Profiling urinary Bile Acids by targeted Liquid Chromatography-Tandem Mass Spectrometry

Inauguraldissertation
zur Erlangung des Grades eines Doktors der Medizin
des Fachbereichs Medizin
der Justus-Liebig-Universität Gießen

vorgelegt von Schauermann, Marcel
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Aus dem Fachbereich Medizin der Justus-Liebig-Universität Gießen
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Contents

1 Introduction

Bile acids are steroidal acids with 24 carbon atoms and a carboxyl group at C_{24}. The four major bile acids in humans are the primary bile acids - cholic acid (CA) and chenodeoxycholic acid (CDCA) - and the secondary ones - deoxycholic acid (DCA) and lithocholic acid (LCA). They differ in the hydroxylation state at C_3, C_7 and C_{12}. The majority of bile acids within the human body are conjugated with the amino acids glycine or taurine, respectively. They can also undergo further chemical conjugation, such as sulfation (Monte et al., 2009; Russell, 2003). Figure 1 reveals the 18 analytes included in our present targeted measurement method to show the molecular structures of typical human bile acids.

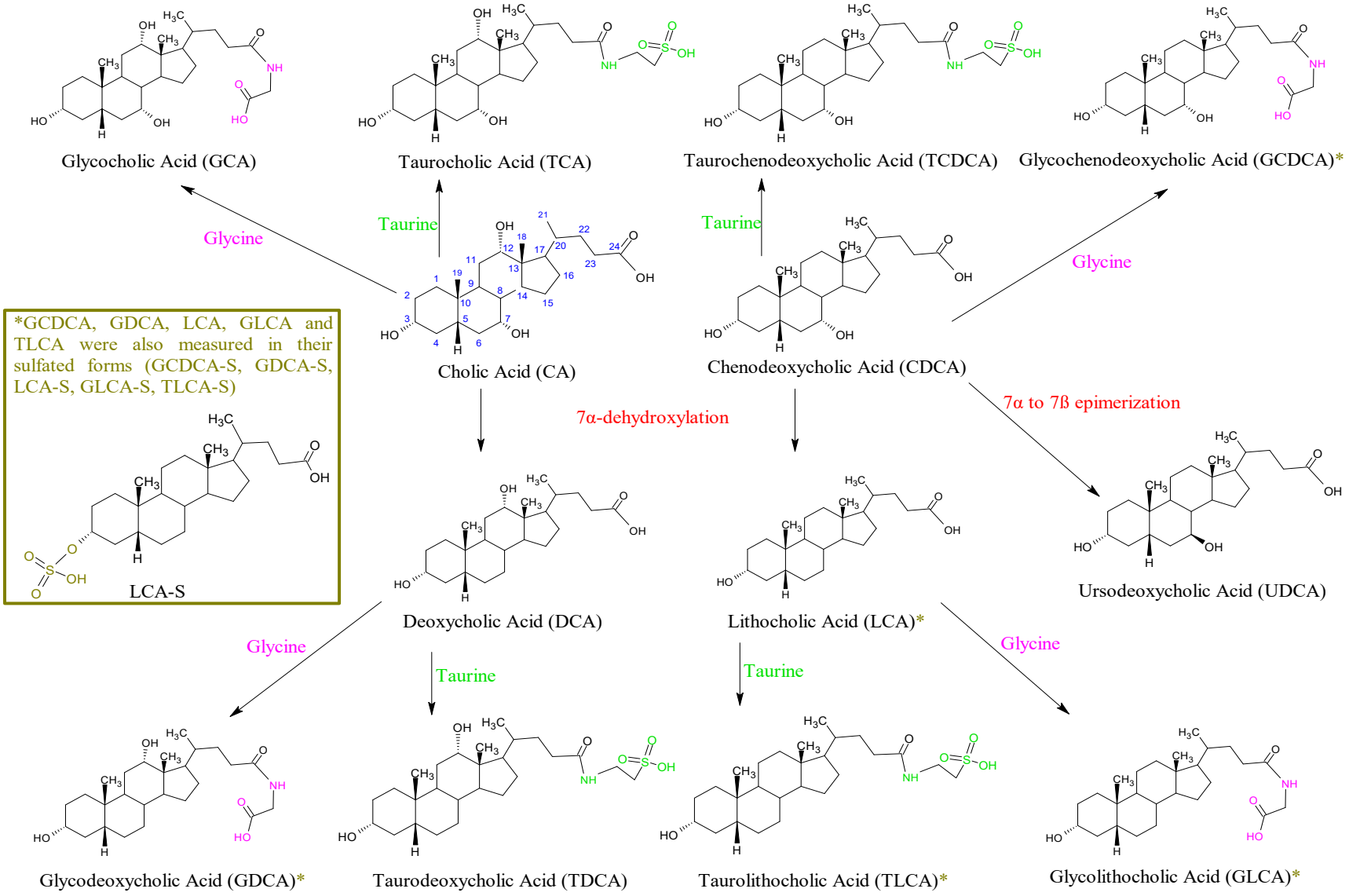


Figure 1. Structures of typical bile acids in human organism. The depicted 13 bile acids plus 5 bile acid sulfates (see box) are included in our targeted LC-MS/MS method.

1.1 Bile Acids Synthesis and enterohepatic Circulation

Proceeding from cholesterol, the primary bile acids CA and CDCA are synthesized in hepatocytes of the liver. In total, 17 different enzymes participate in bile acids synthesis (Russell, 2003). A simplified sketch of bile acids synthesis as reported by Chiang (Chiang, 2013) and Monte et al. (Monte et al., 2009) is shown in Figure 2.

The initiating and simultaneously rate-limiting step is the 7α-hydroxylation of cholesterol by cholesterol 7α-hydroxylase, an enzyme localized exclusively in the liver (Monte et al., 2009). The following steps contain various modifications of the ring structure, such as isomerization (e.g., 3β-OH to 3α-OH and A/B ring connection from trans to cis), double bond reduction, and – in case of CA synthesis – also 12α-hydroxylation. The side chain is oxidated forming a carboxyl group and shortened from 27 carbon atoms (cholesterol) to 24 carbon atoms (unconjugated bile acids). 7α-hydroxy-4-cholesten-3-one, an intermediate during formation of primary bile acids CA and CDCA, has shown to serve as a biomarker reflecting the activity of cholesterol 7α-hydroxylase and thus the state of bile acids synthesis (Sauter et al., 1996).

Before excretion into the bile, the primary bile acids get conjugated with the amino acids glycine or taurine - forming GCA and TCA or GCDCA and TCDCA, respectively (Russell, 2003). Practically every bile acid in human bile is conjugated with an amino acid, since conjugation increases their solubility. While about 75% are glycine-conjugated, circa 25% are taurine-conjugated (Monte et al., 2009). At physiological pH values, these conjugated bile acids occur in bile as negative ions and are also referred to as bile salts (Rassow et al., 2016). With bile they reach the small intestine, where they can be deamidated and undergo 7α-dehydroxylation catalyzed by enzymes of anaerobic intestinal bacteria to form the secondary bile acids (Nicholson et al., 2012; Russell, 2003). A recent study has elucidated the details of the transformation from primary bile acids to secondary bile acids, which in fact requires several steps catalyzed by different enzymes (Funabashi et al., 2020). The enzymes of intestinal bacteria can also catalyze epimerization reactions. A noteworthy example is the formation of UDCA by 7α- to 7β-epimerization of CDCA (Monte et al., 2009).

Due to their amphiphilic character, bile acids can function as emulsifiers and support the resorption of lipophilic food compounds. After intestinal resorption, they get back to the liver via hepatic portal vein and in this way the enterohepatic circulation closes. About 95% of bile

acids within the human body get recycled by intestinal re-absorption within the enterohepatic circulation, while only 5% get excreted. This 5% loss of bile acids is compensated by a daily bile acids synthesis of about 500 mg. For this reason, bile acids synthesis is responsible for 90% of the metabolized cholesterol in the human organism - making it the primary pathway of cholesterol catabolism (Russell, 2003). A small amount of bile acids within the enterohepatic circulation does not get re-extracted by the liver and therefore finds its way into the systemic circulation (Zwicker and Agellon, 2013). Reportedly, 10-30% of bile acids escape hepatic first-pass clearance (Qi et al., 2021). However, systemic circulating bile acids are supposed to rapidly undergo hepatic re-uptake (Cowen et al., 1975).

The synthesis mechanism described above and shown in Figure 2 is referred to as the "classical" (neutral) pathway. There has also been described an "alternative" (acidic) pathway and other additional pathways. In these pathways, the order of the synthesis steps can be different, other intermediates occur and some steps may also take place in extrahepatic tissues. Compared to the "classical" pathway, however, they all play a minor role in bile acids metabolism (Monte et al., 2009).

The bile acids synthesis is regulated by a negative feedback mechanism: High levels of bile acids lead to inhibition of the key enzyme cholesterol 7α-hydroxylase to stop further synthesis. In addition to that, HMG-CoA reductase, the key enzyme of cholesterol synthesis, is also inhibited by high concentrations of bile acids, since lower amounts of cholesterol also result in a decrease of bile acids synthesis (Horn et al., 2019). Not necessarily the synthesis, but at least the concentration of systemically circulating bile acids seems to be affected by β3-adrenergic receptors, since a recent study found a decrease in plasma bile acids due to a β3 agonist (Baskin et al., 2018).

Apart from regulating their own synthesis, bile acids also exert mediating effects on the enterohepatic circulation: They increase bile acids efflux from the liver into bile or into blood and they decrease intestinal absorption and hepatic reuptake within the enterohepatic circulation (Prawitt et al., 2011).

The gut microbiome also plays an important role within bile acids metabolism – and vice versa: Since secondary bile acids are synthesized with the help of intestinal bacteria, the

microbiome has an influence on the composition of the circulating bile acids pool. Microbiotal deprivation in rats causes altered bile acids profiles in different tissues and furthermore even changes gene expression and signaling pathways involved in steroid and bile acid metabolism (Swann et al., 2011). On the other hand, bile acids seem to promote maturation of the intestinal microbiome, since oral administration of certain bile acids forced the pace of microbiotal maturation in newborn mice (van Best et al., 2020). It stands to reason that also in humans the gut microbiome and bile acids interact with each other in both directions.

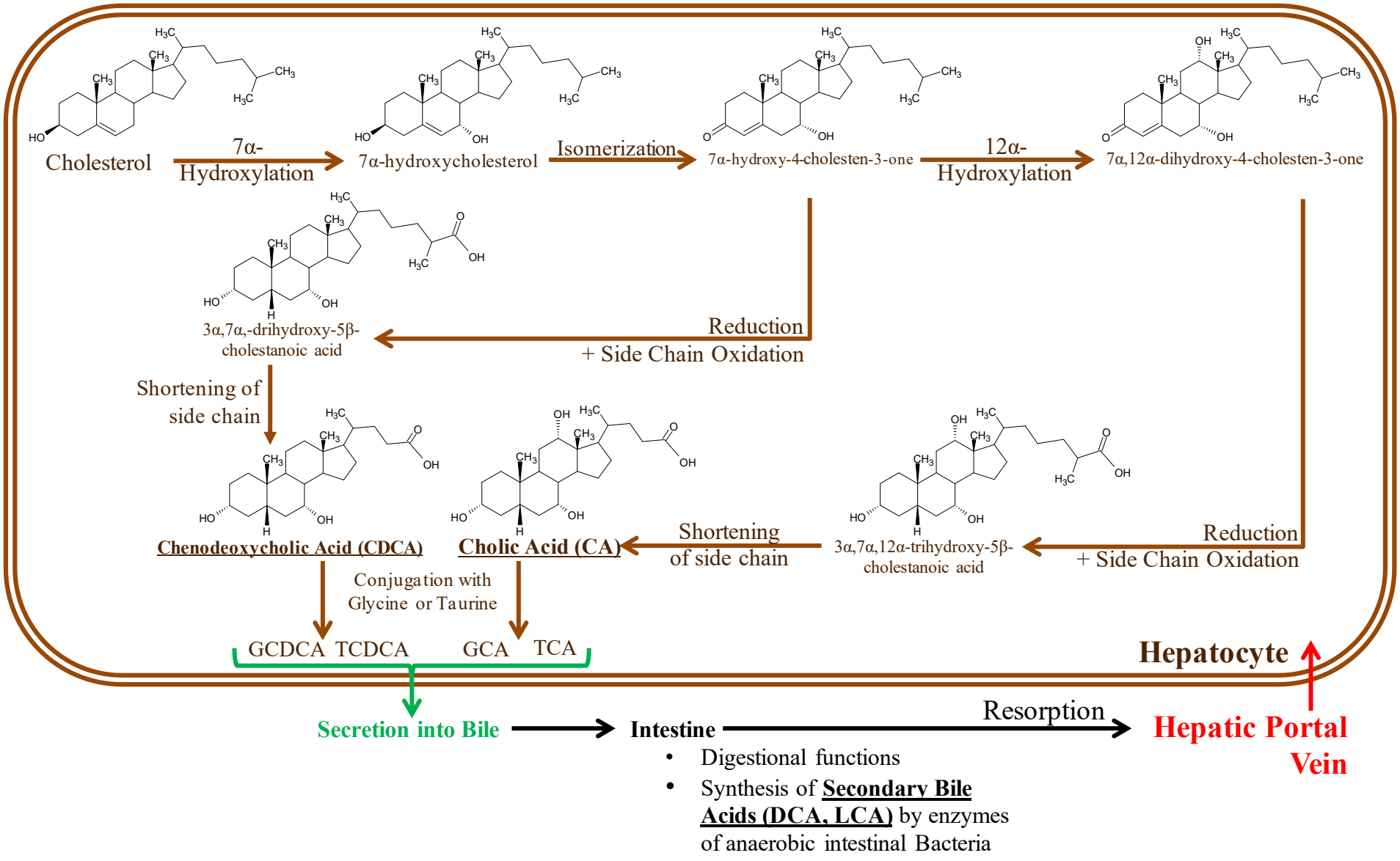


Figure 2. "Classical" pathway of bile acids synthesis designed according to explanations of Chiang (Chiang, 2013) *and Monte et al.* (Monte et al., 2009) *and enterohepatic circulation.*

