nature reviews gastroenterology & hepatology

https://doi.org/10.1038/s41575-024-00914-3

Perspective

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The changing metabolic landscape of bile acids – keys to metabolism and immune regulation

Ipsita Mohanty[1], Celeste Allaband[2], Helena Mannochio-Russo[1], Yasin El Abiead[1], Lee R. Hagey[3], Rob Knight[2,4,5,6] & Pieter C. Dorrestein[1,4,7,8]

Abstract

Bile acids regulate nutrient absorption and mitochondrial function, they establish and maintain gut microbial community composition and mediate inflammation, and they serve as signalling molecules that regulate appetite and energy homeostasis. The observation that there are hundreds of bile acids, especially many amidated bile acids, necessitates a revision of many of the classical descriptions of bile acids and bile acid enzyme functions. For example, bile salt hydrolases also have transferase activity. There are now hundreds of known modifications to bile acids and thousands of bile acid-associated genes, especially when including the microbiome, distributed throughout the human body (for example, there are >2,400 bile salt hydrolases alone). The fact that so much of our genetic and small-molecule repertoire, in both amount and diversity, is dedicated to bile acid function highlights the centrality of bile acids as key regulators of metabolism and immune homeostasis, which is, in large part, communicated via the gut microbiome.

Sections

[1]Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California San Diego, La Jolla, CA, USA. [2]Department of Pediatrics, University of California San Diego School of Medicine, La Jolla, CA, USA. [3]Department of Medicine, University of California San Diego, San Diego, CA, USA. [4]Center for Microbiome Innovation, University of California San Diego, La Jolla, CA, USA. [5]Department of Computer Science and Engineering, University of California San Diego, La Jolla, CA, USA. [6]Department of Bioengineering, University of California San Diego, La Jolla, CA, USA. [7]Department of Pharmacology, University of California San Diego, La Jolla, CA, USA. [8]Collaborative Mass Spectrometry Innovation Center, Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California San Diego, La Jolla, CA, USA. e-mail: pdorrestein@health.ucsd.edu

Introduction

Our understanding of the relationship between bile acids and the gut microbiome has undergone several major paradigm shifts. In many such cases, the main driver of new insights has been the advancement in analytical techniques, expanding the pool of bile acid structures. The first microbial bile acid product discovered was deoxycholic acid (the 7-dehydroxylated product of cholic acid), which is biosynthesized by the gut microbiome[1,2]. Later, lithocholic acid was described as the corresponding 7-dehydroxylation product of chenodeoxycholic acid[2,3]. Both compounds are hydrophobic, and much attention was focused on their toxicity to both bacteria and intestinal cells. The development of high-performance liquid chromatography–mass spectrometry (HPLC–MS) and gas chromatography–mass spectrometry (GC–MS) for the analysis of bile acid structures in human intestinal contents and faeces increased the number of described bile acid structures to nearly 100, and led to the concepts of bile acid 'damage' and 'repair' to describe alterations in bile acids caused by the gut microbiome[4,5]. In this paradigm, a small set of primary bile acids, which are exposed in the intestine to the microbiome, are chemically altered (damaged) and transformed into a larger set that is recycled via the enterohepatic circulation to the liver, where their structure is restored (repaired). However, this model was overturned in the 1990s by the gradual realization that bile acids, including those produced by the gut microbiome, are hormones with signalling properties. Bile acids were discovered to be the previously unknown ligands for the farnesoid X receptor (FXR)[6–8], a protein that, in turn, regulates the activity of many other genes[9–11]. Subsequently, bile acids were found to be ligands for a number of other receptors[12], including transmembrane G-coupled protein receptor 5 (TGR5)[13,14], the c-Jun amino-terminal kinase (JNK) signalling pathway[15], pregnane X receptor (PXR)[16,17], liver X receptor-α[18], vitamin D receptor (VDR)[19], sphingosine-1-phosphate receptor 2 (ref. 20), and the nuclear hormone receptor NR4A1 (ref. 21). Using the analytical technique LC–MS(–MS) and large-scale screens of human intestinal contents, we and others have now discovered that there are hundreds, and perhaps tens of thousands, of different bile acid structures made by the gut microbiome. This astronomical rise in the number of bile acids calls for a new understanding of the relationship between the microbiome and its host.

In this Perspective, we discuss the mass spectrometry (MS)-based technologies and data science-related approaches that are fuelling the discovery of modified bile acids, many of which are introduced by the microbiota. Our Perspective emphasizes the discovery of new amino acid or amine-conjugated bile acids (bile acid amidates), as this is leading to the discovery of a large array of different modifications. However, we acknowledge that there are also other modifications (such as esterification, sulfation and glucuronidation) and organisms or genes responsible for the introduction of these bile acid modifications that have been reported in the past few years and are discussed in detail in other literature[22–28]. Although the world of bile acids includes C_{27} bile alcohols, C_{27} bile acids and C_{24} bile acids, in this article we only consider C_{24} bile acids as those are the predominant bile acids found in humans. We further highlight the distribution of bile acids and bile acid-related genes and gene products in organs outside the enterohepatic system, leading to an encoder–decoder hypothesis in which the microbiome can influence the function of distant organelles, cells, tissues and organs. In this regard, we provide a perspective on outstanding challenges and future research avenues, especially in light of the recent findings.

Bile acid amidates are not exclusively made in the liver

Bile acids originate from the oxidation of cholesterol and are conjugated, a process generally described as being associated with the liver, resulting in glycine and taurine amidates of the bile acids[29] (Fig. 1). Bile acids span many orders of magnitude in concentration, from picomoles to hundreds of millimoles (Box 1). The dominant congeners of bile acids in bile are taurocholic acid and glycocholic acid (also known as cholyltaurine and cholylglycine, respectively) and are thought to be exclusively produced by the liver. However, a revision of the notion that these bile acids are only biosynthesized in the liver is warranted. First, the RNA transcripts for the bile acid-CoA:amino acid *N*-acyltransferase responsible for amidation can be found in other organs in animals, including humans, such as the gallbladder, spleen, ovary and brain, suggesting that organs other than the liver can carry out amidation reactions[30]. Second, glycine-conjugated C_{24} cholic and deoxycholic acid can be synthesized by gut bacteria, which originally was demonstrated only with oceanic flavobacteria. It has been proposed that microorganisms can use them as external biosurfactants to repel competitors[31,32].

That gut bacteria and fungi have been shown to couple glycine and cholic acid to furnish the glycocholic amidate, along with other glycine bile amidates, highlights that there is a blurring of the distinction between host-derived and microbially derived bile acids[33,34]. This picture of host or microbial production has the potential to be even further blurred, as microorganisms were also found to be capable of synthesizing cholesterol and oxysterols[35]. The level of microbial contribution to the cholesterol pool and the animal glycine bile amidate pool is as yet unknown. Either the microbial contribution could be minimal or could be the major source, or it could be that microorganisms stimulate host production, as seen with other important human molecules. For example, commensal microorganisms can make serotonin, and as much as 90% of the total human serotonin content is produced when commensals are present[36–38]. In germ-free mice, the glycocholic acid conjugates were decreased compared with the levels in colonized mice, often below detectable levels[39], highlighting that microorganisms probably substantially complement the production by the host liver, and therefore that glycine bile acid conjugates should no longer be considered only primary host-derived metabolites.

Journey of bile acids along the digestive tract

Considering the modifications that can be introduced to a bile acid, their diversity is astonishing, especially when combinations of modifications are observed. Much of this bile acid diversification after their synthesis occurs when the gallbladder is emptied. When we consume a meal, the gallbladder, where bile acids are stored after synthesis from cholesterol, is emptied into the duodenum. In humans, the most commonly described bile amidates in the gallbladder are the taurine and glycine amidates of cholic, chenodeoxycholic and deoxycholic acid, but many other amidates may be present, including glycine, alanine, arginine, asparagine, glutamine, leucine/isoleucine, lysine, phenylalanine, tyrosine and tryptophan[40]. Upon entry into the duodenum, microbial processing of bile acids begins and continues down the length of the digestive tract to create secondary bile acids, a dynamic series of changes in which the original bile acid molecule can be altered to such an extent that it is no longer recognized as a bile acid. Within the intestinal environment, bile acids undergo many different transformations[41–44] (Fig. 1). One of the re-amidation reactions couples bile acids to dietary proteins, whereupon they promote protein digestion by pancreatic proteases[45]. This observation is corroborated by the involvement of

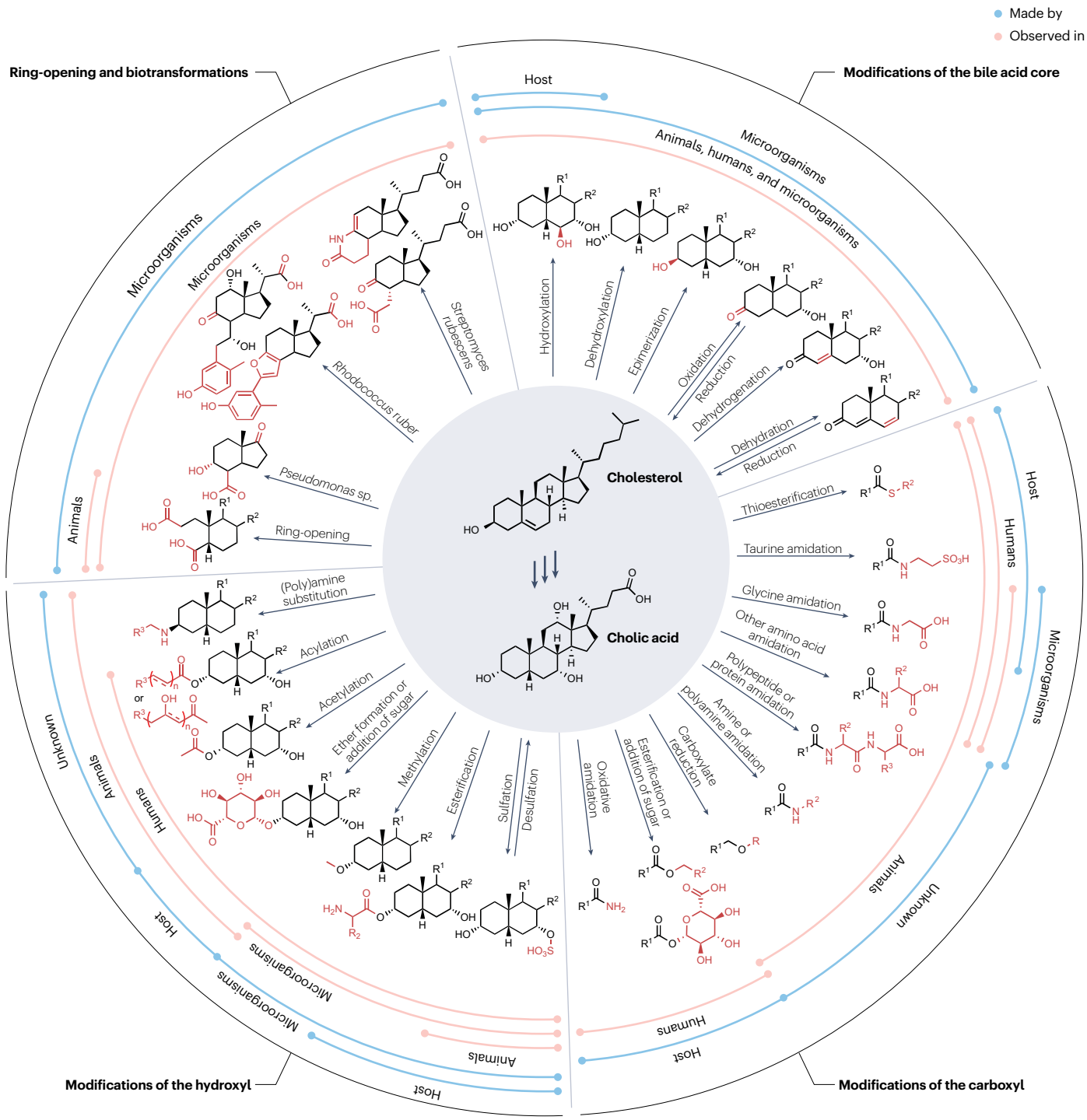


Fig. 1 | Diversity of known bile acid chemical modifications. The outer circle (blue) shows the producer organisms for these modifications, if known. The inner circle (pink) shows where these bile acid modifications have been observed: humans, animals or microorganisms. Substructures (red) represent the modification introduced by the specified reaction. Modifications to the bile acid core: hydroxylation[33,213–215], dehydroxylation[33,216–218], epimerization[153,219–224], oxidation[153,225–227] and reduction[226], dehydrogenation[228], and dehydration[229,230] and reduction[231]. Modifications of the carboxyl: thioesterification[41], taurine amidation[232], glycine amidation[232], other amino acid amidation[40,42,121–124], polypeptide or protein amidation[125], amine or polyamine amidation[124,233,234], carboxylate reduction[235,236], esterification or addition of sugar[74], and oxidative amidation[68]. Modifications of the hydroxyl: sulfation and desulfation[70,71], esterification[77], methylation[76], ether formation or addition of sugar[72,73,75], acetylation[42], acylation[77,80,82], (poly)amine substitution[237]. Ring-opening and biotransformations: ring-opening[67], *Pseudomonas* sp.[63], *Rhodococcus ruber*[64], and *Streptomyces rubescens*[65].

