its Bile Acid Targeted Panel under Good Clinical Practice (GCP) and Good Clinical Laboratory Practice (GCLP), which follows the International Council for Harmonisation (ICH) M10 guideline for bioanalytical method validation, which is recognized by both the US Food and Drug Administration (US FDA) and the European Medicines Agency (EMA). Meanwhile, though Biocrates has demonstrated that the AbsoluteIDQ Bile Acid kit is highly reproducible through a multi-laboratory validation study [99], the kit has not been validated for GCP. In order to measure BA acid concentrations for diagnostic purposes in academic laboratories in the future, additional frameworks must be developed and adhered to.

5. Conclusion

This study underscores the significant variability in reported peripheral BA concentrations and sheds light on the preanalytical and analytical factors contributing to this challenge. While LC–MS is the dominant analytical technique, specific methodological differences in LC–MS, particularly regarding parameters related to the chromatography column and interface, could play a role in the observed variability. Beyond analytical techniques, crucial preanalytical considerations such as the fasting status of the subjects, the blood collection matrix and the starting volume of this matrix are vital for reducing variability. The choice of protein precipitation solvent, with methanol being the most effective, and the use of SPE for cleaner extraction and improved signal-to-noise ratio, are also critical steps in robust sample preparation. While the identified determinants of variability were drawn from across studies, emphasizing the need for investigations within the same patient to confirm their true impact, the implications for future research are clear. Standardization across all stages, from sample collection to analysis, is of utmost importance. This will not only improve the accuracy and reproducibility of BA concentration measurements but also enable more meaningful inter-study comparisons, particularly when studying BA profiles in various disease states.

Despite the current variability, the potential of peripheral BAs as valuable biomarkers for enterohepatic disorders, including MASLD severity, remains promising. By utilizing standardized protocols and leveraging enhanced analytical technologies, we can move closer to unlocking the full diagnostic and prognostic potential of BAs, ultimately translating research findings into improved patient outcomes.

CRediT authorship contribution statement

Sebastian Joseph: Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation. **Sophie de Buyl:** Writing – review & editing, Supervision, Software, Methodology. **Isabelle A. Leclercq:** Writing – review & editing, Supervision, Methodology, Funding acquisition. **Kristian Serafimov:** Writing – original draft. **Olivier Feron:** Writing – review & editing, Supervision. **Laure-Alix Clerbaux:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization.

Declaration of Generative AI and AI-assisted technologies in the writing process

The authors declare that generative AI was not used during the preparation of this manuscript.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary material related to this article can be found online at https://doi.org/10.1016/j.plabm.2025.e00508.

Data availability

The data and code to reproduce the figures can be found online at https://github.com/sebjoseph122/Bile-Acid-Literature-Review.

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