1.2 Chemical Structure of Bile Acids

Bile acids can fulfill their mentioned function as emulsifiers for fatty nutritional compounds because of their characteristical chemical structure. In human, their steroid skeleton normally appears in 5β-configuration, meaning that the steroidal A and B ring are in a cis-position and are therefore not in the same plain (see Figure 3). Consequently, bile acids have a curved structure. Since the hydroxyl groups at positions 3, 7 and/or 12 (hydroxylation state depends on individual bile acid) are connected to the steroid skeleton in α-configuration, they all point in the same direction, forming the hydrophilic α-side of the molecule (because of its 7β-OH, UDCA is an exception). On the contrary, the opposing β-side is rather hydrophobic with its methyl groups at positions 18 and 19 in β-configuration. These stereochemical circumstances lead to the mentioned amphiphilic character of bile acids. The extent of hydrophilicity differs between individual bile acids with LCA being the least hydrophilic. In general, amidated bile acids are more hydrophilic than unconjugated ones (Monte et al., 2009).

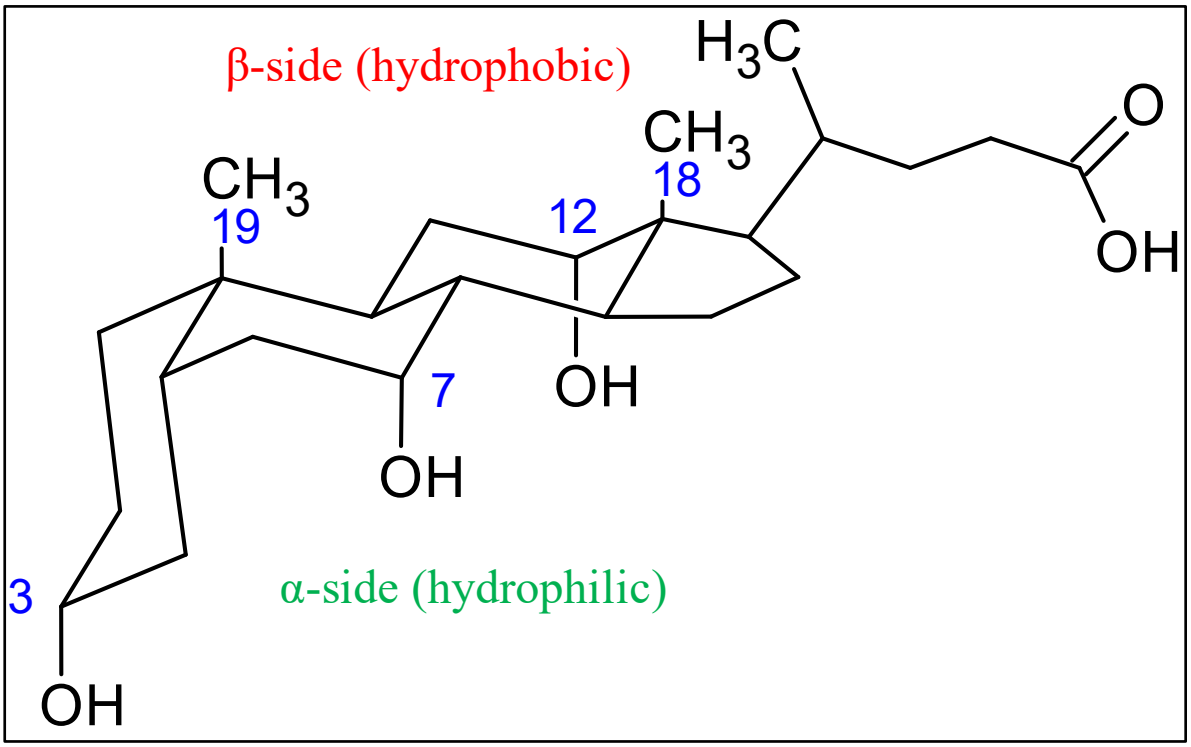


Figure 3. The chair conformation of cholic acid illustrates the presence of a hydrophobic and a hydrophilic side within bile acid molecules.

1.3 Biological Functions of Bile Acids

The "classical" functions of bile acids are attributable to their physical-chemical ability of emulsification. Their amphiphilic character allows them to form micelles together with phospholipids. Being lipophilic on the inside and hydrophilic on the outside, these micelles enable the transportation of lipophilic substances within aqueous solutions. On the one hand, this property of the bile acids can be used to solubilize the bile fluid itself by forming micelles containing bile acids, phospholipids (e.g. lecithin) and cholesterol which prevents the precipitation of cholesterol stones. On the other hand - as mentioned before -, the same mechanism is used for intestinal absorption of fatty nutritional compounds, not least the essential lipophilic vitamins A, D, E and K (Horn et al., 2019).

More recent research has discovered many further functions of bile acids within the human organism considering bile acids not only as emulsifiers, but also as biologically active molecules with hormonal functions in regulation of the metabolism. For a start, this also concerns digestive functions, as bile acids increase the activity of pancreatic lipolytic enzymes cholesterol esterase (Horn et al., 2019; Lombardo, 2001) and pancreatic lipase (Horn et al., 2019). But because bile acids are moreover present in the systemic circulation, their influence can likewise affect any other part of the human organism. As signaling molecules, they can activate mitogen-activated protein (MAP) kinase pathways and they can work as ligands for membrane receptor TGR5 and nuclear receptors (Houten et al., 2006). A mentionable nuclear receptor is the farnesoid X receptor (FXR). FXR participates in regulation of bile acids synthesis, excretion and the enterohepatic circulation (Sydor et al., 2020). Bile acids binding to FXR inhibit the transcription of cholesterol 7α-hydroxylase gene and activate the transcription of a gene encoding a bile acid transporter. This is one way of enabling the bile acids to regulate their own synthesis and transportation (Makishima et al., 1999). Activation of FXR uses different mechanisms to avoid accumulation of bile acids in hepatic and intestinal tissues, which would otherwise lead to cytotoxicity (Houten et al., 2006). According to that, FXR plays an important role in bile acids homeostasis. Furthermore, FXR can lead to transcription of antimicrobial and enteroprotective genes. Thus, bile acids help avoiding bacterial overgrowth and mucosal injury in the small intestine (Inagaki et al., 2006). A bile acid-binding membrane receptor is the G protein-coupled receptor TGR5, which for instance participates in regulation of the immune system (Kawamata et al., 2003). Apart from their other tasks, FXR and TGR5 are both involved in modulation of lipid, glucose and energy metabolism, for example by affecting insulin sensitivity or glucagon-like peptide 1 (GLP1)

secretion (Prawitt et al., 2011). Thus, bile acids take part in pathogenesis of diseases associated with the metabolic syndrome, such as obesity, type II diabetes, dyslipoproteinemia or atherosclerosis. Consequently, bile acids metabolism offers potential targets for new pharmaceuticals (Houten et al., 2006).

As mentioned above, the accumulation of bile acids can lead to cytotoxicity. Moreover, especially hydrophobic bile acids (like LCA) are even presumed to have teratogenic and carcinogenic effects (Monte et al., 2009; Perez and Britz, 2009). In general, the harmful effects of bile acids are reported to increase with hydrophobicity, which again is related to stronger detergent effects. The following order of increasing hydrophobicity has previously been published: UDCA < CA < CDCA < DCA < LCA (Attili et al., 1986; Perez and Britz, 2009).

There are various mechanisms explaining (hydrophobic) bile acids' cytotoxic effects: Being detergents, they may damage cell membranes. They also lead to the formation of reactive oxygen species causing necrosis and apoptosis. Furthermore, they can bind death receptors expressed by hepatocytes (Perez and Britz, 2009). Bile acids are reported to be involved in hepatic deficiencies such as non-alcoholic fatty liver (NAFL), non-alcoholic steatohepatitis (NASH) and even hepatocellular carcinoma (Prawitt et al., 2011; Sydor et al., 2020).

Within systemic circulation, bile acids are supposed to harm the endothelium of kidneys and lungs (Perez and Britz, 2009). Less hydrophobic and consequently less toxic bile acids (like UDCA), however, have been reported to counteract these pathological effects. Pharmaceutical benefits of UDCA include protection against cytotoxicity, enhanced hepatobiliary excretion and antioxidant activity (Perez and Britz, 2009). Hence, UDCA is used in treatment of cholestatic liver diseases. Beyond that, it shows beneficial effects in dissolution of gall stones in clinical praxis (Lazaridis et al., 2001).

Bile acids activity has also been reported within the central nervous system. Besides indirect pathways involving intermediate agents, bile acids can directly participate in central nervous signaling via central FXR and TGR5 receptors, since conjugated and unconjugated bile acids are able to cross the blood-brain barrier (Mertens et al., 2017). As a logical consequence, bile acids seem to be involved in diseases of the central nervous system and their potential treatments. Altered bile acids serum profiles have been described in patients with Alzheimer's

disease (MahmoudianDehkordi et al., 2019; Nho et al., 2019) and UDCA supplementation may have possible affects in amyotrophic lateral sclerosis (Min et al., 2012; Parry et al., 2010).

1.4 Elimination of Bile Acids

Due to recycling within the enterohepatic circulation, only a small fraction of bile acids gets eliminated from the human organism. The majority of lost bile acids is attributable to absent intestinal absorption and following fecal excretion. To a much smaller amount, bile aicds are also eliminated via urine: Bile acids that find their way from the liver into the systemic blood circulation can be filtrated in the kidneys and undergo urinary excretion (Alnouti, 2009; Russell, 2003).