Box 1

Bile acid concentrations

Bile acids are among the most abundant molecules in the body and have large intrapersonal and interpersonal variations[113]. Their reported concentrations range from not detectable (below the picomolar range) to high millimolar. Up to 300 mM concentrations are found in the gallbladder[243]. Passage from the gallbladder to the digestive tract results in dilution to 0–70 µg/ml (0 to ~150 mM) wet weight in faeces[113,244]. Bile acid concentration in the caecum has been reported to be 0.6 mM[245] or >500 mg/kg dry weight[244]. Another dilution is observed as bile acids pass to the plasma/blood, although a concentration of up to ~3 mM has been reported. Some reports mention that the majority of bile acids are sulfated[83], but other work has shown much lower concentrations in the blood. The reported concentrations of bile acids in the blood are in the range 6–200 nM[246,247]. It could be that both reports are correct as the bile acid pool is very dynamic, but this requires a deeper analysis. In general, however, the average total bile acid concentrations are reported to be ~20–50 µM in portal blood and ~5 µM in venous blood[113,248]. The average values reported for individual bile acids in the human metabolome database, which catalogues concentrations from published papers on blood and bile, reveal that cholic acid has the highest concentrations in both blood and bile, with averages of 48.5 µM[249] and 32 mM[250], respectively (see Supplementary Fig. 1). As bile acids leave the enterohepatic system, they can be concentrated, as millimolar quantities have been reported in the urine (for example, ~9 mM, the majority reported to be sulfated[83]). Sulfated and glucuronidated bile acids are believed to be the dominant species in urine (micromolar to millimolar concentrations)[83].

Concentrations of microbial bile acids, such as deoxycholic acid, in mice were found to be ~20 pmol/mg wet weight (20 µM) in faeces, 450–700 µM in the caecum[245] and 150 µM in the small intestine[251]. Another study found concentrations of the microbial bile acids lithocholic acid, deoxycholic acid and hyocholic acid in fasting plasma to be in the range 0.02–0.1 µM[252].

The average concentration of microbial bile acid amidates, such as phenylalanine-conjugated cholic acid, in the jejunum of female mice has been reported to be 147 nmol/g tissue weight (~147 µM, assuming that the density of faeces is close to 1 g/ml), with the highest value of 447 nmol/g (~447 µM) measured in one sample. In the ileum the concentration was 84 nmol/g (~84 µM), decreasing to 4.7 nmol/g (~4.7 µM) in the caecum and to 11.6 nmol/g (~11.6 µM) in the colon[42]. In another study, the concentrations of cholic acid amidates in the caecum of mice were found to be 0.3–11.4 nmol/g (~0.3–11.4 µM), 0.3–0.5 nmol/g (~0.3–0.5 µM), 1.1–11.3 nmol/g (~1.1–11.3 µM), 0.4–1.0 nmol/g (~0.4–1.0 µM), 0.4–8.5 nmol/g (~0.4–8.5 µM), 1.2–6.0 nmol/g (~1.2–6.0 µM) and 0.5–2.6 nmol/g (~0.5–2.6 µM) for the phenylalanine, tyrosine, leucine, tryptophan, serine, alanine and glutamate conjugates, respectively[158]. The trend of changing concentrations down the digestive tract of microbial amidates was also observed in humans[43,44]. Estimated concentrations of glutamine and serine conjugated to a trihydroxylated bile acid in the human intestine were in the range 0 to ~1,000 ng/ml (~1.86 µM) and 0 to ~8,000 ng/ml (~16.15 µM), and in stool from 0 to ~200 ng/ml (~0.37 µM) and 70 ng/ml (~0.14 µM), respectively[43].

Bile acid concentrations often appear to be inconsistent within a study and across different studies, but there are good reasons for this. In humans, the levels of each of the different bile acids can change by at least an order of magnitude throughout the day, and can also be experiment-dependent, with the taurine and glycine conjugates peaking around lunch and dinner and the unconjugated cholic acids, lithocholate, deoxycholate, ursodeoxycholate and chenodeoxycholate peaking around midnight and remaining fairly stable until around awakening but before breakfast[253,254]. Although this highlights diurnal changes in bile acid concentrations, there are other factors that can cause rapid changes in bile acid concentrations aside from eating or fasting. The serum levels of bile acids in humans are rapidly affected by exercise, with measured average concentrations of cholic acid changing from 0.16 µM to 0.06 µM after exercise[255]. Microbial bile acids are also affected as deoxycholic acid changes from 0.32 µM to 0.1 µM[255]. Systematic assessments of bile acids and their levels on the basis of different human lifestyles and diurnal and circadian physiology are still needed to fully understand bile acid dynamics.

bile acids in protein digestion[45], nutrient absorption from the gut, proper functioning of energy supply, temperature regulation and mitochondrial bioenergetics, and also in the proper functioning of the endoplasmic reticulum and Golgi apparatus responsible for protein synthesis[46–49]. Using pull-down assays, it has been shown that many mitochondrial, endoplasmic reticulum and Golgi apparatus proteins bind to bile acids, or related molecules, including bile acid biosynthetic enzymes, hydrolases/lipases and sulfatases[50].

As the bile acids move along the digestive tract through the duodenum, jejunum, ileum, caecum (in rodents as in humans this is a 6-cm region near the appendix but does not appear to be a separately functional organ), and colon, including the appendix[51], they are continuously modified by the gut microbiota, often in a region-specific manner that is also dependent on age (Box 2), diurnal timing, sex and health status. In addition, given the sheer diversity of microorganisms that are associated with humans, the genetic potential to encode enzymes with bile acid-modifying capabilities for human-associated microorganisms is at least 1.5 to two million times larger than the potential associated with the human genome[52]. In the digestive tract, bile acids can modulate intestinal permeability and gastric motility, processes that are regulated by the microbiota, through the stimulation of the enteric nervous system[53–56]. By the time bile acids reach the caecum, taurine and glycine bile acid levels are greatly reduced, and, in their place, bile acids are now decorated with proteinogenic and non-proteinogenic amino acids, dipeptides, (poly)amines, fatty acids, sulfate, cholesterol, saccharides, and other modifications as highlighted in Fig. 1.

In general, it is said that the bulk of unmodified bile acids are reabsorbed in the terminal ileum before reaching the large intestine, but this statement has been made by monitoring only a few bile acids. A misunderstanding is that the depletion of bile acids by the time they

reach the caecum or colon is an analytical artefact in that we are unable to detect bile acids that are modified beyond recognition using the assays that have been widely used in the past few decades[57–59]. There is currently not a single bile acid detection protocol that can fully capture the true diversity of the metabolic changes undergone by bile acids, and, as a result, the ability to comprehensively detect and describe the extensive bile acid metabolic network truly represents a major 21st century analytical challenge.

How many modifications?

To represent a lower bound for the count of modifications associated with known bile acids (excluding stereoisomers), we summarized information from metabolomics and lipidomics databases (Fig. 2a). For the purpose of this estimate, tetracyclic steroidal cores with the carboxy tail that have two methyl groups attached to the carbon skeleton were considered. Their stereochemistry was not considered as not all the structures in the structural databases had stereochemistry

Box 2

Bile acids and age

The intestinal tract in the fetus is generally considered to be sterile and, thus, is unable to produce microbial bile acids; only those bile acids that are biosynthesized de novo by the fetus or that pass through the umbilical cord are present[256]. Surprisingly, fetal and neonatal bile acids show a great diversity in their structure[257], many of which are not present in the adult biliary profile. This population of bile acids participates in shaping the nutritional uptake, energy homeostasis and inflammatory landscape of the developing human before birth[258]. For example, B-ring oxysterols bind to and activate the 7-transmembrane receptor known as Smoothened. When activated, Smoothened can inhibit hedgehog signalling (hedgehog signal transduction is an ancient pathway that regulates the differentiation of embryonic cells). In humans with Smith–Lemli–Opitz syndrome, the overproduction of a B-ring oxysterol 3β-hydroxy-6-oxo-5α-cholesten-7,8-ene results in deficient hedgehog signalling[259]. The fetal meconium is largely composed of unusual polyhydroxylated bile acids that are probably the metabolic reflections of oxysterols initially synthesized for alternative functions.

Even the bile acid-modifying enzymes change after birth. For example, the formation of hyocholic acid involves the hydroxylating enzyme CYP3A7 and starts 20 weeks before birth. After birth, the enzyme CYP3A4 replaces CYP3A7 in hyocholic acid synthesis[260]. We hypothesize that the entirety of the bile acid pool while in the womb and during the first few years is critical in the establishment of the immune system, energy homeostasis and receptor development within the developing fetus and infant.

Upon birth, bile acids such as cholate and chenodeoxycholate are already present in the colostrum and breast milk, serving both to contribute to fat digestion and to aid the child's development[261]. Bile acids drive maturation of the microbiome in the developing infant[262]. Tetrahydroxylated bile acids, which are selectively capable of reversing liver damage in mice[263], rapidly increase in the first 5 days postpartum, followed by a rapid decline in the urine, but continue to be detected in the first year in the blood. Tetrahydroxylated bile acids have prognostic value for cholestasis and are an underappreciated class of bile acids. Early in life, little microbial processing of bile acids is observed, but as the infant matures, the diversity of intestinal bile acid structures increases. A disruption of the bile acid trajectory by antibiotics given to the mother changes the bile acid profile of the developing infant and affects long-term health[264–267]. The timing of the transition from the neonatal bile acid pattern to one characterized by microbial bile acids is associated with islet autoimmune disease, which might contribute to conditions such as type 1 diabetes and other immune disorders[268]. Changes in bile acid repertoires due to the early use of antibiotics should not be overlooked when examining the causative mechanisms for a subsequent rise in immune and metabolic disorders.

In older age, there is a shift in the bile acid population that favours conjugates, traditionally being thought of as the glycine and taurine conjugates, which can fine-tune amphiphilicity and receptor interactions[269–271]. Shifts in microbial bile acid products such as lithocholic acid and deoxycholic acid have been linked to the development of Alzheimer disease[95,272,273]. In general, microbial bile acids seem to be related to age and cognitive risk factors[274]. SXR/PXR activation and increased levels of lithocholic acid, a vitamin D receptor agonist, might contribute to osteoporosis[275–277]. Such observations suggest that the bile acid metabolic network changes, and that changes in this trajectory can have a critical role in the preconditioning of health homeostasis in older age. It is not implausible that alterations in microbial composition early in life (that is, treatment with antibiotics), with subsequent effects on bile acid metabolism[278], might lead to increased occurrence of immunological and/or metabolic disorders (for example, allergies, asthma, inflammatory bowel disease (IBD), atherosclerosis, diabetes and polycystic ovary syndrome) later in life[279]. Antibiotics have been shown to alter the bile acid pool of the large intestine, liver and plasma in mice[280]. These changes decreased insulin sensitivity[281]. In centenarians, certain types of microbial bile acid modifications can lead to improved metabolic health. For example, centenarians have been shown to have increased amounts of microbial-mediated oxidation–reduction reactions and epimerization modifications to bile acids, resulting in larger quantities of isolithocholic acid, 3-oxo-lithocholic acid, allolithocholic acid, 3-oxo-allolithocholic acid, and iso-allolithocholic acid[282]. All of these are extreme hydrophobic bile acids with nanomolar effects on cellular receptors. It might be that such bile acids result in altered mitochondrial function and, therefore, in altered energy homeostasis, as lithocholic acid accumulation in yeast mitochondria results in an anti-ageing cellular phenotype[283]. At least one of these lithocholic acid isomers has been found to inhibit gram-positive microorganisms, which might reduce the risk of systemic infections and aid in the maintenance of health. The presence of the common microbiome 7α-dehydroxylating bacteria and bacteria with bile salt hydrolase in the intestine is sufficient to alter the growth of *Clostridium difficile*[69,284–286]. Thus, as we age and our bile acid profiles change, so too does our bile acid preconditioning to disease susceptibility.