An important pathway for the elimination of bile acids is their sulfation (Alnouti, 2009). This chemical reaction catalyzed by sulfotransferases (Lööf and Hjerten, 1980) (SULT) adds a sulfonate group (SO_3^-) to the bile acids. Activity of bile acids SULT has been detected in various organs, such as liver and small intestine (Lööf and Wengle, 1979) or adrenal tissue (Lööf, 1981). Although several other tissues have been described containing bile acids SULT, the liver is said to be the main location for bile acids sulfation (Alnouti, 2009). It is assumed that the SULT responsible for bile acids sulfation is the same enzyme catalyzing the sulfation of neutral steroids, such as DHEA (Radominska et al., 1990). Due to its substrate DHEA, it was formerly known as DHEA sulfotransferase, while today it is referred to as SULT2A1 (Alnouti, 2009; Radominska et al., 1990). In humans, the preferred position for bile acids sulfation is presumed to be the 3-hydroxy group (Alme et al., 1977; Alnouti, 2009; Hedenborg and Norman, 1984), although in-vitro experiments have also shown sulfation at 7-OH or 12-OH, respectively (Lööf and Hjerten, 1980). Sulfated bile acids in human seem to be exclusively mono-sulfates (Alme et al., 1977; Alnouti, 2009; Hedenborg and Norman, 1984; Lööf and Hjerten, 1980). Regarding the physical-chemical and biological properties, sulfation increases bile acids' solubility and decreases their toxicity (Alnouti, 2009). Besides that, sulfation of bile acids also affects different steps within the enterohepatic circulation. The small intestine's capacity of absorbing sulfated bile acids is low and desulfation does not take place in the small intestine. In the large intestine, however, bile acids can be desulfated, but the absorption rate here is much lower compared to the small intestine. Consequently, the overall enteral absorption of bile acids is lowered due to their sulfation, which increases fecal excretion (Alnouti, 2009). In addition to that, sulfated bile acids are less likely to get re-extracted by the liver than unsulfated bile acids and therefore get into the systemic circulation to a higher amount. But at the same time, sulfated bile acids are more efficiently excreted by the kidneys than unsulfated ones. As a consequence, the amount of sulfated bile acids in serum of healthy subjects accounts for about 15% of total serum bile acids and is lower than

the amount of unsulfated bile acids (Alnouti, 2009). On the contrary, the fraction of sulfated bile acids in urine comes to approximately 37-87% according to previous publications (Almé et al., 1977; Alnouti, 2009; Makino et al., 1975; Meng et al., 1997; Takikawa et al., 1984). More recent publications even report numbers beyond 70%, running up to 89% (Alamoudi et al., 2021; Bathena et al., 2013; Humbert et al., 2012).

In summary, sulfation increases the fecal as well as the urinary excretion of bile acids. Since bile acids in high concentrations can develop toxic effects, their homeostasis should be preserved and their accumulation in the systemic circulation should be avoided. Under physiological conditions, this is guaranteed by hepatic re-extraction and biliary excretion within the enterohepatic circulation. Although sulfated bile acids are more likely to get excreted via feces or urine than unsulfated ones, their major pathway for elimination is still the biliary excretion. This state changes in cholestasis: The stagnation of the biliary outflow leads to a higher amount of bile acids passing over to the systemic circulation. To preserve homeostasis, sulfation and ensuing urinary excretion now turn into the major pathway of bile acids elimination (Alnouti, 2009).

1.5 Bile Acids in Urine

Bile acids leaving the enterohepatic circulation for the systemic circulation can undergo renal filtration and thus end up in urine as mentioned previously. The absolute amount of urinary bile acids under physiological conditions is considered very low (Alnouti, 2009; Qi et al., 2021). One reason for this is that glomerular filtered bile acids mainly get reabsorbed in pars convoluta of the proximal tubule (Trauner and Boyer, 2003). In cholestatic diseases, however, the concentration of bile acids in urine is markedly higher (Alnouti, 2009; Trauner and Boyer, 2003). Thus, urinary bile acids are reported to be elevated in patients suffering from hepatitis, obstructive jaundice, liver cirrhosis and other hepatobiliary diseases (Alamoudi et al., 2021; Bathena et al., 2015a; Ferslew et al., 2015; Makino et al., 1975). Apart from absolute concentrations or excretion rates, there are also changes in the composition of the urinary bile acids pool as well as changes in sulfation and amidation state of urinary bile acids, which seem to even better correlate with hepatobiliary diseases and their severity (Alamoudi et al., 2021; Bathena et al., 2015a).

Due to bile acids' hormonal functions and their resulting involvement in several additional pathologies, it is justified to suppose that alterations of urinary bile acids may also appear in diseases other than those of the hepatobiliary system. Therefore, the measurement of individual bile acids in urine as well as the determination of urinary bile acids profiles as a whole could be of clinical value regarding diagnosis and management of diseases. Consistent with these thoughts, variations in urinary bile acids excretion have been described for certain patients with type 2 diabetes (Taylor et al., 2014).

1.6 Measurement of urinary Bile Acids

When talking about urinary bile acids as potential biomarkers for disease management, one must also think about ways of measuring bile acids in urine. In the early days of steroid analytics, bile acids have been preferably measured by gas chromatography-mass spectrometry (GC-MS) (Griffiths and Sjövall, 2010). In fact, one of the first methods coupling gas chromatography and mass spectrometry to analyze steroids was used for identification of bile acids in 1966 – albeit in feces instead of urine (Eneroth et al., 1966). GC-MS is still considered to perform the best separation of steroids and deliver steroid measurements with unsurpassed specificity (Wudy et al., 2018). Thus, it is also the prime method for investigation of biological material containing compound mixtures of unconjugated bile acids (Griffiths and Sjövall, 2010). However, when it comes to conjugated bile acids, GC-MS reaches its limits. Since conjugated bile acids are less volatile and analytes for gas chromatography need to have a certain volatility, it is necessary to cleave conjugated bile acids prior to GC analysis in order to remove charged groups like amino acids or sulfate groups. For instance, this can be done by hydrolyzation (Griffiths and Sjövall, 2010; Wudy et al., 2018). Liquid chromatography (LC), on the other hand, is capable of measuring conjugated bile acids as intact molecules and does not require any cleavage (Wudy et al., 2018). In analogy to GC, LC can also be coupled to mass spectrometry in order to achieve higher specificity. To make up for the lower chromatographic separation compared to GC, LC is usually coupled to tandem mass spectrometry (LC-MS/MS). LC-MS/MS has evolved into the method of choice for analysis of conjugated steroids (Wudy et al., 2018), making it also suitable for determination of urinary bile acids, because urine comprises a complex mixture of - mostly conjugated – bile acids.

There are also non-mass spectrometric methods for measuring urinary bile acids, such as enzymatic assays and immunoassays. However, they often lack in sensitivity or specificity. Consequently, their use will probably be more in a way of determining certain bile acids that are already assumed to be present in a respective sample, e. g. for monitoring purposes (Griffiths and Sjövall, 2010; Sjövall et al., 2010).

1.7 Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

The principle of all forms of chromatography is based on a mobile phase carrying an analyte solution past a stationary phase. The separation of the analytes depends on chemical and physical interactions between the analytes and the mobile or stationary phase, respectively. Components with stronger affinity to the stationary phase move only slowly with the flow of the mobile phase, while analytes with higher affinity to the mobile phase move faster. As a consequence, each analyte has a certain delay of its flow through the chromatography column, depending on its chemical and physical properties. This delay of flow is called retention. Hence, different analytes with different properties have different retention times depending on the time they require to reach the end of the column (Skoog et al., 2007).

As its name implies, in case of liquid chromatography (LC), the mobile phase is a liquid. The stationary phase is usually a column packed with particles. At the end of the column is a detector receiving signals that can be assigned to the different analytes. By plotting these signals as function of time, chromatograms are formed. Chromatograms can be used in a qualitative way for identification of analytes due to their retention time as well as in a quantitative way for measurement of an analyte's amount by integrating the area under the chromatographic peaks. In LC-MS/MS, the tandem mass spectrometer serves as the detector. This can be utilized to add additional selectivity, since the analytes can not only be identified by their retention time, but also by their mass-to-charge ratio (m/z) (Skoog et al., 2007).

The chromatographic resolution of LC increases with smaller stationary phase particles leading to a bigger surface for physicochemical interaction. To maintain a steady flow and acceptable running times despite densely packed columns with small particles, the liquid phase passes the column using a pressurized stream (high-performance liquid chromatography, HPLC). The most common type of HPLC is partition chromatography, meaning that the stationary phase is a second liquid, non-miscible with the liquid of the mobile phase. To guarantee this immiscibility, the stationary liquid has to be hold in place, which is usually achieved by chemical bonding, e. g. to a silica-based support material. The support material is coated with the actual stationary liquid. Depending on the relative polarity of the coating (stationary phase) and the polarity of the mobile phase, there are two different forms of LC: On the one hand, there is normal-phase LC (NPLC), where the stationary phase is more polar than the mobile phase. And on the other hand, the more commonly used reversed-phase LC (RPLC), which means the mobile phase is more polar than the stationary

phase. NPLC can for example use cyano groups, diol groups or amino groups as stationary phase, while the mobile phase could consist of hexane, ethyl ether or chloroform (Skoog et al., 2007). Whereas RPLC for instance works with a stationary phase formed by alkyl groups (e. g. C_2, C_8 or C_{18}) or phenyl material. Mobile phases in RPLC are polar solutions, often mixtures containing solvents like methanol, acetonitrile and water (Makin et al., 2010; Skoog et al., 2007). LC is regularly operated using a gradient elution. This means, two (or more) solvent mixtures that show significant differences in polarity serve as mobile phase. During the chromatographic separation, these solvents are varied in composition. In this way, analytes that are initially more adherent to the stationary phase get also eluted from the column eventually by turning the mobile phase into a more suitable carrier fluid for these respective analytes. Otherwise, it would take longer run times to elute these analytes (Skoog et al., 2007).

The key components of a mass spectrometer (MS) are an ion source, a mass analyzer and a detector. Since an MS identifies analytes by measuring their mass-to-charge ratio in gas phase, initially the analytes in solution have to be transferred to gas-phase ions. This process takes place in the ion source. Ion sources are divided into hard ionization, which leads to fragmentation of the analytes, and soft ionization with almost no fragmentation. Common types of ion sources for connection of an MS with HPLC are the soft ionization forms of atmospheric-pressure chemical ionization (APCI) and electrospray ionization (ESI) (Wudy et al., 2018). Since the present method uses ESI, the following explanations will focus on this type of ionization. ESI starts with the analyte solution being led through a metal capillary. There is a voltage applied between that capillary and a counter electrode to form an electric field. As a consequence, ions that are already in the solution are leaving the solution in form of a charged aerosol. Each droplet within the aerosol contains several ions. Due to evaporation of the solvent, the droplets get smaller, while their surface charge density gets higher. This results in an increasing instability of the droplets, which reaches its climax, when the so-called Rayleigh-Limit is met and the surface tension is no longer able to antagonize the charge. The droplets then “explode” to smaller droplets (Coulomb fission). This process can repeat itself. Ultimately, there are single analyte ions leaving the ion source and reaching the mass analyzer (Skoog et al., 2007). ESI can be performed in positive or negative mode (Wudy et al., 2018). The applied voltage between capillary and counter electrode determines whether the formed analyte ions are of positive or negative charge.

The mass analyzer filters the ions formed in the ion source depending on their mass-to-charge-ratio. There are different types of mass analyzers, the quadrupole being the most common one. A quadrupole consists of four cylindrical rods. These rods are arranged at the corners of a square and function as electrodes. They make it possible to maintain an electrostatic field (direct current, DC) and a radio-frequency field (RF). The ions coming from the ion source are moving through the quadrupole in sinus-shaped trajectories because of the electric field created by the four rods. By modifying the field's DC and RF values, ions with a certain mass-to-charge ratio move with a stable trajectory, allowing them to pass through the rods and reach the detector which follows the quadrupole. Ions with other m/z values move in unstable trajectories and therefore crash into one of the rods or are expelled from the quadrupole. Thus, they are not detected (Skoog et al., 2007; Wudy et al., 2018).

When it comes to tandem mass spectrometry, a triple quadrupole is the most common analyzer configuration. In fact, only two of these three quadrupoles (Q1-Q3) function as mass analyzers. Q2, however, works as a collision cell. The ions are coming directly from the ion source to Q1, these are the so-called precursor ions. By adjusting the quadrupole's electric field, the precursor ions that are going to be measured are selected by their m/z, while other ions are excluded from the system. The precursor ions that get through Q1 reach the collision cell (Q2). Q2 is filled with an inert gas, usually argon. The ions collide with the argon atoms which leads to fragmentation. As a result, smaller ions (product ions) are formed. The product ions reach Q3, which again works as a mass analyzer where certain product ions are selected and finally get to the detector (Skoog et al., 2007; Wudy et al., 2018). This means, tandem mass spectrometry does not only measure masses (or more exactly: mass-to-charge ratios), but rather mass transitions from precursor ions to product ions. In order to increase specificity, there are usually two characteristic mass transitions investigated for each analyte (Wudy et al., 2018). The most sensitive mass transition is used for quantification of the analyte ("Quantifier"), while another transition is used for confirmation of the measurement ("Qualifier").
If one specific precursor ion is selected in Q1 and one specific of its product ions is allowed to pass through Q3, it is called "selected reaction monitoring" (SRM). The application of SRM to multiple analytes is called "multiple reaction monitoring" (MRM) (Wudy et al., 2018).

A schematic illustration of an LC-MS/MS system using gradient elution and a triple quadrupole mass spectrometer is depicted in Figure 4.

The connection of LC and tandem MS (LC-MS/MS) is able to achieve a selectivity that one of the methods alone cannot achieve (Skoog et al., 2007; Wudy et al., 2018).

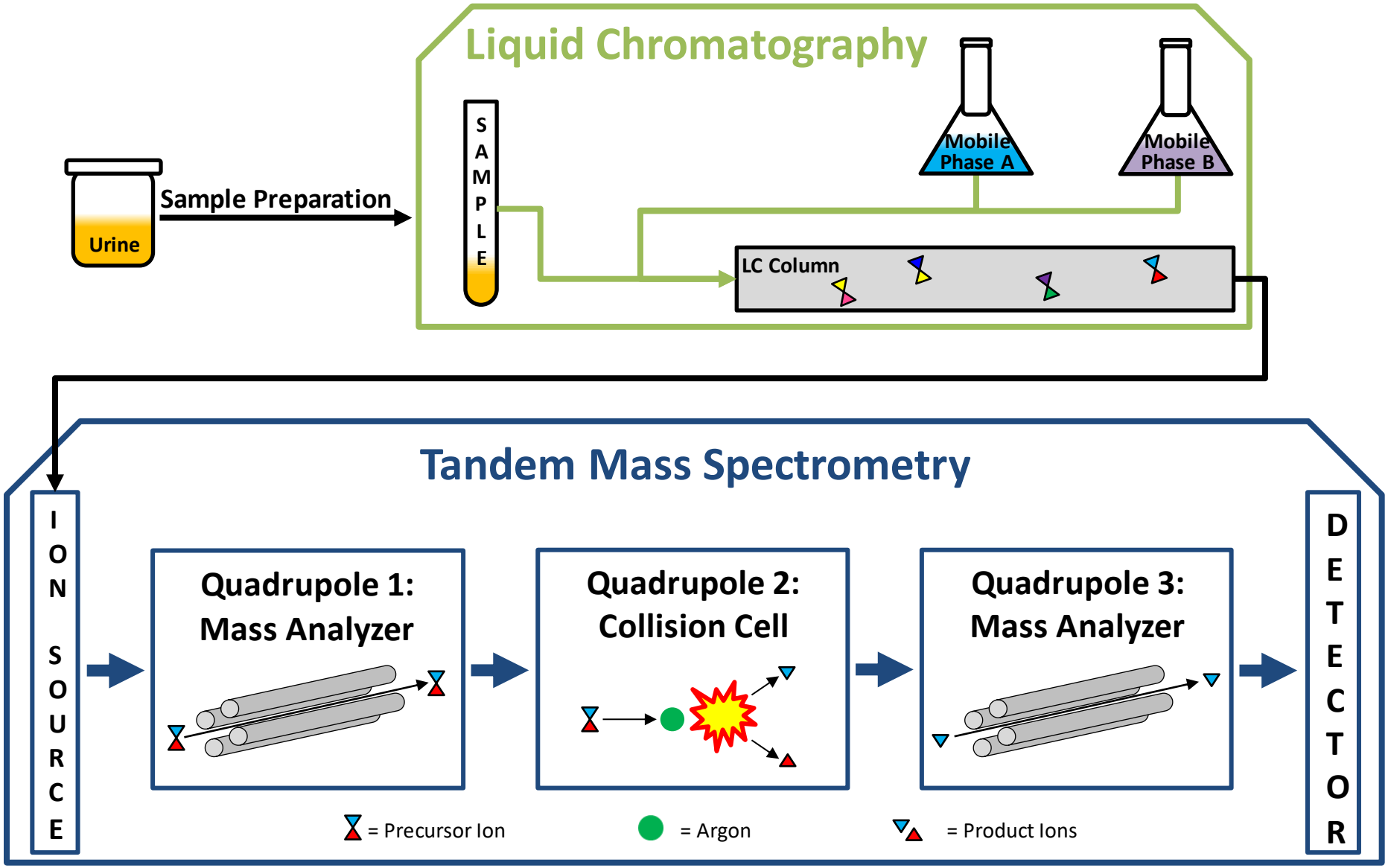


Figure 4. Schematic illustration of a liquid chromatography-tandem mass spectrometry system. Chromatography is done using gradient elution (mobile phases A and B). Tandem mass spectrometry is carried out on a triple quadrupole mass spectrometer.

1.8 Goals of this Study

The present study aims for various goals. First of all, a new method for identification and quantification of urinary bile acids using targeted liquid chromatography-tandem mass spectrometry (LC-MS/MS) should be developed and validated. A second goal consists in applying this method to urine of 317 subjects aged from 3 to 18 years. 80 of these subjects were in healthy physical condition, while 237 suffered from non-syndromic obesity (BMI > 97^{th} percentile). The obtained data of healthy subjects are going to serve as possibly the first estimate for reference values of urinary bile acids excretion in children, adolescents and young adults and thereby should lay the foundation for future research in this field. The study aims to compare the amounts of different bile acids species in urine along with illuminating the role of their sulfation and their amidation with glycine or taurine, respectively. Furthermore, possible sex or age differences as well as differences between the healthy and obese study groups should be investigated. In a brief digression, we also want to examine possible nutritional influences on urinary bile acids.

2 Materials and Methods

2.1 Chemicals and Materials

All reference standards were commercially purchased. GCA and TCA were from EMD Chemicals Inc. (San Diego, USA); LCA-S was from Santa Cruz Biotechnology Inc. (Dallas, USA); CA, CDCA, GCDCA, TCDCA, DCA, GDCA, TDCA, LCA, TLCA, UDCA and TLCA-S were from Sigma-Aldrich-Chemie GmbH (Steinheim, Germany); GLCA was from Steraloids Inc. (Newport, USA) and GCDCA-S, GDCA-S and GLCA-S were from Toronto Research Chemicals Inc. (Toronto, Canada). The same applies to internal standards, where d_4CA was bought from CDN Isotopes (Quebec, Canada); d_4GCDCA, d_4GDCA-S and d_4GUDCA-S from Sigma-Aldrich-Chemie GmbH (Steinheim, Germany) and d_5GCDCA-S from Toronto Research Chemicals Inc. (Toronto, Canada).

Activated charcoal, ammonium hydroxide (25%) and zinc sulfate heptahydrate were acquired from Carl Roth GmbH + Co. KG (Karlsruhe, Germany). Polyether sulfone membrane filters were from Sarstedt AG & Co. (Nümbrecht, Germany). Gradient grade methanol for liquid chromatography, LC-MS grade water and hexane for liquid chromatography were purchased from Merck KGaA (Darmstadt, Germany). Ultragradient grade acetonitrile for HPLC came from Chemsolute (Th. Geyer GmbH & Co. KG, Renningen, Germany). Ammonium acetate was gained from Sigma-Aldrich-Chemie GmbH (Steinheim, Germany) and SepPak Plus Short C18 cartridges from Waters Corporation (Milford, USA).

2.2 Urine Samples

The urine samples we analyzed after method validation were aliquots of 24-hour urine samples.

We obtained 80 samples of healthy children from the "DONALD" study in Dortmund, Germany ("Dortmund Nutritional and Anthropometric Longitudinally Designed Study"). This study is a longitudinal cohort study recruiting healthy individuals to examine potential associations between nutrition, metabolism and development from infancy to adulthood (Buyken et al., 2012).

In addition to that, we received 237 samples (80 samples from Medical University of Silesia in Katowice, Poland; 79 samples from Marmara University Faculty of Medicine in Istanbul, Turkey; 78 samples from University of Messina in Messina, Italy) of obese children from the international research project "Personalized Approach to Non-Syndromic Childhood Obesity using Multi-Omics Disease Signature" (hereafter referred to as MULTIOMICS project) (Gawlik et al., 2021). This cohort includes children with a body mass index over the 97th percentile, but without any further medical issues.

2.3 Preparation of Charcoal-stripped Urine

For method validation and to create calibration curves, we prepared a steroid-free biological matrix by charcoal-stripping human urine. In order to do this, we added charcoal to urine at a ratio of 1 g of charcoal per 10 mL of urine. The resulting liquid was stirred for two days and then centrifugated for 10 minutes at 3800 rpm. After that, the supernatant was collected and underwent the whole procedure once again. Following the second centrifugation, the supernatant was filtered with a polyether sulfone membrane filter and finally stored at -20°C.

2.4 Liquid Chromatography

Liquid chromatography was carried out with an Agilent 1200SL HPLC system (Waldbronn, Germany) using a Nucleoshell Phenyl-Hexyl column (50 × 4.6 mm, 2.7 µm) from Macherey-Nagel (Düren, Germany). Mobile phase consisted of two solvents: buffer solution (adjusted to pH 7 with 10 mM ammonium acetate) consisting of 85% H_2O and 15% acetonitrile (A) and a composite of 50% methanol and 50% acetonitrile as organic solvent (B). The settings for one chromatographic run can be seen in Table 1.

Start [min]	Duration [sec]	Flow [mL/min]	%A	%B
00:00	60	0.5	80.0	20.0
01:00	150	0.5	60.0	40.0
03:30	270	0.5	1.0	99.0
08:00	30	0.6	1.0	99.0
08:30	30	0.5	80.0	20.0
09:00	60	0.5	80.0	20.0

Table 1. Settings for gradient elution liquid chromatography. One chromatographic run took 10 minutes, including clean-up and re-equilibration.

2.5 Mass Spectrometry

Liquid chromatography was followed by tandem mass spectrometry with a TSQ Quantum Ultra triple quadrupole mass spectrometer from Thermo Fisher Scientific (Dreieich, Germany) using electrospray ionization (ESI) in the negative mode. Regarding the ESI settings, sheath gas pressure as well as auxiliary gas pressure were set to 30 arbitrary units, best performance could be accomplished at capillary temperatures of 325°C and 250°C and the applied voltage was -4000 V.

The tandem mass spectrometry was performed with multiple reaction monitoring (MRM) and scan time took 60 ms.

2.6 Sample Preparation for LC-MS/MS

A flow sheet providing an overview of the sample preparation process is shown in Figure 5. In a first step, the urine was centrifugated for 3 minutes at 3800 rpm. 2 mL of urine were then incubated for 15 minutes with 50 µL of an internal standard mixture containing each single internal standard with a concentration of 2500 ng/mL. Afterwards, protein precipitation was done using 1 mL of an aqueous $ZnSO_4$ solution (89 g/L) mixed 1:4 with acetonitrile. The samples were again incubated for at least 15 minutes and subsequently centrifugated for 10 minutes at 3800 rpm. After centrifugation, the protein free supernatant was collected and mixed with 3 mL of water. The SepPak C_{18} cartridges for solid phase extraction were activated with 3 mL of methanol followed by 3 mL of water. The activated cartridges were then loaded with the samples and thereafter washed with 3 mL of water and next with 6 mL of hexane. 5 mL of methanol were finally used to elute the steroid content from the cartridges. The methanolic fraction was evaporated with nitrogen at 40°C, before reconstitution was done using 250 µL of a solution containing 50% methanol, 40% water and 10% ammonia solution (2.5%). To achieve higher purity for LC-MS/MS, after 15 minutes of incubation the reconstituted samples were again centrifugated for 3 minutes at 15000 rpm. The supernatant was transferred to vials for LC-MS/MS analysis.

To obtain calibration curves and also for quality control samples during method validation, the same preparation procedure was done using charcoal-stripped urine spiked with defined concentrations of reference standards for the 18 bile acids.