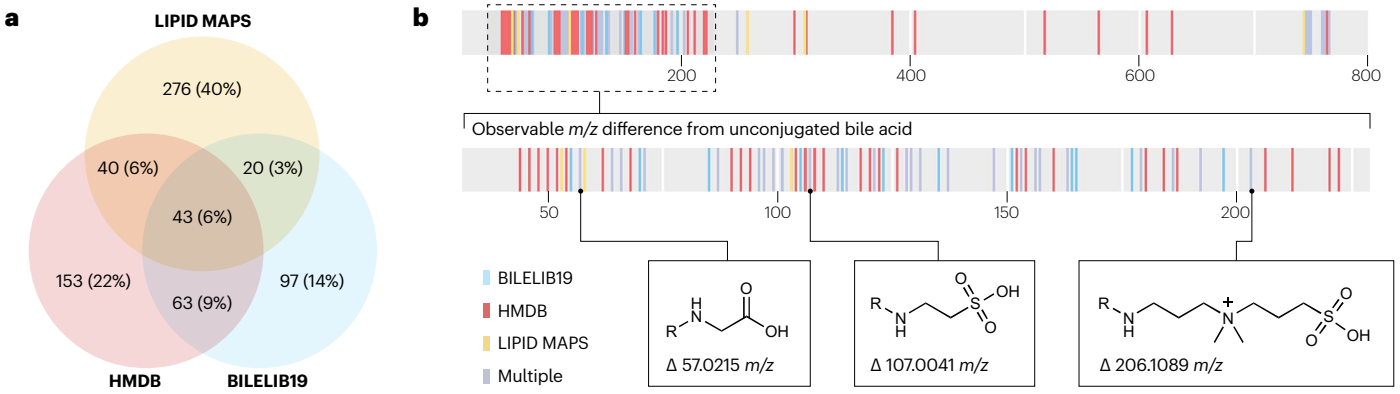


Fig. 2 | Modifications of known bile acids. a, Bile acid overlaps in the structural and mass spectrometry databases for GNPS/MassIVE BILELIB19 (ref. 40), Human Metabolome Database (HMDB)[61] (only considering detected bile acids), and LIPID MAPS[62] were derived from deposited SMILES codes (links to databases are provided in the Data availability section). For the purpose of this figure to visualize the data in the three repositories, all SMILES[238] with the (SMARTS string-defined) substructure "C-,=C12-,=C-,=C-,=C-,=C-,=C-,=1-,=C-,=C-,=C3-,=C4-,=C-,=C-,=C-,=C-,=4(-,=C-,=C-,=C-,=3-,=2)-,=C" and a carboxylic acid ester ("C(=O)O"), (thio)ester ("C(=O)S"), or amide ("C(=O)N") in their carboxy tail were considered as bile acids. Substructure searches were conducted via RDKit (www.rdkit.org). Diastereomeric information was not considered for the comparisons across bile acids. **b**, Mass shifts were calculated by subtracting the mass of the unconjugated bile acid (for example, $C_{24}H_{40+x}O_{2+x}$ with x is the number of hydroxyl modifications on the respective bile acid for C24 bile acids; the formula was adjusted for C22–C28 bile acids (as defined in LIPID MAPS), respectively) from the mass of the (potentially) conjugated bile acid. Only mass differences >40 *m/z* are shown.

information. We retrieved 223 different bile acid structures from the BILELIB19 (ref. 40) reference dataset, which is part of the Global Natural Product Social Molecular Networking (GNPS) untargeted MS data analysis infrastructure[60], 299 bile acids with 23 quantified in blood and/or bile (Box 1; see Supplementary Fig. 1; Fig. 2a) from the Human Metabolome Database (HMDB)[61], and 379 such bile acids from the LIPID MAPS database[62]. Combining the bile acids from all three resources, 692 different bile acids were retrieved. They included 20 ring or core modifications and 110 carboxy tail modifications. These were observable as 97 unique mass shifts relative to the unconjugated bile acids (Fig. 2b).

This is a lower bound of modifications, as these resources are incomplete and are limited by our ability to identify the massive number of bile acids which we are currently incapable of detecting. The biological patterns that drive bile acid modifications and/or absorption are still poorly understood and thus still represent another enormous research opportunity. Also, there is no single resource available that actively creates a digital archive of published bile acid-related studies or emerging studies, or that has aggregate information of all known bile acids and their levels in relation to different biological conditions that the community can browse or reuse. Although bile acids that are synthesized by the host from cholesterol or derived from the diet constitute the defining pillars of the overall bile acid metabolic network, the majority of structurally unique bile acids in the network of bile acids are created by microorganisms[39,42] (Fig. 1).

Expanding to thousands of structures

Given the potential for the existence of thousands of bile acid modifications, the question arises: how can a few host-synthesized bile acids expand into thousands of structures? Our understanding of the nature, role and types of bile acid structures is currently undergoing a revolutionary change, as the number of known structures is ever expanding. The source of this diversity stems from what can be thought of as two 'halos' of change of the core and then decoration or modification of the steroid core (Fig. 1). All bile acids are derived from the cholesterol at its core, and, as such, contain a stable structure consisting of four rings as well as a hydroxyl group at C-3. The first halo consists of changes directly to the four rings. Such changes occur at the 'macro' level of analytical chemistry, and most of the known structures of bile acids fall into this halo. The major human gallbladder bile acids include ring hydroxylation at specific positions and stereochemistry – 3α, 7α and 12α – along with conjugation with the amino acid taurine or glycine at the side chain carboxylic acid. Then, the microbiome alters these bile acids by multiple biochemical processes (Box 3). In addition, as bile acids are released into the environment, they can also undergo biotransformations by microorganisms (observed for *Streptomyces rubescens*, *Pseudomonas* sp., and *Rhodococcus ruber*), resulting in very different metabolites with only the C and D rings of the bile acid core intact[63–66] (Fig. 1). Although these individual microorganisms have been found in the environment and/or have been isolated from wastewater, such bile acid transformations have not yet been observed to be mediated by the microbiota. We hypothesize that many such or related reaction products are likely to be produced by the microbiota in animals, including humans. In support of this hypothesis, 3,4-seco-12α-hydroxy-5β-cholan-3,4,24-trioic acid, as the product of a steroid core ring opening of deoxycholic acid, has been isolated from the bile of a common ringtail possum[67].

Beyond the first halo, there is an extensive outer halo of bile acid structures found at the 'micro' level of analytical chemistry, where partial information exists on thousands of structures, few of which have been completely characterized. This outer halo consists of a large pool of metabolites either attached to ring substituents or, in much larger part, based on new bile amidates obtained by re-conjugation to the side chain carboxylic acid. Host-conjugated taurine or glycine is readily removed from the initial amidates, and the microbiome rapidly and readily replaces these two amino acids with any and all amino acids, along with a large number of other metabolites in animals and humans[42] (Figs. 1, 2b). The ammonia amide conjugate of the carboxylate is created by the action of the copper-dependent human peptidylglycine α-amidating monooxygenase of glycine bile

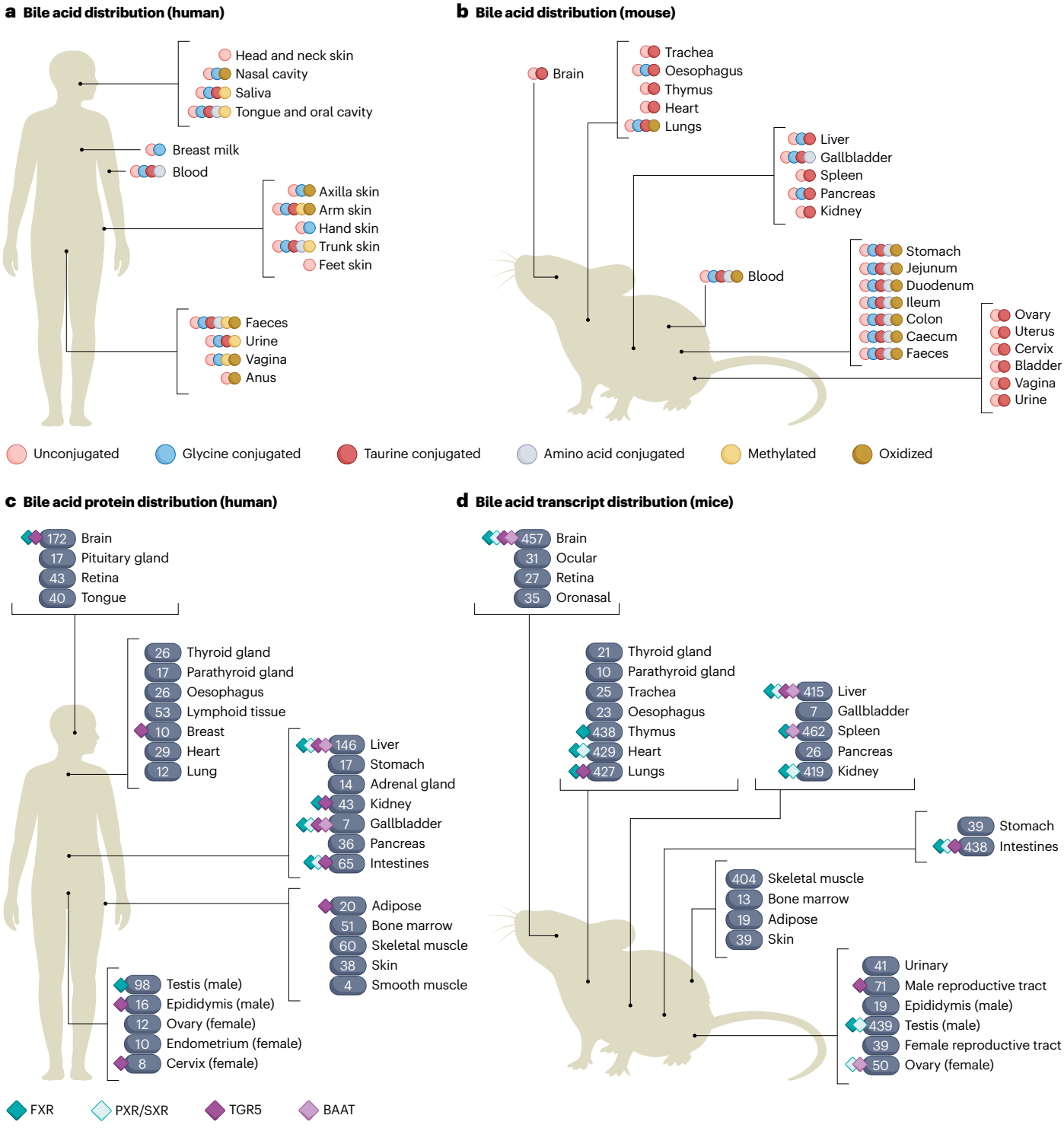
acid amidates[68]. Any attachments to the terminal carboxylic acid of bile acids results in a multiplication of the potential structures found in the inner halo. Re-conjugated bile acids produced by microorganisms can be present at quantities higher than the host-derived taurine amidates[69]. The inner halo of hydroxyl groups can also be expanded by attachment to sulfates[70,71], sugars[72–75], acetyl and methyl groups[42,76], methyl amino acids[77] and fatty acids[78] (for discussion and references see section 'Driving the rapid pace of discovery') in both ether[79–81] and ester linkages[77,80,82]. Although these forms tend to be less studied as the standards are not readily available, the sulfated and glucuronidated forms are dominant in human urine[83–85] (Box 1; see Supplementary Fig. 1), highlighting the need for more comprehensive bile acid detection methods. The astonishing discovery of an undetected massive outer halo of micro-scale but biologically active compounds is challenging our previous understanding of the purpose of bile acids and their intimate relationship with the microbiome.

Box 3

Bile acid modifications

Bile acids fundamentally originate from cholesterol. Modifications can take place in various parts of the bile acid structure, including directly in the bile acid core, in the carboxyl group, and in the hydroxyl groups. These modifications are known as:

- (Re)Deconjugation — taurine or glycine conjugated to bile acids by the host enzyme bile acid-CoA:amino acid *N*-acyltransferase[231] can be removed[157], a reaction that is catalysed by bile salt hydrolases (BSHtr) characterized from multiple gut bacteria (discussed in the section 'A new function of bile salt hydrolases'). BSHs can also catalyse re-conjugation of bile acids with amino acids or amines[158,168]. Conjugation of bile acids with polyamines has also been observed[233], with an additional study supporting the observations that the polyamine precursor, γ-aminobutyric acid, is conjugated to deoxycholic acid and exists in humans, and that bile acids conjugated with polyamines are conjugated by human gut microbes[234].
- Hydroxylation — which can occur in several positions of the bile acid core[213,214]; for example, conversion of deoxycholic acid to hyodeoxycholic acid (which is a C-6 hydroxylation) catalysed by *Collinsella stercoris*[33]. Another biotransformation of methyl cholate to a tetrahydroxy bile acid with a hydroxy group inserted on C-15β was shown in the fungus *Aspergillus niger*[215]. The enzymes responsible for this reaction have not been characterized.
- Dehydroxylation — an example is the removal of the 7α-hydroxyl group mediated by a relatively small number of anaerobes, which nevertheless are extremely active[153,216–218]; the dehydroxylation at C-7 is carried out by seven different enzymes, catalysing a total of eight reactions, and the 12α-hydroxyl group is removed by 12α-dehydroxylation mediated by the gut bacteria *C. stercoris* and *Ruminococcus gnavus*[33] and *Bacteroides* sp.[287].
- 7α-Dehydration — loss of the 7α-hydroxyl group as a water molecule inserting a double bond at C-6, which is known to be catalysed by the enzyme 7α-dehydratase in *Clostridium hylemonae* TN271 (refs. 229,230). The reverse reaction (reduction) by a flavin-dependent squalene desaturase was found in the human gut bacterium *Clostridium scindens* ATCC 35704 (ref. 231).
- Ketone formation — widely observed conversion of hydroxyl groups into ketones benefits those bacteria that have an intact Wood–Ljungdahl pathway, which utilize the captured protons to convert CO_2 into acetate[225]. For example, the hydroxysteroid dehydrogenase enzyme, 12α-HSDH, from *Collinsella tanakaei* YIT 12063 and *C. stercoris* DSM 13279 oxidizes 12α-hydroxyl to 12 ketone[153] and in *Dorea* sp. AM58-8, BaiA, a promiscuous bile acid 3-dehydrogenase enzyme, produces three keto bile acids[226]. The reduction of the keto bile acids can be furnished by the same enzyme[227].
- Dehydrogenation — removal of hydrogen to give a double bond, catalysed by several stereospecific NAD(H)-dependent 7β-hydroxy-3-oxo-Δ4-cholenoic acid oxidoreductases in *C. scindens* VPI 12708 (ref. 228).
- α-Oxidation — commonly seen with 23*R*-hydroxylation (a bile acid found in humans)[288], this modification has only been observed in rodent and human bile, but the enzymes responsible have not yet been determined.
- Hydroxyl group epimerization — flipping the orientation of the ring hydroxyl groups from the usual α-position (below the rings) to the β-position (above the rings) stretches out the overall shape of the four rings, which in turn alters their interactions with transporters and receptors[153,219,220]. There are a relatively small number of gut bacterial species converting bile acid 3α-hydroxy groups into 3β-hydroxyl groups; they include *Eggerthella lenta*[221], *Clostridium perfringens*[222,223], *R. gnavus* and a Lachnospiraceae species[224].
- Side-chain epimerization — where the 17β conformation of the side chain derived from cholesterol is flipped to 17α[289].
- Loss of the side chain — this degradation process shown in the terrestrial bacterium *Azoarcus* sp. strain Aa7 removes the side chain at C-17, leaving behind a ketone group[290].
- Opening of the B ring — hydroxylation at C-9 leads to the spontaneous opening of the B-ring by a reverse vinylogous aldol reaction, creating a new series of 9,10-secosteroids observed in *Pseudomonas* sp.[291].
- Reduction of the carboxylic acid group at C-24 (refs. 235,236).
- Opening of the A-ring — which has been suggested to be achieved by an enzymatic pathway similar to the Baeyer–Villiger oxidation. Initially, a 3-oxo bile acid is converted to a pair of regioisomers, which could serve as a precursor for the 3,4-seco bile acid biosynthesis[67].
- Alterations to the A-ring and B-ring junction – the 7α-dehydroxylation of the cholic acid in humans yields deoxycholic acid as a major product. A side reaction in the process flattens the four sterol rings and creates a parallel set of bile acids known as allo-bile acids[292].