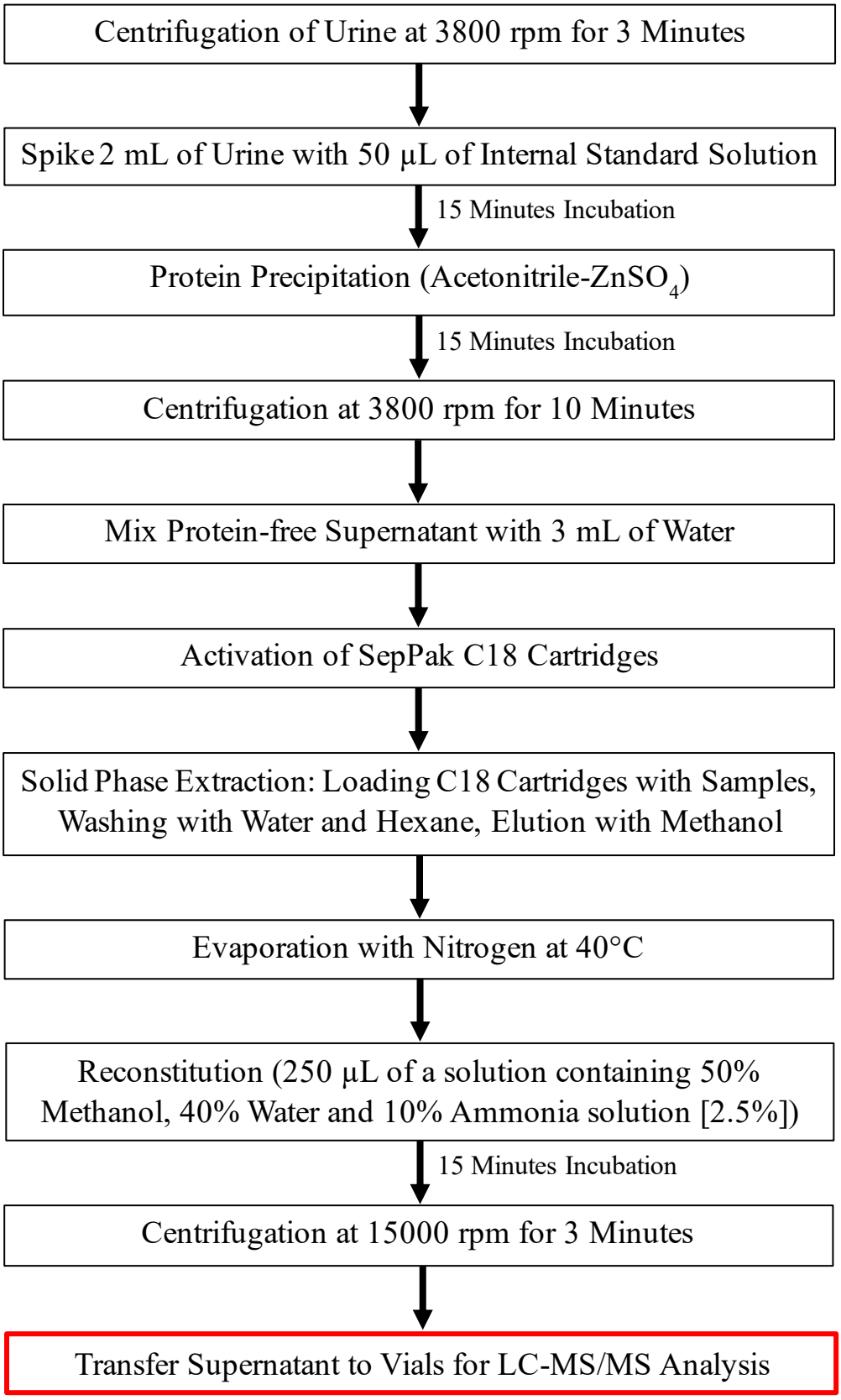


Figure 5. This flow sheet presents the sample preparation process step by step.

2.7 Method Validation

The requirements and acceptance criteria for method validation were chosen according to the "Guidance for Industry: Bioanalytical Method Validation" by the U.S. Food and Drug Administration (FDA) from 2001 (*Guidance for Industry: Bioanalytical Method Validation*, 2001).

Calibration Curves

To obtain calibration curves for sample analysis, 10 calibration points were set at defined concentrations for each analyte. The calibration samples underwent every step of sample preparation. Instead of using urine, the calibration samples were prepared by using steroid-free matrix (charcoal-stripped urine, see above) spiked with a defined concentration of analytical standard for each analyzed bile acid.

Limits of Quantification and Detection

The limit of quantification (LOQ) of a compound was defined as the lowest concentration where five analytical-standard-spiked samples (charcoal-stripped urine spiked with analytical standard) could achieve precision and accuracy in a ± 20% range. The limit of detection (LOD) is the concentration of a compound, for which the signal-to-noise ratio is high enough to reliably identify a chromatographic peak.

Precision and Accuracy

Formulas	Acceptance Criteria
$\boldsymbol{precision}\ (\%) = \frac{\boldsymbol{stdev}}{\boldsymbol{mean\ calculated\ amount}} \times \mathbf{100}$	***Precision: ≤ 15%***
$\boldsymbol{accuracy}\ (\%) = \frac{\boldsymbol{mean\ calculated\ amount}}{\boldsymbol{specified\ amount}} \times \mathbf{100}$	***Accuracy: 100% ± 15%***

Precision and accuracy were used as parameters for correctness and reproducibility of the method within one analytical run (intra-day) as well as between multiple analytical runs (inter-day). Intra-day precision and accuracy were calculated using five replicates at three different quality control concentrations (QC1, QC2, QC3) during one sample work-up. For inter-day precision and accuracy, data of five QC1-3 replicates from three different sample work-ups on three different days were used. According to the FDA, the acceptance criterion

for precision is a deviation of no more than 15% from the coefficient of variation (CV). Regarding accuracy, the measured results should lie within 15% deviation of the actual concentrations. The quality control samples were once again prepared by spiking charcoal-stripped urine with defined concentrations of analytical standards.

Matrix Effect

To detect possible effects of the biological matrix urine on the results, the matrix effect was investigated. The matrix effect was calculated comparing the results of two different sample preparations, preparation A and preparation B. Preparation A was done by only using reference standard solutions and internal standards without sample work-up and with no biological matrix. In preparation B, the same concentration of standards and internal standards was added to steroid free urine matrix (charcoal-stripped urine) after solid phase extraction. The chromatographic area ratio of preparation B (≙ y-axis) was plotted against the area ratio of preparation A (≙ x-axis). If the slope of the resulting linear function is higher than 1.00, the matrix leads to enhancement of the obtained results. A slope lower than 1.00 consequently indicates suppression of the results by the matrix. The criterion for assuming no significant matrix effect is a slope lying in a range of 1.00 ± 0.15.

Recovery

By observing the recovery, possible loss or gain of bile acids during sample work-up was investigated. For that purpose, a sample preparation with regular sample work-up (preparation C) was compared to a sample preparation where the charcoal-stripped urine was spiked with the reference standards after solid phase extraction (preparation B). To calculate the percentage recovery, the quotient between preparation C and preparation B was formed at three different concentrations (QC1, QC2, QC3) and multiplied with 100. The acceptance range was set to $100\% \pm 15\%$.

2.8 Statistical Analyses and Graphics

Transfer to nanomolar Units

Our LC-MS/MS method delivered results for bile acids concentration in ng/mL (≙ µg/L). Statistical analysis and presentation of results were done using nanomolar units - with nmol/L being the basic unit for urinary bile acids concentration. Starting from there, we also expressed our nanomolar results creatinine-related [nmol BA/mmol Crea] and as daily excretion rates [nmol/d]. Unit transfer was calculated using the formula below:

Formula for unit transfer

$$\boldsymbol{concentration}\ [\boldsymbol{nmol/L}] = \frac{\boldsymbol{concentration}\ \left[\frac{\mu\mathbf{g}}{\boldsymbol{L}}\right]}{\boldsymbol{molar\ mass}\ [\frac{\boldsymbol{g}}{\boldsymbol{mol}}]} \times \mathbf{1000}$$

Our 18 analytes' molar masses were used as specified by the manufacturers of the respective chemical standards. The molar masses are shown in Table 2.

Analyte	Molar Mass [g/mol]	Manufacturer***
CA	408.57	Sigma-Aldrich
GCA*	487.60	EMD
TCA*	537.70	EMD
CDCA	392.57	Sigma-Aldrich
GCDCA*	471.61	Sigma-Aldrich
TCDCA*	521.69	Sigma-Aldrich
DCA	392.57	Sigma-Aldrich
GDCA*	471.61	Sigma-Aldrich
TDCA*	521.69	Sigma-Aldrich
LCA	376.57	Sigma-Aldrich
GLCA*	455.61	Steraloids
TLCA*	505.69	Sigma-Aldrich
UDCA	392.57	Sigma-Aldrich
GCDCA-S**	573.65	TRC
GDCA-S**	573.65	TRC
LCA-S**	500.60	Santa Cruz
GLCA-S**	557.65	TRC
TLCA-S**	607.73	Sigma-Aldrich

Table 2. Molar masses of measured analytes as specified by the manufacturers.
**Analyte delivered as sodium salt*
***Analyte delivered as disodium salt*
****Manufacturers: Sigma-Aldrich = Sigma-Aldrich-Chemie GmbH (Steinheim, Germany); EMD = EMD Chemicals Inc. (San Diego, USA); Steraloids = Steraloids Inc. (Newport, USA); TRC = Toronto Research Chemicals Inc. (Toronto, Canada); Santa Cruz = Santa Cruz Biotechnology Inc. (Dallas, USA)*

Analyses of the Study Groups

Each study group's characteristics as there are 24-hour urine volume, age, sex, height, weight, BMI and urinary creatinine concentration were presented as mean ± standard deviation as well as lower quartile (P25), median (P50) and upper quartile (P75). By applying Pearson's chi squared test for the nominal scaled characteristic of sex or the Wilcoxon test for the remaining ratio scaled characteristics, respectively, the groups were tested for potential significant differences. All these calculations were done by using the statistics program "R" (R version 4.2.1) and the graphical user interface "RStudio" (RStudio version 2022.02.3+492) (Grace and Hudson, 2016) (see also: https://www.r-project.org/foundation/; accessed on 03/10/2023).

Analyses of Bile Acids Results

The presentation of the raw data for urinary bile acids in the three mentioned units was primarily done by using tables listing each analyte's mean ± standard deviation as well as its percentiles P05, P10, P25, P50, P75, P90 and P95. These calculations were done in Microsoft Excel (Microsoft Office 2019, Microsoft Corporation, Redmond, USA). Excel was also used to create scatter plots with linear regression trendlines, boxplots for group comparison as well as for generating chromatograms out of our chromatographic raw data. Calculations for percentages of amidation and sulfation and the depiction of amidation shares in form of pie charts were Excel-operated, too.

Calculations regarding potential significant age, sex or group differences, respectively, were carried out in form of linear regression analyses in "R". The depiction of the six most abundant bile acids as percentile curves was also executed in "R".
Please note: For all significance tests regarding urinary bile acids, the bile acids results underwent logarithmic transformation by calculating their natural logarithm. This was done to emulate a normal distribution and was also applied to the creation of percentile curves. Sulfation and amidation percentage form an exception: Since these results were percentage figures, the linear regression analyses for potential significant group differences was done without preceding logarithmization.

Creation of Graphics

Apart from the already mentioned graphics during statistical analyses, Figures 1 and 3 were created using "ChemSketch" (ACD/ChemSketch (Freeware) 2020 1.1 (File Version C15E41, Build 115819, 18 May 2020); Advanced Chemistry Development, Inc., Toronto, Canada). Figures 2 and 4 were created with Microsoft Office 2019 (Microsoft Corporation, Redmond, USA).

3 Results

3.1 Liquid Chromatography

We could receive reliably identifiable chromatographic peaks for each of our 18 analytes. Especially for analytes that were measured at the same mass transitions, we could achieve the necessary chromatographic baseline separation. Those were UDCA/CDCA/DCA, GCDCA/GDCA, TCDCA/TDCA and GCDCA-S/GDCA-S (see also in Table 3 below). The respective chromatograms of the 18 bile acids using analytical standards in charcoal-stripped urine are shown in Figure 6. Depicted in Figure 7 are exemplary chromatograms of a typical urine sample from the reference group, presenting the six most abundant bile acids.

Figure 6. Chromatograms of all 18 measured bile acids using analytical standards.
The figure shows the chromatographic peaks of the 18 analytes measured in charcoal-stripped urine, spiked with analytical standards of each bile acid. The presented chromatograms were generated at calibration point 6 (respective concentrations, see below in section 'Calibration Curves').
x-axis ≙ time [min]
y-axis ≙ intensity
black box ≙ quantifier mass transition [m/z]

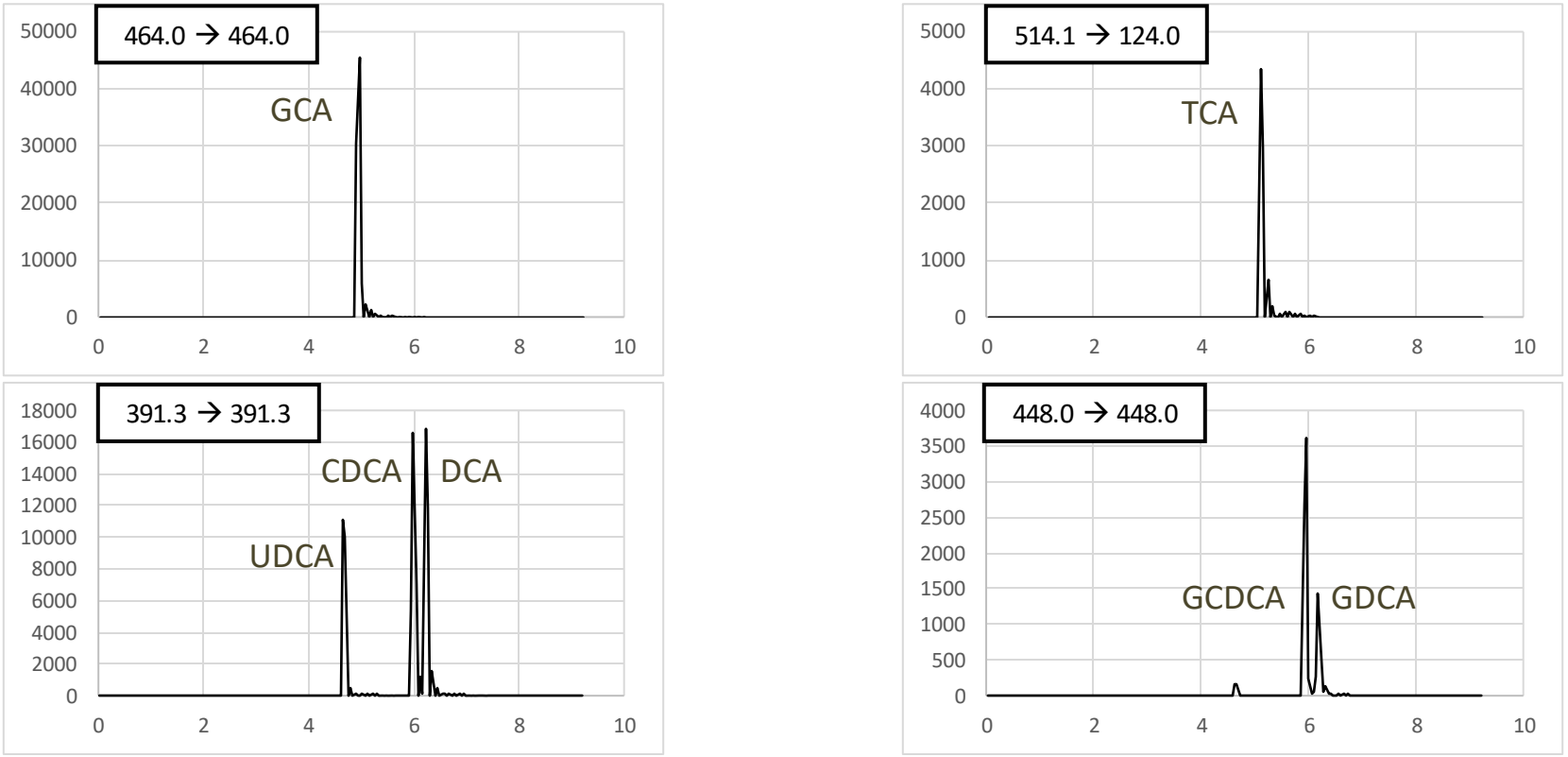


Figure 6 (1/3)

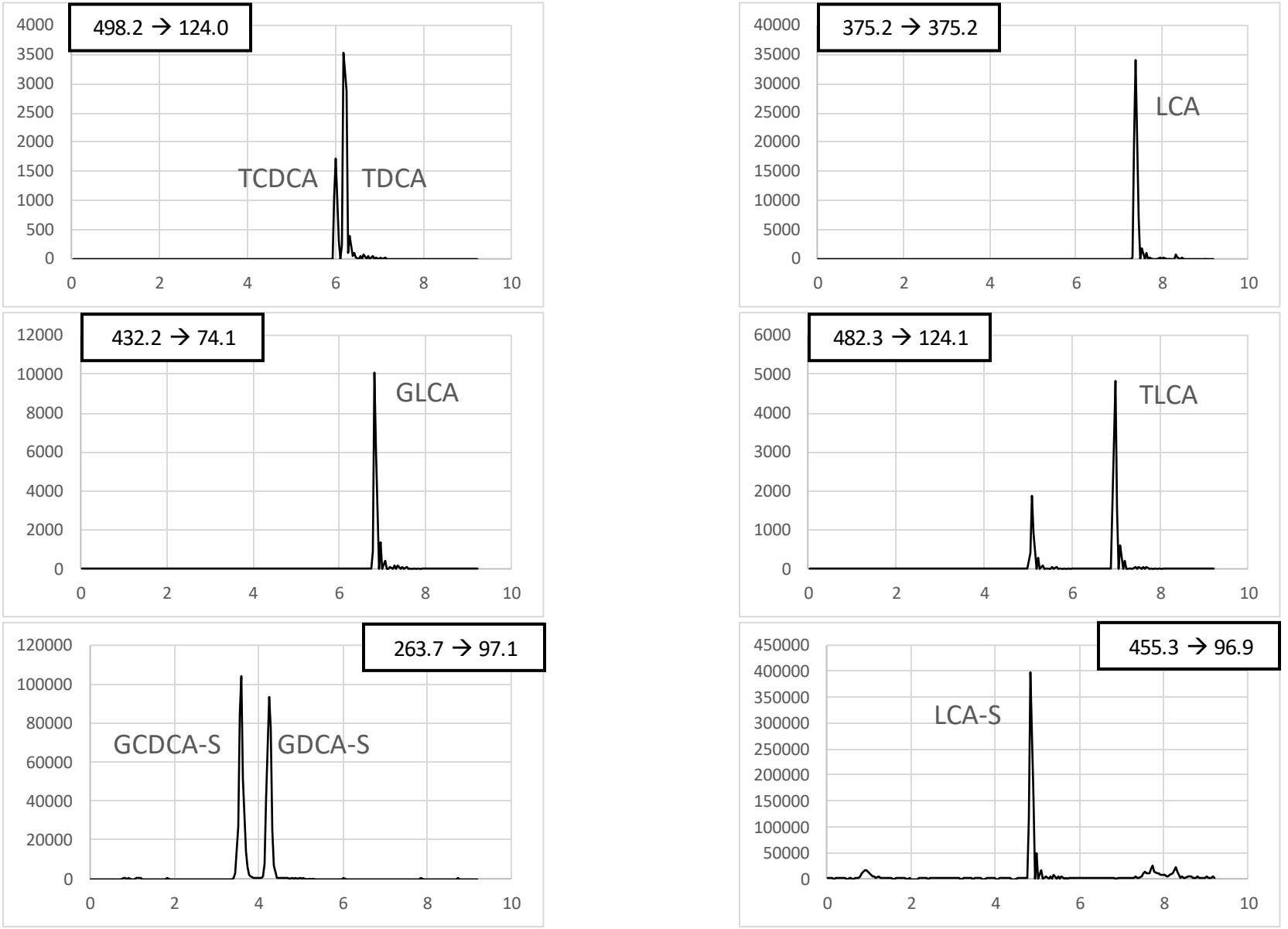
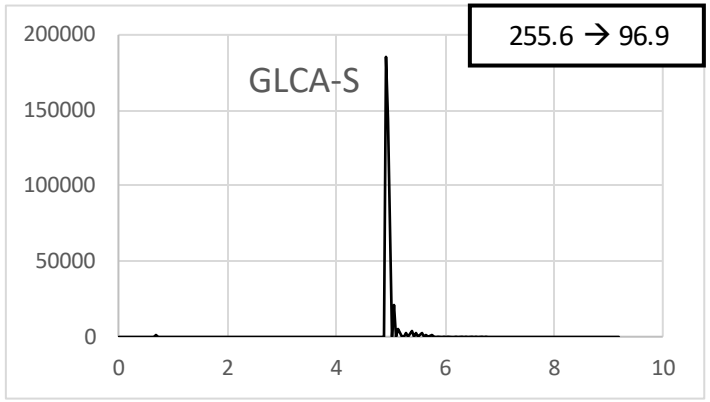

498.2 → 124.0
TCDCA
TDCA
375.2 → 375.2
LCA
432.2 → 74.1
GLCA
482.3 → 124.1
TLCA
263.7 → 97.1
GCDCA-S
GDCA-S
455.3 → 96.9
LCA-S

Figure 6 (2/3)


255.6 → 96.9
GLCA-S
200000
150000
100000
50000
0
0
2
4
6
8
10

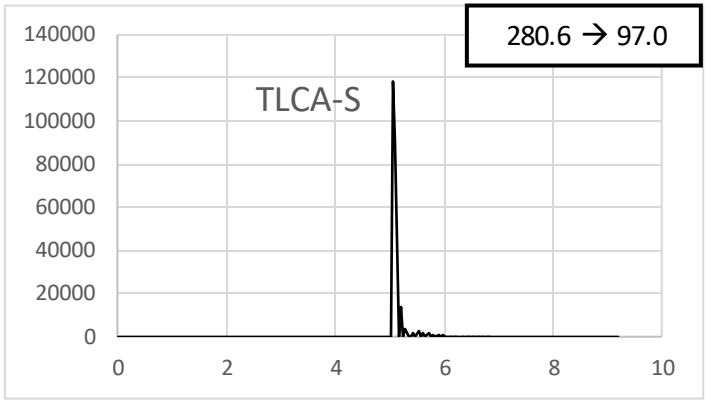
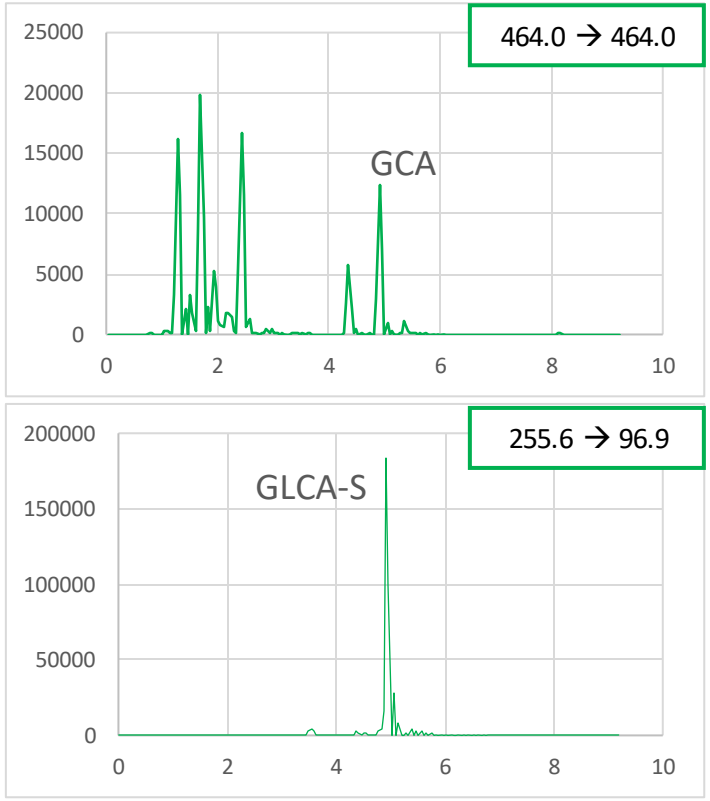
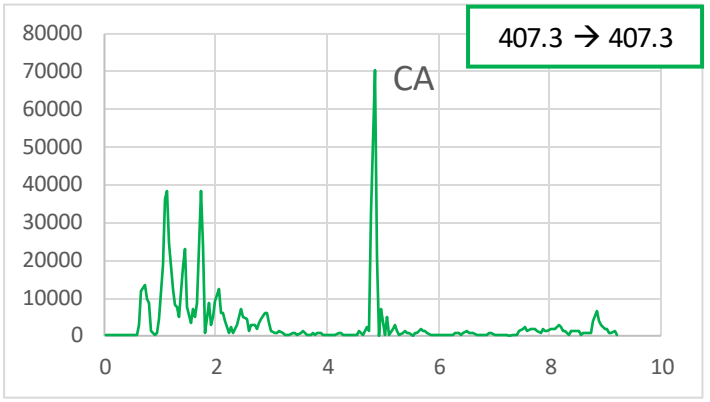
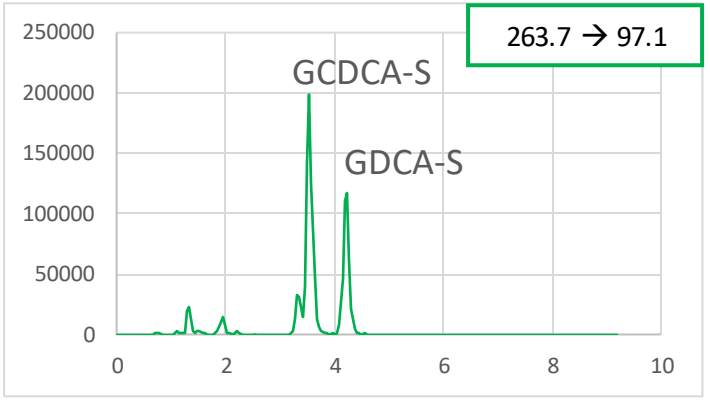