The fate of microbial bile acids

The first target of these modified bile acids is the microbiome itself. Bacterial species are riddled with auxotrophies, in which individual members contain, lack or emphasize certain metabolic pathways and outputs at the expense of others[86]. The re-conjugation of metabolites to the bile acid structure specifically targets those bacteria that contain the bile acid membrane transport systems, hydrolases and enzymes capable of interacting with bile acids – properties that make up a definition of the microbiome and exclude other bacterial species. One of the most studied representative systems is the BaiG bile acid uptake transporter[87]. The second target is the intestine, with multiple cell types, including an extensive array of neurons. It is not surprising that the

Fig. 3 | Distribution of bile acids and related transcripts or proteins. Distribution of observed bile acids as documented by mass spectrometry reanalysis of data user interface (ReDU)[139] in humans (part **a**) and mice (part **b**). Briefly, sample information and metadata available in the public repositories were filtered for '*Homo sapiens*' and '*Mus*/*Rattus*'. A search list adapted specifically for bile acids was used to subset a list of files for which bile acids (only those that are curated in the Global Natural Product Social Molecular Networking (GNPS) spectral library) were detected. The total count of such files across different body regions as defined by the UBERONBodyPartName ontology[239] in ReDU was calculated, grouped based on the bile acid modification (unconjugated, glycine-conjugated, taurine-conjugated, other amino acid-conjugated, methylated and oxidized to form ketones) and described for humans and mice. For protein and transcript locations, all matches discussed are for 'bile' in the Human Protein Atlas[30] (part **c**) and mouse gene expression (part **d**) databases[240]. Numbers in parts **c** and **d** represent the numbers of bile acid-associated genes or gene products observed in respective body locations. Overlaid is the information about the bile acid receptor proteins, FXR, SXR (humans), PXR (mouse) and TGR5, and the *N*-acyltransferase enzyme bile acid-CoA:amino acid *N*-acyltransferase (BAAT). Data and codes used for the reanalysis of public data to create this figure are made available in the 'Data availability' section. It should be noted that differential distributions between organisms should not be inferred from this figure, but the figure should be viewed rather as documenting where transcripts, proteins and bile acids have been detected. The figure is also an incomplete picture, as many organs or biofluids have not been studied by transcriptomics, proteomics or metabolomics, let alone under all conditions that might alter the detection, but it gives an overview of just how widely the bile acid metabolic and gene network is distributed throughout the body.

bile acid–gut–brain axis, rising to the brain and back down to organs of immunity, is a major target of microbial products. In the river-like flow of bile salts across the intestinal wall to reach the portal circulation, neurons wait like fishermen, continuously sampling the waters. The third target is the portal vein, which opens the doorway to the systemic delivery of bile acids in organs beyond the liver. As bile acids move down the digestive tract, they are absorbed into the circulatory system to reach tissues, organelles and cells connected to the vascular system and define a key highway for gut microbiome-to-organ communication. There are bile acid-associated proteins, transporters and receptors in all tissues that have been analysed by proteomics, consistent with a systemic role of bile acids, as opposed to those proteins that only serve a role in the enterohepatic circulatory system[30] (Fig. 3c,d). Some of the bile acid receptors that have been extensively studied in the liver and intestine, such as FXR, PXR and TGR5, can also be detected in distant tissues such as the brain, adipose tissue, gallbladder and breast[6,14,16,30] (Fig. 3c,d).

Interestingly, in humans, the largest number of bile acid-associated proteins are found in the brain, followed by the liver and testes[30]. The diversity of possible bile acid proteins and transcripts in the different organs supports the hypothesis that bile acids have different effects on the brain, testes and thymus function, probably via microbiota crosstalk[88,89]. Microbial bile acids are critical for the proper functioning of the immune system. In the gut, microbial bile acids are involved in T cell regulation and differentiation (discussed in detail in the section 'Bile acids are immune regulators')[90–92]. In addition, the large number of bile acid genes in other organs such as the spleen and thymus (as detected in a mouse transcriptomic atlas)[30] potentiates their influence on immune response in these organs. The thymus and spleen are two critical organs for the defence against pathogens and tumours, and response to tissue damage. There is a key thymus–endocrine–liver pathway, but the role of bile acids in this pathway, if any, has not been investigated[93]. Removal of the spleen in patients with cirrhosis, as part of an observational study seeking to understand the spleen's involvement with the microbiome and metabolome, to reverse liver cirrhosis led to alterations in the levels of bile acids to levels closer to those in healthy volunteers, suggesting that there is also a potential spleen–gut pathway[94].

Bile acids themselves have been detected in human brains (and are altered in patients with Alzheimer disease), and are also readily detected in brains from healthy mice[55,95–98] (Fig. 3a,b). Bile acids are neuroactive in the brains[96]. Both cholate and chenodeoxycholate are agonists of *N*-methyl-D-aspartate receptor, an excitatory neurotransmitter in the human brain, and γ-aminobutyric acid type A ($GABA_A$), a receptor that inhibits ganglia[99]. The brain can hydroxylate cholesterol at C-24, forming the 24*S* stereoisomer. As a result, brain tissue contains about 80% of the total 24*S*-hydroxycholesterol content in the body[100]. Once formed, 24*S*-hydroxycholesterol joins the bile acid synthetic pathway, including via systemic redistribution[100]. The brain, like the liver, has a source of cholic acid and chenodeoxycholic acid, possibly delivered from the enterohepatic system, and is probably able to make bile acid amidates, as the enzyme bile acid-CoA:amino acid *N*-acyltransferase (BAAT) is expressed in the brain as well[101] (Fig. 3d). This suggests that bile acids represent a unique communication channel between the gut–microbiota–brain axis and the rest of the nervous system[98]. The understanding of the gut's relationship to other potential organ axes remains under-explored with respect to bile acid biology.

Although an argument can be made that contamination of the tissues or samples with blood affects the reliability of bile acid detection in each of the tissues, a strong argument can be made against this idea, in that there are unique bile acid profiles across different body parts that are distinct from those in the blood. In addition, sample collection strategies such as skin swabs do not contain blood[39,42,102] (Fig. 3a,b). Thus, a gut–organ axis of a diverse bile acid metabolic network exists. This could be thought of as a highway for gut-to-other-organ signalling. Documented examples include the gut–lung axis[103], gut–liver axis[104], gut–brain axis[98], gut–muscle axis[105,106] and gut–bone axis[107,108]. There is also likely to be a gut–skin axis[109], but perhaps the skin itself is capable of synthesizing bile acids via microbial transformations[110]. Many skin microorganisms also carry bile salt hydrolases (BSHs), the enzymes responsible for deconjugating and amidating bile acids. It is not yet established whether these BSHs found in skin microorganisms have similar functions to the homologous BSH enzymes from gut-derived microorganisms that have been characterized. Thus, a bile acid metabolic network that functions for any of the organs in the body is probably the rule rather than the exception. It is becoming increasingly clear that the gut microbiome can utilize bile acids to influence the function of distant organs, at least in part, through the modifications the microorganisms themselves introduced into bile acids.

Driving the rapid pace of discovery

Advances in analytical chemistry and DNA sequencing technologies, modern computational solutions, publicly available data resources, and an interest in understanding the functional role of the microbiota are all reigniting interest in bile acids and their discovery. One of the main drivers of discovery of new bile acids is the explosion in the development of

experimental and bioinformatic workflows in MS and the search for bile acids outside the blood, plasma, urine or liver matrices, where glycine and taurine bile amidates, sulfates and their hydrolysed versions dominate. These developments have resulted in the discovery of hundreds of additional bile acids, many of which are produced by the human microbiota, in the past 3 years alone, as discussed below. Bile acids have a critical role in wide-ranging health outcomes[111], including liver diseases, atherosclerosis, Alzheimer disease, IBD, depression, sleep disruption, diabetes, polycystic ovarian syndrome[112] and obesity[113,114], and are used as treatments in modern health care[115–119]. The most recent example is the approval by the FDA in September 2022 of the orphan drug status of the combination of phenylbutyrate and taurursodiol as a potential candidate for the treatment of amyotrophic lateral sclerosis following the finding that this combination prolonged life by 10 months in patients with amyotrophic lateral sclerosis (although the efficacy is still being evaluated)[120]. Given these instances of the importance of bile acids in health care, it is surprising that the scientific community is still in the puzzle piece collection phase for the discovery of new bile acids.

New scientific approaches and technologies are driving the discovery of the puzzle pieces that make up the bile acid metabolic network and will be key to understanding the functional role of bile acids (Table 1). Reports describing bile amidates other than those with taurine and glycine in the 1960–1990s do exist: amino acids such as arginine and ornithine in rat liver[121], ornithine in bile from patients with cholelithiasis[122], lysine and ornithine from ox bile[123], (2-aminoethyl) phosphonic acid from bovine bile[124] and the dipeptide glycyl taurine from rabbit bile[125]. Lithocholic acid also binds to lysine residues in proteins[126]. However, it was only following the most recent introduction in 2012 of a spectral alignment algorithm developed in our own laboratory, together with collaborators, using molecular networking[60,127] that three new bile acid amidates were discovered and linked to microbial production. These amidates were also found in animals and humans (tyrosine, phenylalanine and leucine)[42]. In addition to the three new microbial bile amidates with amino acids validated in the study using synthetic standards, other bile acids were observed that are yet to be annotated, including sulfated bile acids and acetylated bile acids in germ-free mice only. When the three new amidates were cultured with microorganisms, a hydroxyl group was oxidized to a ketone within the cholic acid core. All of these findings highlight that even one study can show that there are still many uncharacterized bile acids. This computational MS observation resulted in the rapid and ongoing development of new MS analytical and algorithmic strategies, which are already leading to the discovery of hundreds of new bile acids[40,128–134].

These same microbial bile acid conjugates were also found in human data using the MS search tool MASST[128]. MASST enables searching for unknown MS fragmentation data in a spectral repository, another key advance that searches for new microbial or animal-derived molecules in human studies, facilitating translation[128]. Although MASST searches cannot differentiate between stereoisomers and regioisomers, such as muricholic acids or cholic acids, MASST readily identifies the tri-hydroxylation state and the conjugations attached to the bile acid. MASST also provides information on the datasets in which they were found. The chromatographic retention times of the majority of bile amidates with amino acid detected with MASST were subsequently matched against those amidates found in human and rodent samples[40,42,128]. Not only were these conjugates found in humans, but they were also associated with patients with pancreatic-insufficient cystic fibrosis, diabetes and intestinal bowel disorders, especially the dysbiotic state of Crohn's disease. The first new dihydroxy bile acid – deoxycholic acid-phenylalanine amidate – was observed in faecal samples in a human twin study on non-alcoholic fatty liver disease[128]. Using in silico predictions of candidate bile acids, detection of 626 potential candidates were found to match[130], two of which were validated via organic synthesis.

A third strategy called reverse metabolomics was able to find not only many new amidated bile acids, but also lipid esters[40]. In total, MS–MS data for 174 of the 176 bile acid amidates, which were synthesized combinatorially to be reference standards, were found in public data from humans, animals and microorganisms. They were observed mostly in the caecum and faeces but also in the gallbladder, duodenum, jejunum, ileum, skin and to a much lesser degree in blood, urine and the liver, which were dominated with MS–MS matches to glycine and taurine conjugates. Of the 96 cholic and deoxycholic acid fatty acid esters synthesized, matches to five were observed using reverse metabolomics – the C14:0, C18:1, C18:2, C18:3, C20:4 fatty acid esters to cholic acid – which is consistent with other observations of fatty acid acylated bile acids in human faeces[82]. As previously described, MASST does not (yet) differentiate between bile acid isomers, and therefore the lipids could be attached to other trihydroxylated or dihydroxylated bile acids that are isomers of cholic acid or deoxycholic acid. Fatty acid acylated bile acids could potentially be esterified by lipases in vitro, but the link between this family of enzymes and acylation of bile acids in biological specimens has not (yet) been established[135]. In total, 62 bile acid conjugates (amidates) were confirmed to be present in humans and associated with Crohn's disease[40,42]. These previously overlooked bile acid amidates are strongly associated with Crohn's disease[40,42]. Microorganisms from the bacterial classes Actinobacteria, Bacilli, Clostridia, Fusobacteria and to a lesser degree Bacteroidia were all able to amidate the bile acids cholate and deoxycholate with 15 different proteinogenic and non-proteinogenic amines and amino acids[40]. As reported in a preprint article[134], replicating the synthetic strategy described previously, via combinatorially synthesizing reference standards and LC–MS analysis, 40 and seven different conjugated bile acids were found in faecal and plasma samples from extremely premature infants, respectively.

Other strategies (for example, culturing in combination with manual analysis) have similarly uncovered evidence of new microbial bile acids, including a study that included the addition of cholic acid, chenodeoxycholic acid, or deoxycholic acid to the human microbiota[33]. The findings of this study were consistent with the observations of our group and others that Firmicutes, Bacteroidetes and Actinobacteria produce 15 proteinogenic amino acid bile acid amidates, representing 44 bile acids, including the glycine amidate[40,42]. This work supported the observations described previously that the microbiota produces proteinogenic amino acid and bile acid conjugations, although it should be noted that, due to the absence of synthetic standards, the possibility that the bile acids are connected as esters on a hydroxyl group cannot be excluded, highlighting the importance of verification using standards. In another study, in which chenodeoxycholate and 3-oxo-chenodeoxycholate were added to fresh human faecal material, tens to low hundreds of new bile acids were generated[77]. This work also provided support for eight proteinogenic amino acid conjugations and another ten putative conjugations. Through manual interpretation of fragment ions, but without validation using synthetic standards, the researchers proposed that some of these conjugations are possibly conjugated as esters instead of amidates.

Excitingly, some additional dedicated MS workflows using ion mobility MS or polarity-switching multiple reaction monitoring (MRM)

MS, in combination with computational strategies, are now being introduced to detect new bile acids. These techniques have enabled the observation of serine, valine, threonine, cysteine, asparagine, aspartate, lysine, glutamine, glutamate, methionine, histidine, arginine, isoleucine and tryptophan bile acid conjugates from microorganisms, mice and human faeces[133]. A strength of polarity switching is that it increases the number of diagnostic MS–MS fragments, which increases the diversity of discoverable modified bile acids, even in the absence of any standards. Still, diastereomers cannot be distinguished, limiting annotations to mono-, di-, tri- and tetra-hydroxylated bile acid conjugates, as opposed to identifying a specific bile acid core with locations of the hydroxyl groups and their stereochemistry which is already useful as it allows one to find modified bile acids, but it is important to be aware of its limitations. Even when one standard is available, polarity switching cannot exclude the possibility that the retention times overlap with those of other isomers. In contrast, ion mobility-based MS

Table 1 | Computational and experimental approaches used in bile acid discovery

Approach	Application	Output or results	Additional requirements
Computational approaches			
Molecular networking[60]	Hypothesis-free visualization of MS–MS spectral/molecular families	Global analysis; discovery of new analogues by propagation of annotations	Orthogonal techniques required to confirm the structure of metabolites
MASST[128]	Exploration of public data repository with MS–MS spectra for translational biological inferences between different organisms	Public datasets where the query MS–MS spectrum is detected; metadata of these datasets (from ReDU[139])	Limited by the availability of public (meta) data, and biological interpretations depend on systemic metadata associated with a study
MassQL[141] (preprint)	Development of MS-based query to search for specific chemical classes	MS–MS spectra that adhere to the restrictions outlined in the MassQL query	Prior knowledge of fragmentation spectra is required
Reverse metabolomics[40]	Searching of MS–MS spectra against the public repository to understand its disease state, organism distribution and source observations (e.g., blood, faecal, brain)	Data table of occurrence of the MS–MS spectrum in public data and then also reporting sample information or metadata for each of the samples (e.g., NCBI taxonomy, Uberon ontology, disease ontology)	Access to the MS–MS spectrum (e.g., via synthesis, isolation to get the molecules from which a MS–MS spectrum is generated); high-resolution mass spectrometer
Experimental approaches			
Untargeted LC–MS (discovery MS)	Broad metabolite profiling in complex biological samples (emphasizing relative quantification, not annotation)	Raw data files in vendor-specific format with profile of accurate masses along with the chromatographic separation	Liquid chromatography system; high-resolution mass spectrometer; feature extraction tool (e.g., MZmine 3 (ref. 207), MS-DIAL[208], XCMS[209], MetaboScape, OpenMS[210])
Untargeted LC–MS–MS (discovery MS)	Broad metabolite profiling in complex biological samples (emphasizes annotations, although noisier due to poorer peak shapes, can be used for relative quantification)	Raw files in vendor-specific format with profile of accurate masses along with the chromatographic separation with additional MS–MS spectral data allowing metabolite annotations by matching to MS–MS reference libraries of known bile acids	Liquid chromatography system; high-resolution mass spectrometer with MS–MS capabilities; advanced computational tools needed to deconvolute MS–MS spectra and aid in metabolite annotation
Targeted LC–MS (quantitative MS)	Quantitative measurement of known bile acids	Quantitative values for analysed bile acids	Liquid chromatography system; mass spectrometer; retention time information for bile acids to be quantified; analytical standards for calibration curves; isotope-labelled standard for increased quantitative reliability
Targeted LC–MS–MS (quantitative MS)	Sensitive and highly selective quantitative measurement of known bile acids	Quantitative values for analysed bile acids	Mass spectrometer with MS–MS capabilities (e.g., QQQ-MS); retention time information for bile acids to be quantified; fragmentation behaviour information of bile acids to be analysed; analytical standards for calibration curves; isotope-labelled standard for increased quantitative reliability
Polarity switching MRM[211]	Facilitation of real-time switching of polarity to capture both positive and negative ions from a single sample injection	Raw files in vendor-specific format with positive and negative scans allowing more information on analysed metabolites	A mass spectrometer that can do polarity switching
Ion mobility MS[212]	Separation of isomeric ions, in addition to chromatographic retention time prevalent in bile acids	Raw files in vendor-specific format with additional ion mobility dimension separating molecules using their collisional cross section	Ion mobility unit; mass spectrometer; software capable of deconvoluting MS data with an ion mobility dimension (e.g., MZmine 3 (ref. 207), MS-DIAL[208], XCMS[209], MetaboScape, OpenMS[210])
Culturing techniques	Providing specific building blocks to cultured cells enabling a more targeted testing of biochemical hypothesis for downstream analytical workflows	Testable hypothesis; further targeted/untargeted analytical analysis needed (specifics depend on designed experiment)	Prior information about growth conditions of microorganisms required

LC, liquid chromatography; MRM, multiple reaction monitoring; MS, mass spectrometry; NCBI, National Center for Biotechnology Information; QQQ-MS, triple quadrupole MS.

provides improved resolution to the specificity of the bile acid cores that are attached to the conjugations, as different stereochemistry and conjugations have different drift times. The combination of retention time and ion mobility has enabled the separation and identification of nearly 300 different bile acids, including the leucine–isoleucine isomers. This has been used to reveal the effects of antibiotics in mice and faecal transplants in humans on the changes in bile acid amidates[136]. It could be envisioned that a combination of both approaches might become a key strategy to discover all possible bile acids in a sample, especially as most bile acids are not readily available as pure standards.

Finally, although not exclusively designed to find bile acids, a *N*,*N*-dimethylethylenediamine tagging strategy that enhances detection of metabolites with a free carboxylic acid in combination with molecular networking[60,127] led to the discovery of phenylacetyl acylated deoxycholic acids, which was further validated with synthetic standards[137].

We expect that a combination of new methodologies, including new metabolomics repository scale analysis tools such as repository scale molecular networking[138], ReDU metadata tracking[139], MASST[128], microbeMASST[140], MassQL[141], reverse metabolomics, total metabolomics strategies[60,142–146], and other creative molecular networking and modification-tolerant data mining tools such as Mass2Motif[147], MS2Deepscore[148], SIMILE[149] and hybrid searches[150], will be used to complement molecular networks to discover new bile acids. These analytical approaches will not only enable the discovery of new bile acids but also aid in our ability to understand their associations with microbial production or host production, and to trace the organ or biofluid distribution and relation to phenotypes.

Embracing data science

Total bile acid content is used as a key clinical measurement. However, total bile acid measurement is based on a few bile acids considered to be the only major components[59]. Although such a narrow selection of bile acids can provide practically useful data, it is not clear how representative they will be of the total bile acid content, because the types of bile acids vary so dramatically. A simplified view of the bile acids from representative public untargeted metabolomics human data in urine, blood and faeces reveals a highly heterogeneous distribution of bile acid classes (Fig. 4). Similar observations of heterogeneity were found in a study profiling the faecal and plasma bile acid composition in more than 200 individuals with obesity[113], and in another study in which distinct bile acid profiles were demonstrated in intestinal samples from 15 healthy individuals[43]. Many additional bile acid patterns can be expected to be observed depending on the level of resolution with which the data are analysed; the maximum diversity analysis can be performed at the individual molecule level or by grouping bile acids. Given that detection strategies might not always distinguish between bile acid isomers, we should report them as a group of related bile acids. This also supports the need to build a bile acid ontology that embraces the true bile acid complexity and ensures consistency in the usage of this ontology by the community. An example of similar complexity is the microbiome, for which questions are asked at different phylogenetic levels, such as phylum, genus, species or strain, depending on the hypothesis to be tested. We expect that, similarly, bile acid analysis will require different levels of resolution depending on the questions asked. Such levels of resolution can vary from the clinically useful measurement of a few major bile acids, as used today, to a far deeper identification that embraces the true diversity of bile acids and their detailed association with phenotype and mechanism. Future analyses at higher resolution will also enable better integration with microbiome data, which is a key driver of the bile acid metabolic network fluxes.

A new function of bile salt hydrolases

Visualizing bile acids as the basis for a chemical language requires a substantial increase in the number of known structures[151,152]. Most of this pool of previously hidden structures will be present in such low amounts that they lie at or beyond the current detection limits of analytical instruments and/or are in high enough concentrations but are outside the purview of discovery even with modern algorithms. As a carrier of information, the core structure of the bile acid (the bent A/B bile acid ring structure and the amphipathic nature of the bile acid nucleus) provides master key access to sites and organelles within the host, while the 'message' is attached to the flexible side chain in an easy-to-remove linkage. Here, the common host bile acids that are conjugated to taurine or glycine can be seen as inert biochemical precursors that become activated when exposed to the microbiome. The initial step is the removal of the taurine or glycine conjugate, a process accomplished by microbial hydrolases with the protein encoded by the *bsh* gene. BSHs belong to the protein family of enzymes that also include aminotransferases. There are an astonishing number of such hydrolases with great structural diversity, having only a recognizable active site in common. An examination of 16,936 bacterial genomes found a putative *bsh* gene in 2,456 of the genomes[153–155]. Although BSH is responsible for taurine or glycine removal, the reaction can work in reverse, and the term 'hydrolase' can be expanded to call these same enzymes 'transferases'. Working as a transferase, the BSH enzymes can reattach substrates (including all the amino acids) to the bile acid, which will serve as the carrier of the attachment[17,152,156,157] (Fig. 5a). In this transfer reaction, taurine serves a function analogous to that of coenzyme A, where it functions as a cofactor in BAAT (Fig. 5a). It has also been observed that when unconjugated bile acids are fed to microorganisms that contain BSHs, they have the ability to amidate using any number of amines or amino acids, although the mechanisms of activation of the bile acids are not yet known[33,40,158]. Growing evidence suggests that the microbially produced bile acids made by the BSH should not be dismissed as just minor, low-concentration variants with no biological importance (Box 1; see Supplementary Fig. 1). For example, in faecal samples from patients with IBD[40], 25% of the samples contained microbial bile acid amidates that were present at the same or higher concentrations as the host-synthesized taurine and glycine amidates[40]. We and others have also found cholic acid-phenylalanine amidate concentrations in healthy female mice of 82 mg/kg in the jejunum (highest measured concentration 248 mg/kg in the jejunum), 46 mg/kg in the ileum, 3 mg/kg in the caecum and 6 mg/kg in the colon[42]. Larger amounts of the same conjugate have been detected in the upper colon (where mice have a caecum) of healthy humans[43]. BSHs have different hydrolytic specificities and Yao and colleagues, by knocking out the bacterial gene for bile salt deconjugation in a mouse model, demonstrated that this single change was responsible for multiple transcriptional changes in host metabolism, circadian rhythm and immune pathways. BSH and/or their hydrolytic and amidate products also affect T cells, host stem cell differentiation, microbial biofilm formation and germination of *Clostridium difficile* spores[17,40,69,159,160]. It is likely to take the scientific community decades to fully tease apart the functional roles of the ever-expanding global pool of bile acids that have been, and will be uncovered, over the next decade.

A single organism with a single BSH is fully capable of altering host metabolic, circadian rhythm gene expression and immune pathways.