280.6 → 97.0
TLCA-S
140000
120000
100000
80000
60000
40000
20000
0
0
2
4
6
8
10

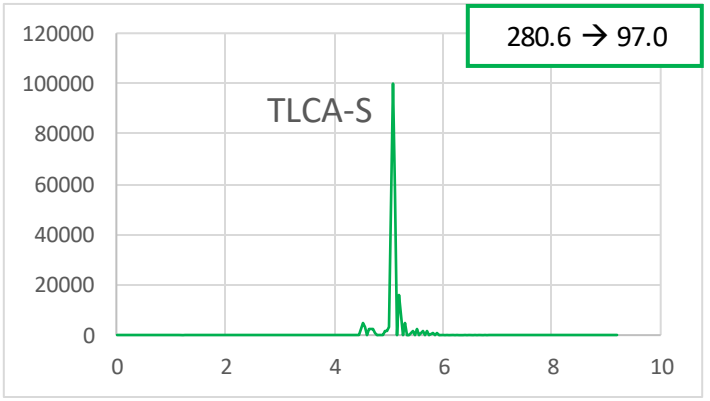
Figure 6 (3/3)

Figure 7. Real sample: chromatograms of the six most abundant bile acids, measured in a 24-hour urine sample of a proband from the healthy group (5.95 years, male).
x-axis ≙ time [min]
y-axis ≙ intensity
green box ≙ quantifier mass transition [m/z]









3.2 Mass Spectrometry

The best mass spectrometry performance for the 18 analytes could be achieved at the mass transitions and parameters listed in Table 3.

Compound	Precursor ion [m/z]	Product ion [m/z]	Tube lens voltage [V]	Collision energy [eV]
CA	407.3	407.3	166	25
GCA	464.0	464.0	167	10
TCA	514.1	124.0	169	50
CDCA **DCA** **UDCA**	391.3	391.3	167	37
GCDCA **GDCA**	448.0	448.0	108	25
TCDCA **TDCA**	498.2	124.0	166	43
LCA	375.2	375.2	162	37
GLCA	432.2	74.1	125	29
TLCA	482.3	124.1	170	48
GCDCA-S	263.7	97.1	96	35
GDCA-S	263.7	97.1	78	35
LCA-S	455.3	96.9	143	37
GLCA-S	255.6	96.9	81	37
TLCA-S	280.6	97.0	167	37
d_4CA	411.2	347.0	104	34
d_4GCDCA	452.3	74.0	129	31
d_5GCDCA-S	266.2	98.0	66	52
d_4GDCA-S	265.7	98.1	86	55
d_4GUDCA-S	265.7	98.1	89	55

Table 3. Mass spectrometry information for all 18 analytes, including MRM transition (precursor ion → product ion), tube lense voltage and collision energy.

3.3 Method Validation

All aspects of method validation, including precision and accuracy (intra-day or inter-day, respectively), recovery and matrix effect met the FDA-default standards (*Guidance for Industry: Bioanalytical Method Validation*, 2001).

Table 4 shows the validation data for each analyte in detail.

Method Validation

			CA	GCA	TCA	CDCA	GCDCA	TCDCA	DCA	GDCA	TDCA	LCA	GLCA	TLCA	UDCA	GCDCA-S	GDCA-S	LCA-S	GLCA-S	TLCA-S
	Internal Standard		d4CA	d4GCDCA	d4GCDCA	d4GCDCA	d4GCDCA	d4GCDCA	d4GCDCA	d4GCDCA	d4GCDCA	d4GCDCA	d4GCDCA	d4GCDCA	d4GUDCA-S	d5GCDCA-S	d4GDCA-S	d4GCDCA	d4GCDCA	d4GCDCA
	LOD	[ng/mL]	0.63	0.63	1.25	1.25	0.63	1.25	1.25	0.63	1.25	1.25	0.63	1.25	0.63	1.25	1.25	0.63	1.25	1.25
	LOQ	[ng/mL]	6.25	6.25	6.25	6.25	18.75	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25
	Linearity		> 0.99	> 0.99	> 0.99	> 0.99	> 0.99	> 0.99	> 0.99	> 0.99	> 0.99	> 0.99	> 0.99	> 0.99	> 0.99	> 0.99	> 0.99	> 0.99	> 0.99	> 0.99
	Range	[ng/mL]	6.25-1250	6.25-1250	6.25-125	6.25-125	18.75-125	6.25-125	6.25-125	6.25-125	6.25-125	6.25-125	6.25-125	6.25-125	6.25-125	6.25-1875	6.25-1875	6.25-1250	6.25-1250	6.25-1250
	LOQ	Precision %	8.78	5.60	8.72	5.99	2.38	13.36	3.75	4.63	12.53	3.78	9.25	9.41	10.55	4.14	7.31	4.28	4.44	4.20
		Accuracy %	86.56	83.48	82.77	80.58	99.16	99.34	83.73	89.14	83.10	87.28	81.09	87.64	81.15	105.67	88.98	94.49	80.41	86.92
Intra-Day	QC 1	Precision %	4.49	5.50	4.83	10.30	1.77	10.51	3.08	4.50	11.07	5.92	5.42	10.84	9.53	7.27	5.64	2.50	6.22	4.98
		Accuracy %	85.75	99.69	103.01	87.19	108.26	100.18	95.74	87.44	101.21	93.68	90.01	93.71	85.97	95.53	105.21	88.06	93.02	90.38
	QC 2	Precision %	8.00	2.75	2.80	5.26	2.67	3.89	5.96	2.33	4.47	6.05	3.90	6.29	4.86	5.23	4.11	3.57	2.66	2.27
		Accuracy %	99.66	108.33	108.37	93.66	97.22	98.29	96.84	92.77	98.40	100.57	95.19	103.56	97.39	98.31	92.13	102.83	99.91	108.42
	QC 3	Precision %	6.81	4.31	6.55	4.84	3.36	6.22	2.49	4.32	1.02	6.24	3.58	2.19	2.80	7.48	6.55	5.00	4.87	4.92
		Accuracy %	108.76	100.90	107.75	105.56	106.68	107.52	107.08	107.62	108.90	103.45	106.54	100.65	101.78	106.08	102.26	100.86	90.04	89.35
Recovery %	QC 1		97.29	102.53	103.70	113.99	106.76	113.43	110.41	100.40	94.81	98.31	101.20	97.39	101.13	102.87	92.34	104.93	97.70	98.24
	QC 2		101.59	106.72	107.37	108.20	104.88	105.58	100.68	106.82	105.97	99.95	98.86	107.86	104.96	103.97	93.14	103.59	104.89	106.30
	QC 3		97.10	95.55	99.49	93.25	96.63	92.98	99.54	97.39	102.21	97.12	99.12	99.12	100.85	102.45	97.59	92.06	90.49	92.31
Inter-Day	QC 1	Precision %	4.34	3.76	11.47	4.69	9.12	7.24	3.99	5.14	5.56	8.98	2.58	3.57	2.14	5.23	1.30	6.89	6.98	5.57
		Accuracy %	90.26	95.55	92.92	86.76	98.72	99.38	96.62	90.64	95.25	102.88	90.73	91.76	88.00	98.09	106.80	89.82	94.43	93.52
	QC 2	Precision %	5.95	4.83	6.12	3.28	4.19	6.95	1.30	7.57	1.31	0.78	3.33	3.88	2.41	3.18	5.90	2.72	3.14	5.79
		Accuracy %	102.23	105.51	108.47	97.35	101.05	96.62	98.15	100.84	99.86	100.59	96.91	99.24	95.85	101.57	98.81	106.16	102.55	107.07
	QC 3	Precision %	9.12	4.56	2.91	0.66	2.73	3.07	2.31	3.17	2.11	6.56	8.29	3.92	3.43	3.15	3.39	0.42	7.70	9.12
		Accuracy %	107.86	100.37	109.92	104.78	108.41	104.97	106.78	110.99	108.03	99.63	104.80	105.42	98.18	104.01	105.60	100.80	98.81	99.82
Matrix Effect	Slope		0.98	1.05	1.03	1.02	1.01	1.05	1.09	0.98	1.05	0.94	1.01	0.94	1.12	0.98	1.09	1.07	1.10	1.06
Spec. Amount	QC1	[ng/mL]	9.38	9.38	9.38	9.38	25.00	9.38	9.38	9.38	9.38	9.38	9.38	9.38	9.38	9.38	9.38	9.38	9.38	9.38
	QC2	[ng/mL]	312.50	312.50	31.25	31.25	31.25	31.25	31.25	31.25	31.25	31.25	31.25	31.25	31.25	468.75	468.75	312.50	312.50	312.50
	QC3	[ng/mL]	937.50	937.50	93.75	93.75	93.75	93.75	93.75	93.75	93.75	93.75	93.75	93.75	93.75	1406.25	1406.25	937.50	937.50	937.50

Table 4. Overall data of method validation. The concentrations are presented in ng/mL.

Calibration Curves

All calibration curves could reach a linearity >0.99.

The 10 calibration points for the 18 analytes were set as listed in Table 5.

Samples with concentrations that exceeded the calibration range were re-measured in dilution.

	Calibration levels [ng/mL]		
	other analytes*	**CA + GCA + LCA-S + GLCA-S + TLCA-S**	**GCDCA-S + GDCA-S**
cal 1	0.31	3.13	4.69
cal 2	0.42	4.17	6.25
cal 3	0.63	6.25	9.38
cal 4	1.25	12.50	18.75
cal 5	3.13	31.25	46.88
cal 6	6.25	62.50	93.75
cal 7	12.50	125.00	187.50
cal 8	31.25	312.50	468.75
cal 9	62.50	625.00	937.50
cal 10	125.00	1250.00	1875.00

*** "other analytes" means TCA, CDCA, GCDCA, TCDCA, DCA, GDCA, TDCA, LCA, GLCA, TLCA and UDCA**

Table 5. Calibration points for generating calibration curves.
For each analyte, 10 calibration points (cal 1 – cal 10) were set. For analytes with higher expected urinary concentrations, the range of the curves was chosen wider.

Limits of Quantification and Detection

For all bile acids, the defined criteria for the LOQ could be met at a concentration of 6.25 ng/mL - except for GCDCA, where the LOQ came to 18.75 ng/mL. The LOD, meaning the lowest concentration to identify the 18 analyzed bile acids reliably, was 0.63 ng/mL or 1.25 ng/mL, respectively.

Precision and Accuracy

Intra-day precision of our 18 analytes ranged from 1.02% to 11.07%, while inter-day precision lay between 0.42% and 11.47%. Intra-day and inter-day accuracy ranged from 85.75% to 108.90% and 86.76% to 110.99%, respectively. Conclusively, the criteria of acceptance defined by the FDA could be met.

Matrix Effect

Calculated as described above, the slopes of the linear functions representing the matrix effect for all 18 analytes varied between 0.94 and 1.12. Therefore, the defined criteria could be achieved for all analyzed bile acids. This means, there could not be observed a significant matrix effect.

Recovery

For all 18 analytes, the percentage recoveries comparing a normal sample work-up with a work-up where the steroid-free urine was spiked after solid phase extraction lay between 90.49% and 113.99%, meeting the defined criteria. Thus, it can be concluded that the sample preparation does not lead to any loss or gain of bile acids, respectively.

3.4 Measurement of Urine Samples

3.4.1 Study Groups

The study group we received from DONALD study contained 24-hour urine samples from 80 healthy children. The age span encompassed 2.98 years to 18.37 years with a median age of 10.55 years. Including 40 boys and 40 girls, there was a balanced sex ratio. The median height was 145.5 cm (span from 92.8 cm to 194.5 cm). Weight varied between 13.45 kg and 86.30 kg with a median weight of 35.5 kg. BMI ranged from 13.4 kg/m^2 to 27.1 kg/m^2 with a median of 16.8 kg/m^2. The median 24-hour urine volume came to 862.5 mL (span from 220 mL to 3570 mL). Creatinine concentration varied between 2.1 and 14.5 mmol/L (median 6.8 mmol/L).