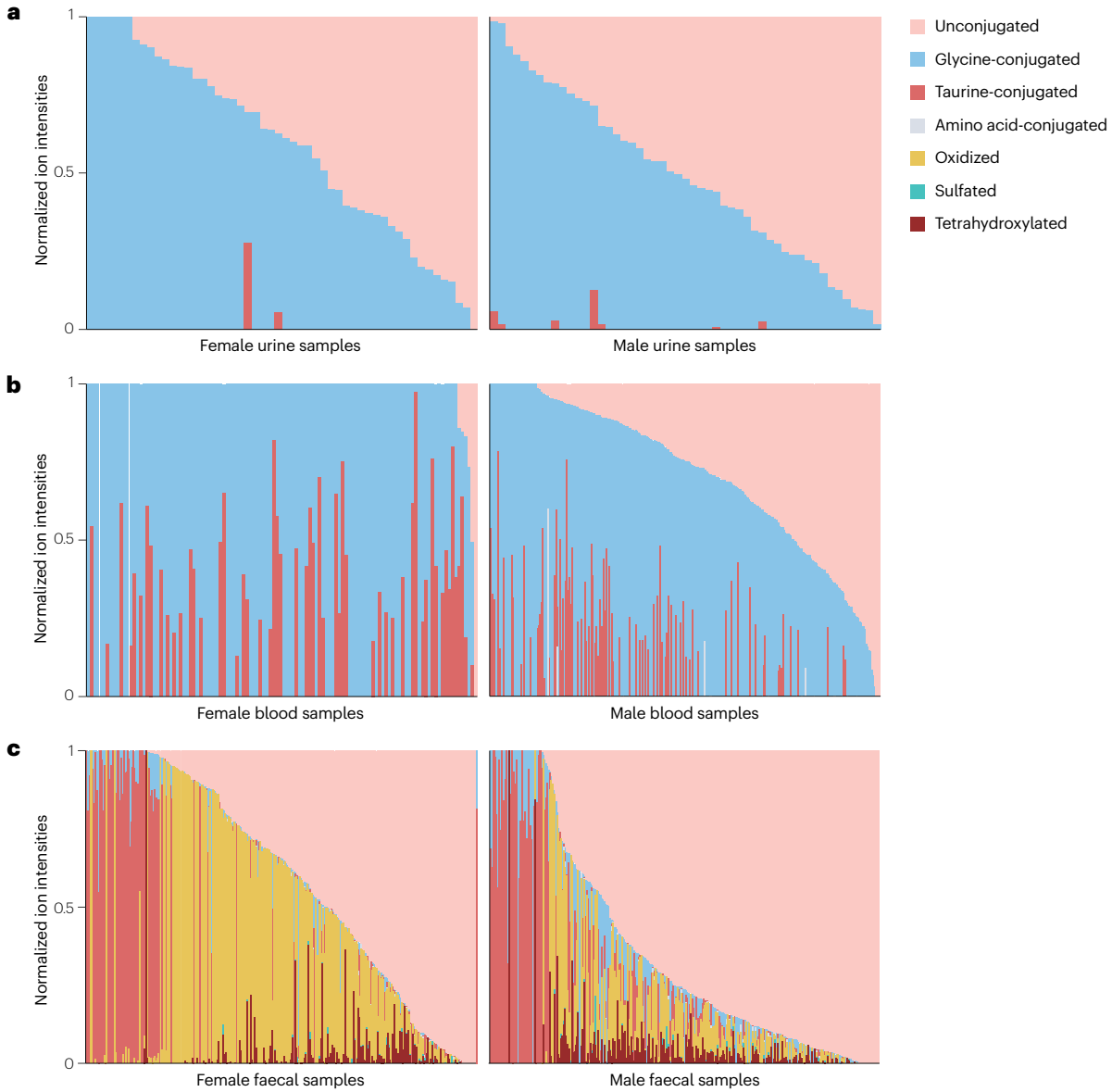


Fig. 4 | Proportion of bile acids in humans. Data available in ReDU[139] represented as normalized ion intensities across three biological matrices: urine (part **a**), blood (part **b**), faeces (part **c**). Each stacked bar represents a unique individual. Colours represent the seven bile acid groups: unconjugated, glycine-conjugated, taurine-conjugated, amino acid-conjugated, oxidized, sulfated and tetrahydroxylated. The information visualized is from untargeted MS–MS datasets acquired on a high-resolution mass spectrometer, a Q-Exactive, and with associated metadata filtered from ReDU. Reprocessing these files with living data in Global Natural Product Social Molecular Networking (GNPS)[60] yielded spectral matches to bile acids in the GNPS libraries. The precursor ion intensities of these bile acids were extracted from the classical molecular network and are represented as sum normalized ion intensities of all female and male samples across three biological matrices.

When colonized with different strains of gut bacteria of the genus *Turicibacter*, gnotobiotic mice revealed very different bile acid and lipid profiles[161]. Metabolic changes that are the direct result of a BSH include changes in glucose, triglyceride and cholesterol levels, as well as the uptake of oxygen[17]. Although the general shape of BSHs is fairly conserved across microbial species, they vary widely in substrate preference and efficiency[22]. Thus, we should begin to think of varying patterns of BSHs in relation to the larger bile acid metabolic network when doing functional bile acid metabolic network assessments, especially once the substrate specificities are better understood. For example, there are varying BSH patterns observed in sequencing data from faecal samples in different countries[154], possibly due to different dietary patterns that promote the growth of different microorganisms, but such patterns can also be expected to change with health status, medication use and other characteristics that influence the microbiome. The vast repertoire of potential BSHs and homologue genes remains uncharacterized[154,162].

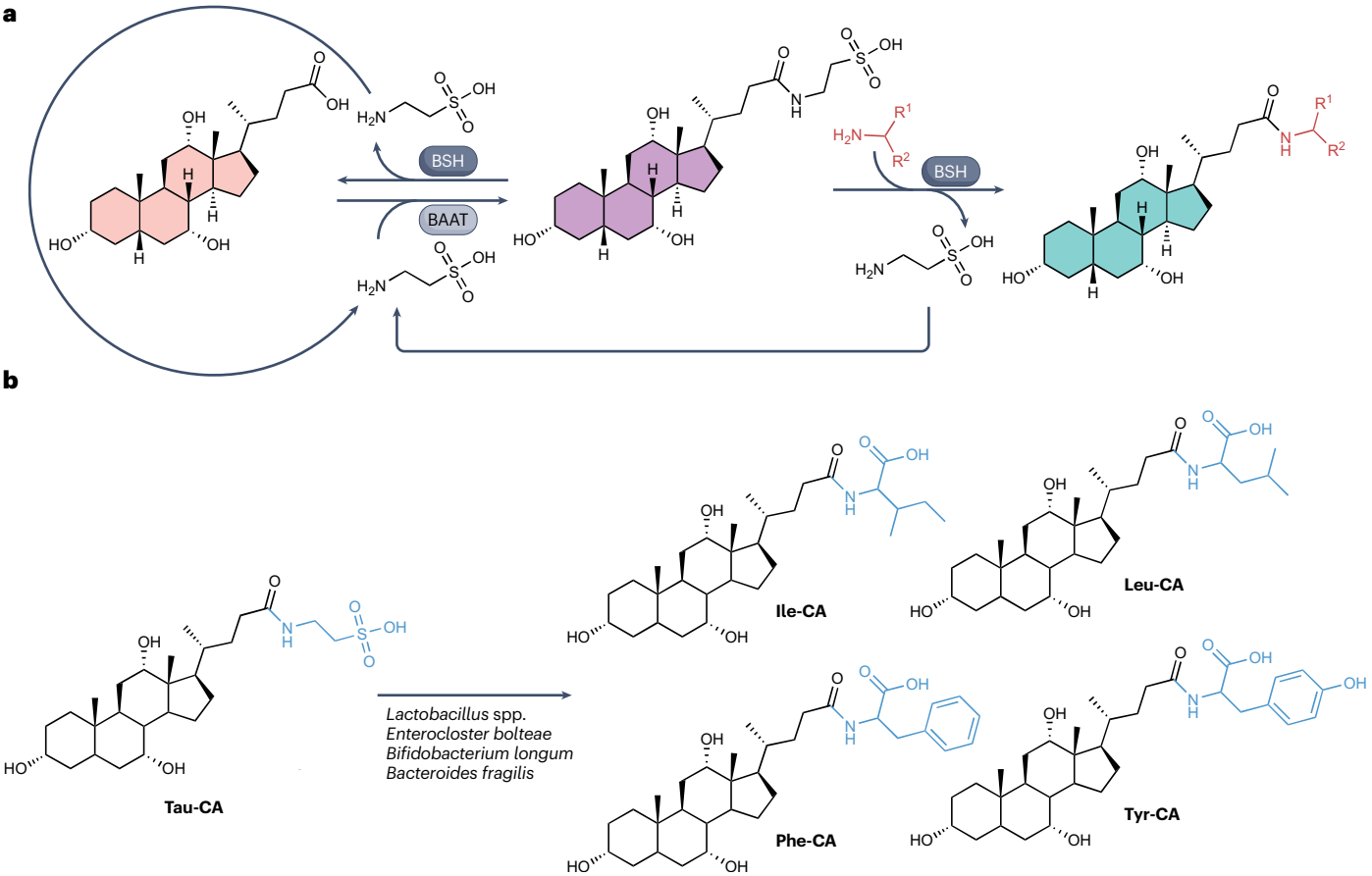


Fig. 5 | The known deconjugation and re-conjugation of bile acids by BAAT and BSH enzymes. a, Representative reactions carried out by bile salt hydrolase (BSH) and bile acid-CoA:amino acid *N*-acyltransferase (BAAT). Although other amidates, aside from taurine, can serve as substrates for the transamination reaction carried out by BSH, the taurine conjugates are the key molecules converted to the microbial bile acid amidates. Different BSH enzymes have different substrate specificities for amines and bile acids in catalysing the transamination reaction. **b**, Representative structures of amino acid conjugated to cholic acid (CA) catalysed by bacterial BSH belonging to the genera *Lactobacillus, Enterocloster, Bifidobacterium* and *Bacteroides*. Ile, isoleucine; Leu, leucine; Phe, phenylalanine; Tau, taurine; Tyr, tyrosine.

Much remains to be understood, implying many opportunities for the scientific community. For example, depending on the BSH or transferase, taurine, glycine or other amidates are more efficiently utilized and the free acid can be converted. However, the mechanism of free carboxylate activation is unknown. Even though certain bile acid core structures, amidates and free amines can serve as preferential substrates for BSH transferases, we consider that host-derived taurine amidates will be found to be some of the most important substrates for the transferase reaction. This is consistent with the observation that taurocholic acid acts as a substrate for BSH from the bacterial species *Lactobacillus* spp., *Enterocloster bolteae* (formerly *Clostridium bolteae*), *Bifidobacterium longum* and *Bacteroides fragilis* to furnish amino acid-conjugated bile acids, as highlighted by representative examples from the literature[42,158] (Fig. 5b). In addition, germ-free and/or antibiotic-treated mice predominantly have taurine amidates and reduced glycine amidates, which we now know can also be made by microorganisms[39,102,163]. In other words, without microorganisms to carry out the transferase reaction, bile acid taurine conjugates tend to accumulate. The release of free taurine after the microbial BSH-catalysed deconjugation of taurocholate and re-amidation with other amino acids also helps in promoting host health by maintaining the balance between taurine and taurocholate, and is guided by the microbiome[164]. The amino acid taurine itself serves as an energy source for the microbiota, provides cytoprotection and aids in neuromodulation[165], with its highest concentrations observed in the brain and heart of animals and humans[166] – organs in which bile acid-related genes and receptors have also been detected. Organisms with different clades of *bsh* gene sequences act on different conjugated bile acids[17,69,158,167,168]. It is known that some microorganisms do not contain the genetic sequence for a classical BSH, yet are still capable of forming bile acid amidates, suggesting that other mechanisms of bile acid conjugation remain undiscovered[168]. To that effect, there are bile acid aminotransferase homologues observed in microorganisms, and hydrolases, lipases and proteases that can form amide (or ester) bonds[155,169].

These observations suggest that BSH research needs to be reinterpreted in the context of this new information, in which transferase activities mediated by BSH represent key mechanisms for the diversification of bile acids. Each BSH produces its own unique set of modified bile

acids leading to thousands of different bile acids. We can speculate that other enzymes carry out similar de-conjugation and re-conjugation reactions and even form esters instead of amidates of the free carboxylic acid[17,77,170]. This is an opportunity for the community to research the patterns of all BSHs known to date, in addition to how they contribute to the metabolic bile acid network via de-conjugation and re-conjugation specificities — a key requisite to enable mechanistic explanation for the role of all BSH transferase enzymes in health outcomes. This begs

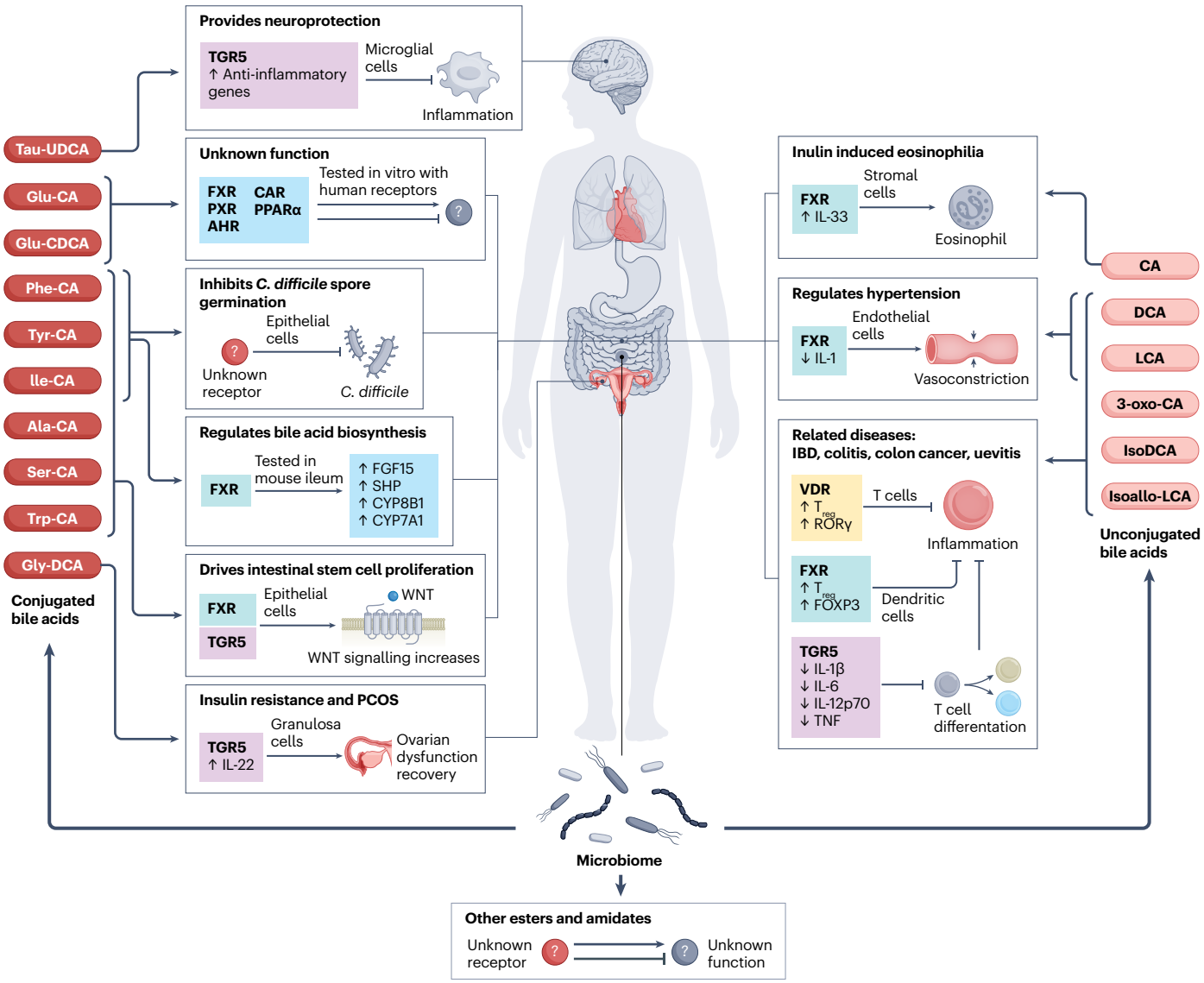


Fig. 6 | Bile acids and receptor interactions. Interactions of known microbially modified bile acids (conjugated and unconjugated) with common host ligand receptors and the phenotypic response, when characterized, are highlighted. The unconjugated bile acids iso-allolithocholic acid (isoallo-LCA), 3-oxo cholic acid (3-oxo-CA), isodeoxycholic acid (isoDCA), deoxycholic acid (DCA) and lithocholic acid (LCA) bind to the nuclear receptors VDR, FXR and TGR5 and induce cytokines production and increases differentiation of regulatory T (T_{reg}) cells, reducing inflammation[90,91,179,241]. DCA and LCA activate FXR, which downregulates IL-1, leading to vasodilation in endothelial cells[242]. Cholic acid (CA) binds to FXR and increases production of IL-33, activating eosinophilia in stromal cells[175]. Taurine-conjugated ursodeoxycholic acid (Tau-UDCA) is an agonist of the membrane receptor TGR5, leading to an increase in intracellular cAMP levels in microglia, which induces anti-inflammatory markers providing neuroprotection in conditions such as Alzheimer disease, Parkinson disease and amyotrophic lateral sclerosis[98,178]. Glycine-conjugated deoxycholic acid (Gly-DCA) induces production of IL-22 on binding to TGR5, causing recovery of ovarian function in mice with polycystic ovary syndrome[177]. In the case of microbially conjugated bile acids, the studies highlighting ligand interactions are limited, given the novelty of such discoveries, but highlighted in this figure are some important observations from recent studies. Although only tested in mouse ileum so far, tyrosine (Tyr), phenylalanine (Phe) and isoleucine (Ile) conjugated to CA have binding affinity for FXR and regulate bile acid biosynthesis genes[42]. Elevated amounts of these amino acid amidates furnished by treating mice with a cocktail of BSH enzymes also led to inhibition of *Clostridium difficile* spore germination and prevented its colonization[69]. The mechanism for the inhibition of spore germination and the bile acid receptors involved are still unknown. Glutamate (Glu) conjugated to CA and chenodeoxycholic acid are agonists of multiple nuclear receptors in vitro in human cells; however, the phenotypic response has not yet been explored[158]. The amino acids alanine (Ala), serine (Ser), tryptophan (Trp), Tyr, Phe and Ile bind to FXR and TGR5, increasing WNT signalling and stem cell differentiation[160].

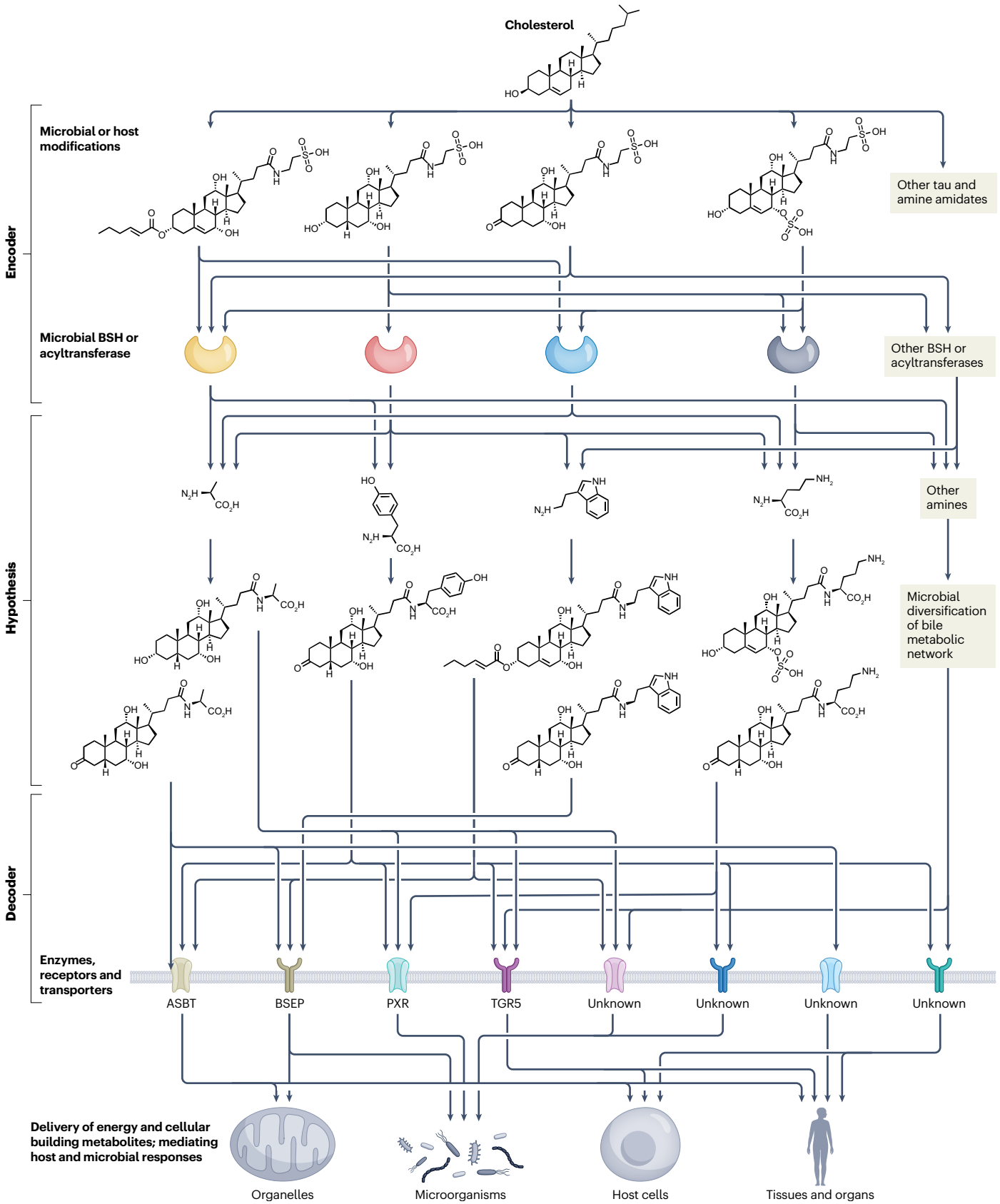

Cholesterol
Encoder
Microbial or host modifications
Other tau and amine amidates
Microbial BSH or acyltransferase
Other BSH or acyltransferases
Hypothesis
Other amines
Microbial diversification of bile metabolic network
Decoder
Enzymes, receptors and transporters
ASBT
BSEP
PXR
TGR5
Unknown
Unknown
Unknown
Unknown
Delivery of energy and cellular building metabolites; mediating host and microbial responses
Organelles
Microorganisms
Host cells
Tissues and organs

Fig. 7 | The bile acid encoder–decoder hypothesis. In this hypothesis, cholesterol is modified by host and microbial enzymes to encode the message that is then decoded by as yet unknown and known receptors (PXR, TGR5) and transporters (apical sodium-dependent bile acid transporter (ASBT) and bile salt export pump (BESP)) to enable communications at the organelle, cell (microbial or host cells), and tissue and organ levels. BSH, bile salt hydrolase.

the question as to why we need so many different bile acids. There is a clear need to functionally understand the roles of the many newly discovered and yet-to-be-discovered bile acids. Large-scale functional screens with as many bile acids as possible will be necessary to discover their functional roles and to complement hypothesis-driven analysis of the thousands of bile acids with unassigned functional roles at this time, including their functions as possible immune regulators.

Bile acids are immune regulators

Similar to many other cholesterol-derived steroids, modified bile acids, including microbially modified bile acids and amidates, are found to regulate the immune system (Fig. 6). An important role of bile acids in innate immunity and inflammation control is illustrated by the observation that bile acid receptor activation results in the reduced expression of human NF-κB-dependent signalling pathways[171,172]. In addition, major bile acid sensors (including FXR and TGR5) are found in the intestinal interface between the microbiome and the host[173]. Bile acids — such as chenodeoxycholic acid, deoxycholic acid and cholic acid — bind to these sensors and regulate innate immune cell responses in macrophages, dendritic cells and natural killer T (NKT) cells[8]. Upon binding, deoxycholic acid reduced the secretion of inflammatory cytokines IL-1β, IL-6, IL-12p70 and TNF through TGR5 signalling in mice[174]. Mice fed an inulin-rich diet showed changes in the levels of bile acids, especially cholic acid, which in turn caused a type 2 inflammation in the intestine and lungs, characterized by IL-33 production, activation of group 2 innate lymphoid cells and eosinophilia[175]. In non-tumour liver tissue from patients with primary liver cancer, chenodeoxycholic acid levels correlate with CXCL16 expression and NKT cell accumulation, whereas glycolithocholate shows an inverse correlation[176]. Specifically, in mice with polycystic ovary syndrome phenotype, glycodeoxycholic acid recovers ovarian function by inducing intestinal group 3 innate lymphoid cell IL-22 secretion through GATA binding protein 3 (ref. 177). Taurine-conjugated ursodeoxycholic acid is an agonist of the membrane receptor TGR5, leading to an increase in intracellular cAMP levels in microglia, which induces anti-inflammatory markers providing neuroprotection in diseases such as Alzheimer disease, Parkinson disease and amyotrophic lateral sclerosis[178].

In adaptive immunity, responses are modulated by regulatory T (T_{reg}) cells, and several bile acids have been demonstrated to alter T_{reg} cell populations in the intestine. A combination of two secondary unconjugated microbial bile acids — lithocholic acid and 3-oxo-lithocholic acid — regulate the levels of colonic nuclear hormone receptor RORγ$^+$ T_{reg} cells (Fig. 6) through the VDR bile acid receptor[90]. In this study, germ-free mice mono-colonized with BSH-knockouts of *B. fragilis* had both altered levels of bile acids and reduced colonic RORγ$^+$ T_{reg} cells, showing that certain primary and secondary bile acid species preferentially regulate this cell type. The secondary bile acid isodeoxycholic acid also enhances expression of T_{reg} cells while concurrently increasing induction of the transcription factor FOXP3 in mice[179]. Another study found an increase in ileal FOXP3$^+$ T_{reg} cells in mice administered a combination of 3-oxo-lithocholic acid and iso-allolithocholic acid[91]. Both bile acids were detected in the faeces of patients with colitis and completely disappeared in germ-free mice. A follow-up study by Li and colleagues identified the genes in gut bacteria from the phylum Bacteroidetes that catalyse the conversion of 3-oxolithocholic acid to iso-allolithocholic acid[21]. The study also showed a reduction in levels of iso-allolithocholic acid and the genes responsible in patients with IBD, reaffirming the role of this bile acid in increasing the differentiation of anti-inflammatory T_{reg} cells. In contrast to T_{reg} cells, 3-oxolithocholic acid inhibits the differentiation of pro-inflammatory T helper 17 (T_H17) cells[21]. Other studies have also found a decrease in T_H17 cell differentiation by both isolithocholic acid[180] and lithocholic acid-3-sulfate[181]. Taurine-amidated ursodeoxycholic acid also reduces the differentiation of T_H17 cells, and dampens the innate inflammatory response to IFNγ[182], leading to an intriguing question based on the information contained in this Perspective: are these observed phenotypes due to the production of new microbially produced amidates via the conversion of taurine-activated bile amidates by BSHs or other microbial modifications? Oral administration in mice of *Bacteroides uniformis* led to production of more bile acids, such as isolithocholic acid and isochodeoxycholic acid, which regulated the NF-κB and mitogen-activated protein kinase (MAPK) signalling pathways in colonic tissues and the differentiation of T_H17 cells[183].

The functional role of microbial bile amidates is even less understood and much more research is needed. Amino acids threonine-amidated to cholic acid and glutamate-amidated to deoxycholic acid and chenodeoxycholic acid bind to PXR[40]. Such binding has the potential to decrease T cell proliferation by decreasing the expression of CD25 and IFNγ and decreasing phosphorylated NF-κB and MEK1/2 (ref. 184). In the same way that canonical bile acids activate FXR and regulate immune functions, microbial bile amidates such as glutamate, isoleucine, leucine, methionine and tryptophan amidated to chenodeoxycholic acid have now been reported to bind to FXR[42,158,160] and could potentially have critical roles in immunity. A large number of amino acid-amidated bile acids bind to the aryl hydrocarbon receptor[158], which has multiple roles in T cell and dendritic cell signalling[185]. Cholic acid amidates with phenylalanine, tyrosine, tryptophan and glutamate modulate signalling via human and mouse FXR and TGR5, and also promote WNT signalling and intestinal stem cell proliferation[160].

Immune regulation is a key component of enterohepatic system–organ communication, and bile acids serve as bidirectional mediators between the host and the microbiome[186]. Given that so many bile acids are being discovered, and only a small proportion of these have been tested for immune-regulatory properties, evaluation of bile acids for immune-regulatory properties and their receptor interactions will need to continue into the future.

Bile acids as mediators of communication

Outside the intestine, there is another site in vertebrates that includes a similar microbiome: scent glands, in which a host-specific microbiome is carefully cultured in tissues. In these regions, the host supplies nutrition to a bacterial population that, in turn, forms a complex and extensive chemical mixture of volatile and semi-volatile compounds used in depositing scent marks. What is surprising about the components of scent is that none of the structures present is derived from the host[187–189]. Instead, all are bacterial metabolites, which combine to create an extensive chemical message readily interpreted by conspecifics

Box 4

Microscale bile acid cargos in health and disease

The hypothesis of a bile acid carrier that delivers microbiome-mediated micro cargos throughout the body has major implications for normal host health. There are multiple ways in which this pathway can be disrupted or disconnected. Genetic defects in the pathway necessary to convert cholesterol into bile acids, albeit rare, disrupt the bile acid structure as a carrier. Invariably, these defects are either fatal to the developing fetus or, if seen in the neonate, are associated with brain developmental defects[293,294] (emphasizing the importance of a gut–brain axis) and a failure to thrive. The standard of treatment for bile acid defects is to administer normal human bile acids, which are recognizable by both the host and the microbiome, which restores communication. The rise in the use of antibiotics, particularly in the developing neonate and infant, breaks the link between the microbiome and its host. The loss of this linkage (and cargo for the bile acid carriers) has been associated with a corresponding rise over the past 70 years in multiple downstream effects featuring negative health outcomes[295,296]. For example, current epidemiological studies link alterations in the microbiome to obesity, asthma, allergy, metabolic disease and colorectal cancer[297]. The narrowing of the microbiome diversity seen in the modern Western diet concurrently limits diversity in the potential nature of the bile acid cargo. The loss of a macronutrient (for example, vitamin C in scurvy) has an effect seen on the scale of months. One would anticipate that the loss of a micronutrient that is no longer synthesized by the microbiome would have far more subtle effects and appear in the host over a longer timescale. The microbiome is progressively altered with age[298], and a more limited delivery of cargo might parallel declines in health associated with ageing. In addition, use of probiotics has been considered to support favourable gut microbial alterations and help in maintaining host health[299]. This can be viewed as a means of establishing a microbiome that provides the bile acid carriers with cargos needed for host metabolism.

as being derived from the same vertebrate[190]. Here, the 'sender' is a vertebrate, the 'message' is written by bacteria, and it is received by another vertebrate.

By analogy, we propose that the human host synthesizes a small pool of bile acid structures, and releases them into the intestinal environment, where the microbiome converts the bile acids into an extensive 'first half' of a chemical message of a size equal to or greater than the sizes of a thousand, if not tens of thousands, of compounds. Here, the sender is the host, the message is written by bacteria, and the message is intended for and delivered back into the host. In any communication system, there are two halves, the sender and the receiver. How does the microbiome get the attention of the host, that is, the second half of the message? By providing something that it needs. The microbiome generates an extensive buffet of essential nutrients and signal compounds that, in turn, are conjugated to, carried, and delivered by the reusable stable bile acid platform. From the viewpoint of the vertebrate host, the most interesting of the small molecules moved by the bile acid carriers are those derived predominantly or exclusively from the microbiota, using their million-fold greater metabolic genomic capacity. Following absorption by the host, bile acids become ubiquitous throughout the body, delivering their micro cargos to individual cellular organelles. As a result, the different kingdoms of vertebrate hosts and bacterial microbiomes are interlocked in continuous dynamic chemical communication with each other.

The bile acid encoder–decoder hypothesis

It is now well established, and has been discussed comprehensively in many reviews, that microbially modified bile acids can act as signalling molecules and activate host–ligand receptors, which regulates host health and metabolism in multiple ways[191,192], including in conditions such as IBD, colitis and neurodegeneration (Fig. 6). The recent burgeoning of studies continuously expanding the repertoire of new bile acids paves the path to exploration of their binding affinities to both known and yet unknown bile acid receptors and the subsequent implications for human health and disease. Addressing the need for such huge diversity of bile acids and their interactions with specific receptors to regulate host health, we hypothesize that microorganisms use a bile acid communication highway to signal and communicate across scales, from organelles, to cells (both microbial and host cells), tissues and organs, with each bile acid carrying specific information. We propose that the host and the microbiota encode the diverse array of bile acids with messages that are then decoded by bile acid receptors and transporters (Fig. 7). We propose a micro-mechanism (Box 4) of bile acid nutrient transport in which, at concentrations far below the critical micellar concentration (the concentration at which detergents such as taurine bile acid conjugates form micelles), each bile acid is a unique carrier, and this uniqueness in each bile acid is encoded by bile acid enzymes, transporters and receptors[12] that have often been demonstrated to show different selectivities, in essence decoding the information of the bile acids themselves. While a macro-mechanism of bile acid-mediated nutrient absorption occurs using millimolar amounts of bile acids in micelles that 'trap' nutrients to be transported systemically, we hypothesize that this view needs to be greatly expanded by a micro-mechanism in which picomolar to micromolar amounts of bile acids participate not only in nutrient delivery via the macro-mechanism to organelles, cells, tissues and/or organs, but also in specific and targeted communication and possibly targeted signalling communication from the gut to other organs or in delivery of nutrients across membranes. Thus, although micelles are indeed part of a system of nutrient transport, micelle formation requires high bile acid concentrations that are orders of magnitude above the concentrations needed for the bile acid transporters and receptors to recognize bile acids (active in the picomolar to micromolar range). The full scope of specificity of encoding and decoding is not yet known, but the process of conjugating bile acids has been shown to facilitate uptake and requires the correct stereochemistry[193,194].

A key question that remains is how can the delivery of nutrients, more specifically building blocks to make proteins or lipids and energy, be coordinated and specific to only the organelles, cells, tissues and organs that need them, avoiding delivery to all at the same time?

This is a fundamental question in biology that has not been answered. We suggest that bile acids serve as active delivery vehicles moving nutrients, or form communication lines, to specific cells (microorganisms and host) and function in a web of communication between

the gut, brain, all other organs and the immune system. That bile acids might have the ability to selectively deliver messages and cargo to targets has already been reported for trojan drugs, where the drug of choice is attached to bile acids as a means to enhance its efficacy[195]. This targeted cargo delivery through a cofactor encoding and decoding hypothesis is consistent with the observations of a differential bile acid distribution across different body regions and the presence of different bile acid-related proteins in different tissues. Activity-based probe and subsequent pulldown assays have revealed that many of the proteins that interact with bile acids also interact with proteins from the microbiota[71], mitochondria, endoplasmic reticulum and the Golgi apparatus[50], which are key organelles in energy metabolism and delivery of proteins to lysosomes and the plasma membrane, or in secretion. It is unclear how bile acids alter mitochondrial energetics[196–198]; however, metabolomics studies in patients with chronic fatigue syndrome, which is associated with mitochondrial dysfunction, found that chenodeoxycholic acid is one of the key metabolic compounds in diagnosing the disease, and that low concentrations of mitochondrial taurine conjugates regulate bile acid production, and thus low levels of taurine in the diet lead to mitochondrial disease[197–201]. Perhaps bile acids can serve a role similar to that of carnitines, which enable transport of attachments across the membrane, are involved in systemic transport, or serve as cofactors in reactions such as those in which coenzyme A is involved. It is also not yet known how endoplasmic reticulum stress regulates bile acid synthesis, or the role bile acids play in Golgi membrane fission[202–204].

It is likely that we do not yet know all the different genes and proteins that are part of or control the encoding and decoding of information. Moreover, imagining bile acids as the central components in a communication system implies that new puzzle pieces of what constitutes the 'message' remain to be discovered. Although we anticipate that bile acids are key messages in the encoder and decoder hypothesis, other classes of molecules might also fulfil this role[205,206]. To our knowledge, there are currently no data that support an argument against the encoder–decoder hypothesis, but even if such data were to become available, it will be critical to explain why there are so many bile acids needed in the biology of complex organisms such as humans. It is, however, clear that, as central regulators of biology, there is a need to discover all bile acids present in humans and to understand how the patterns of bile acid modifications change. A detailed understanding of their roles will lead to improved control of the gut–microbiome–brain–other-organ metabolic network, which is influenced by diet, medications and other exposures, microbiota, sex, age, season and time of day, and data science will be instrumental in understanding the encoding and decoding strategies employed by the human body (Fig. 7).

Future outlook

At least 692 different bile acids have already been discovered, not considering stereochemistry (Fig. 2a,b), and it is reasonable to predict that thousands, potentially tens of thousands, of additional new bile acids will be discovered in the coming years owing to the fast advances in MS-related analytical tools. To keep track of this projected astronomical increase in the number of known bile acids, a uniform digital inventory of curated bile acids will be beneficial and essential for the research community. In addition, an open access database specific to bile acids and their related literature, similar to GNPS and HMDB, among others, will accelerate the pace of bile acid research. The discovery of bile acids as ligands of orphan receptors and their role as hormones challenged the traditional idea that bile acids are produced only in the liver, stored in the gallbladder, and released into the intestine for fat digestion. Consistent with a more systemic role of bile acids, the search for bile acids in locations other than the enterohepatic circulation was pushed forward and supported the role of bile acids in a wider gut microbiome–organ axis. However, there is still immense scope for further research in tracing bile acid locations and routes of transport, especially with the discovery of new bile acid conjugates. In addition to finding the bile acids themselves, their biosynthetic enzymes and genes encoding them also deserve attention. It will be important to establish the pathways by which bile acids are made, and where and when they are made. While the first two studies on the aminoacyl transferase activity of BSH was a monumental step in this direction (as discussed in the section 'A new function of bile salt hydrolases'), further experiments are required to explore the substrate specificity of BSHs and the number of different BSHs involved.

Existing bile acids, both host and microbial-derived, have been shown to serve as agonists for multiple transcription factors and receptors, which is also discussed extensively in this Perspective. With the discovery of new bile acids and their microbially derived amidates, it is pertinent to test the interaction of these new bile acids with existing receptors and against other orphan receptors. This knowledge would also percolate down to our understanding of the physiological importance of bile acids. An important avenue for future research involves exploring and providing more functional insights into the encoder–decoder hypothesis put forth in this Perspective. The use of labelled precursors coupled with untargeted metabolomics can be used to trace the role of these new bile acid conjugates as mediators in the crosstalk between the microbiome and the host, and to evaluate their influence on nutrition, energy and immune regulation of the host. A somewhat tantalizing prospect that presents itself is the idea of leveraging bile acids as the medium of communication to provide specific cargos (metabolites of interest) and genetically modified BSHs in vivo to furnish 'designer' bile acid conjugates. Although bile acid chemical biology started more than 170 years ago, we still do not completely understand their diversity, let alone their functions. We anticipate that this Perspective will provide motivation for additional future research in this area, as bile acids have fundamental roles in human biology.

Data availability

Structural databases referred to in Fig. 2 and Supplementary Fig. 1 can be accessed and downloaded from https://hmdb.ca/downloads (HMDB), https://gnps-external.ucsd.edu/gnpslibrary (BILELIB19) and https://www.lipidmaps.org/data/structure/download.php (LIPID MAPS). The source code used for generation of Fig. 2 and Supplementary Fig. 1 can be accessed at https://github.com/YasinEl/Bile_Acid_Review_2022; Figs. 3a,b and 4 can be accessed at https://github.com/mohantyipsita/Bile_Acid_Review_2022; Fig. 3c,d can be accessed at https://github.com/callaband/Bile_Acid_Review_2022.

Published online: 04 April 2024

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Acknowledgements

P.C.D. and R.K. acknowledge support from NIH U19 AG063744, and support for the collaborative microbial metabolite centre (1U24DK133658); R.K. also acknowledges DP1AT010885.

Author contributions

P.C.D., I.M. and C.A. researched data for the article, made a substantial contribution to discussion of content, wrote the article, and reviewed/edited the manuscript before submission. H.M.-R. and Y.E.A. researched data for the article, made a substantial contribution to discussion of content, and reviewed/edited the manuscript before submission. L.R.H. made a substantial contribution to discussion of content, wrote the article, and reviewed/edited the manuscript before submission. R.K. made a substantial contribution to discussion of content and reviewed/edited the manuscript before submission.

Competing interests

P.C.D. is an adviser and has equity in Cybele and is a scientific co-founder and holds equity in Enveda, Arome and Ometa with prior approval from UC San Diego. All other authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41575-024-00914-3.

Peer review information *Nature Reviews Gastroenterology & Hepatology* thanks Changtao Jiang, Matej Orešič, Eric Pamer and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.