Deriving from the MULTIOMICS project, we examined 237 samples of 24-hour urine from children with non-syndromic obesity. Their age covered a span from 7.95 years to 17.10 years with a median age of 12.66 years. With 112 female and 125 male probands, this study group contained slightly more boys (53% male, 47% female). The height fluctuated between 124.5 cm and 183.0 cm with a median height of 158.0 cm. The median weight accounted for 75.5 kg (span from 35.10 kg to 151.70 kg). The obese probands' BMI lay in the range of 22.5 kg/m^2 to 56.8 kg/m^2 (median 30.2 kg/m^2). The 24-hour urine volume ranged from 280 mL to 4500 mL, resulting in a median volume of 1000 mL. The information of 24-hour volume is missing for one sample in the MULTIOMICS group. Creatinine concentration was unknown in case of 12 samples. The remaining samples covered a span of 0.5 to 22.8 mmol creatinine/L (median 7.8 mmol/L).

Comparing the groups' characteristics, only the sex ratio did not significantly differ between the two study groups ($p = 0.671$). Apart from that, there were significant differences ($p < 0.05$) between the two groups regarding the remaining aspects. The obese probands showed markedly higher weight and BMI values. The probands in this group were also significantly older and taller and excreted higher urine volumes within the 24 hours of collection as well as higher creatinine concentrations. When correcting for age, the creatinine concentration showed no significant group difference.

A summarized comparison between the two study groups is visualized in Table 6.

		N	HEALTHY *N=80*	OBESE *N=237*	p-value
Volume [mL]		316	562.5 862.5 1185 971.3 ± 597.5	800 1000 1650 1300.2 ± 703.2	**<0.001**[1]
Age [years]		317	5.75 10.55 15.26 10.65 ± 4.89	10.41 12.66 14.14 12.37 ± 2.32	**0.003**[1]
Sex	f	317	0.50 ($^{40}/_{80}$)	0.47 ($^{112}/_{237}$)	0.671[2]
	m		0.50 ($^{40}/_{80}$)	0.53 ($^{125}/_{237}$)	
Height [cm]		317	118.1 145.5 166.1 143.5 ± 27.2	146.2 158 165 155.8 ± 13.0	**<0.001**[1]
Weight [kg]		317	21.8 35.5 53.9 38.7 ± 18.9	61.5 75.5 90.8 76.4 ± 20.2	**<0.001**[1]
BMI [kg/m^2]		317	15.4 16.8 19.3 17.4 ± 2.8	27.2 30.2 33.4 30.9 ± 4.9	**<0.001**[1]
Creatinine [mmol/L]		305	4.8 6.8 9.1 7.3 ± 3.2	5.5 7.8 10.3 8.4 ± 4.1	**0.041**[1]

Table 6. Comparison of study groups.
For continuous variables, the top line shows the lower quartile, the median and the upper quartile. The bottom line represents mean ± SD.
The variable "sex" is shown as proportion of the respective study group.
N = number of non-missing values.
Tests used for p-value: [1]Wilcoxon test; [2]Pearson's chi-squared test.

3.4.2 Urinary Bile Acids in healthy Children

For establishment of preliminary reference values for urinary bile acids in children, we measured 24-hour urine samples of 80 healthy children from the DONALD study. The results are presented in three different units: Urinary bile acids concentration [nmol/L], creatinine-related bile acids concentration [nmol BA/mmol Crea] as well as daily urinary bile acids excretion [nmol/d].

Urinary Bile Acids Concentrations [nmol/L]

The data for urinary bile acids concentrations in healthy children [nmol/L] are depicted in Table 7.

In general, the most abundant bile acids measured were the sulfated ones. These bile acids delivered the highest concentrations as well as only a small number of results below the limit of quantification. With a median of 588 nmol/L, GCDCA-S was the most abundant within the 18 analyzed bile acids. The second most common bile acid was GLCA-S with a median of 354 nmol/L - followed by GDCA-S (319 nmol/L) and TLCA-S (158 nmol/L).
These four bile acids added up to 83% of all measurable bile acids, although they represent only four out of 18 analytes.
LCA-S formed an exception among the sulfated bile acids. For this analyte, 85% of the samples lay below the limit of quantification, resulting in a median of 0 nmol/L. Lying at 102 nmol/L, even the highest result for LCA-S was distinctly lower than the medians for the other four sulfated bile acids.

The most common unsulfated bile acids were CA and GCA. With medians of 135 nmol/L or 100 nmol/L, respectively, they follow upon TLCA-S.

For five of the bile acids (GCDCA, TDCA, LCA, GLCA and TLCA), the results of all 80 healthy samples lay below the limit of quantification. A similar situation appeared for CDCA and UDCA, where only 3 samples or 1 sample, respectively, lay over the limit of quantification.

	Mean ± SD	.05	.10	.25	.50	.75	.90	.95
CA ($^{77}/_{80}$)	220.8 ± 245.2	20.0	30.5	42.3	135.1	324.3	580.3	668.9
GCA ($^{79}/_{80}$)	138.0 ± 109.6	32.9	41.3	62.1	100.2	168.8	287.5	352.2
TCA ($^{40}/_{80}$)	9.3 ± 10.7	0.0	0.0	0.0	5.9	16.1	23.0	25.7
CDCA ($^{3}/_{80}$)	1.0 ± 5.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GCDCA ($^{0}/_{80}$)	0.0 ± 0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
TCDCA ($^{19}/_{80}$)	4.3 ± 8.2	0.0	0.0	0.0	0.0	0.0	17.9	19.4
DCA ($^{28}/_{80}$)	11.0 ± 20.7	0.0	0.0	0.0	0.0	18.9	29.1	37.4
GDCA ($^{31}/_{80}$)	10.7 ± 18.4	0.0	0.0	0.0	0.0	17.6	29.3	42.7
TDCA ($^{0}/_{80}$)	0.0 ± 0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
LCA ($^{0}/_{80}$)	0.0 ± 0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GLCA ($^{0}/_{80}$)	0.0 ± 0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
TLCA ($^{0}/_{80}$)	0.0 ± 0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
UDCA ($^{1}/_{80}$)	0.2 ± 2.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GCDCA-S ($^{80}/_{80}$)	670.2 ± 444.1	186.4	250.5	340.0	588.4	863.3	1312.2	1456.1
GDCA-S ($^{75}/_{80}$)	459.5 ± 498.2	0.0	36.0	116.8	319.3	655.7	957.6	1342.5
LCA-S ($^{12}/_{80}$)	4.2 ± 13.5	0.0	0.0	0.0	0.0	0.0	16.8	23.0
GLCA-S ($^{77}/_{80}$)	580.6 ± 637.6	24.3	38.8	160.8	353.9	748.9	1299.1	1709.5
TLCA-S ($^{78}/_{80}$)	234.3 ± 232.5	12.7	31.6	69.6	158.0	324.9	538.7	598.1
Total ($^{80}/_{80}$)	2344.1 ± 1472.4	697.8	801.9	1193.1	1867.2	3269.6	4732.6	5338.0

Table 7. Urinary bile acids concentrations [nmol/L] in healthy children (DONALD study, N=80).
The table lists mean ± standard deviation as well as 7 different percentiles.
In brackets after each analyte: number of quantifiable samples (samples > LOQ).

To visualize the age pattern, the scatterplots in Figure 8 show urinary bile acids concentrations plotted against the children's age. Furthermore, a distinction is made between male (blue dots) and female (pink dots) probands. In this way, potential age and sex relations of bile acids concentration may be seen in the scatterplots.

Figure 8 (1/3) Scatterplots of urinary bile acids concentrations [nmol/L] in healthy children. Bile acid concentration is plotted against the children's age. CDCA, GCDCA, TDCA, LCA, GLCA, TLCA and UDCA were left out, since each of these analytes delivered less than 4 results beyond the limit of quantification. Pink dots: female; blue dots: male, black line: linear regression trendline.

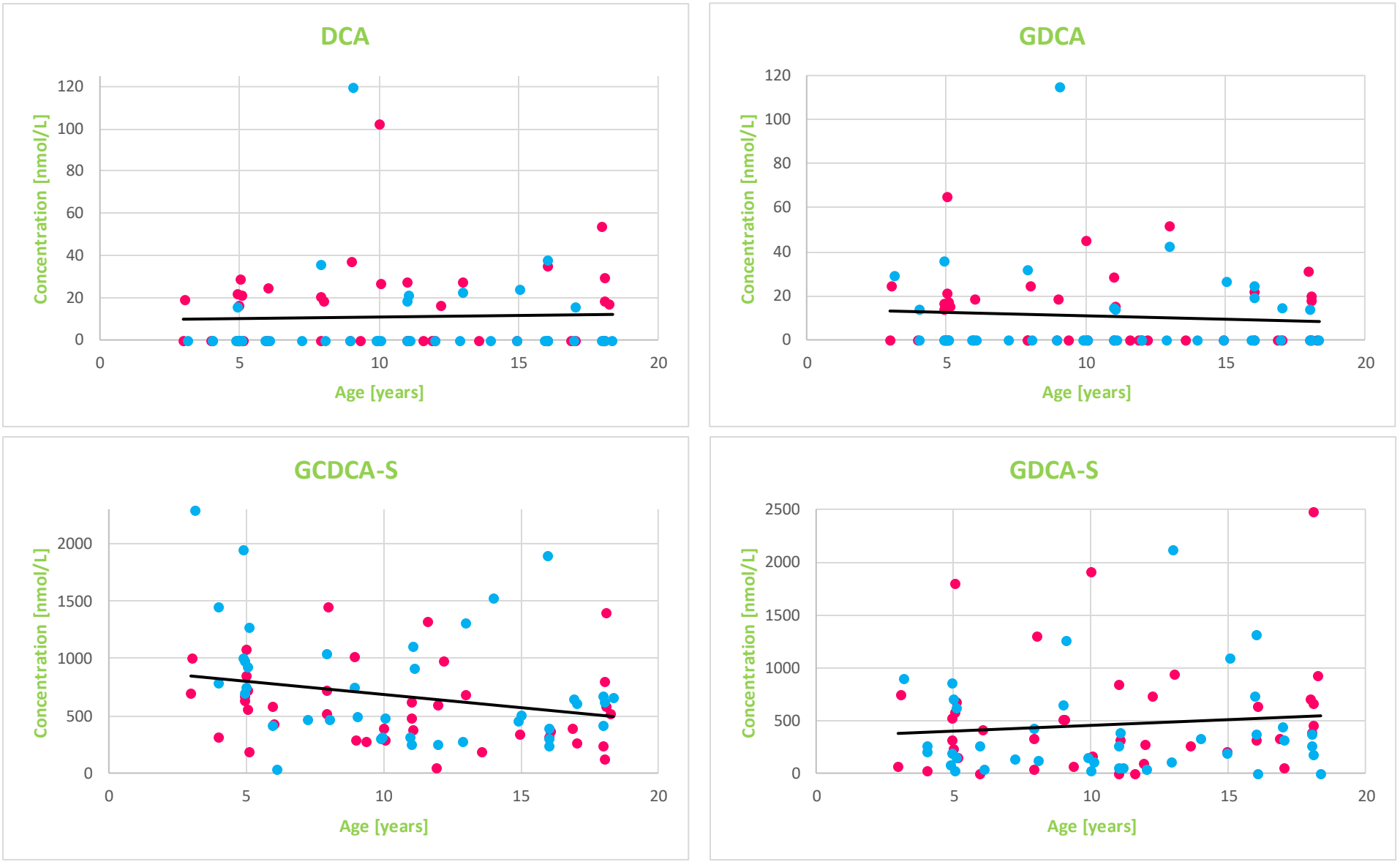

DCA
Concentration [nmol/L]
Age [years]
GDCA
Concentration [nmol/L]
Age [years]
GCDCA-S
Concentration [nmol/L]
Age [years]
GDCA-S
Concentration [nmol/L]
Age [years]

Figure 8 (2/3)


LCA-S
Concentration [nmol/L]
Age [years]
GLCA-S
Concentration [nmol/L]
Age [years]
TLCA-S
Concentration [nmol/L]
Age [years]
Total 18 Bile Acids
Concentration [nmol/L]
Age [years]

Figure 8 (3/3)

The scatterplots in Figure 8 show no aligned age trend throughout the different analytes. Looking at the linear regression trendlines, some analytes seem to slightly increase or decrease with age, while others' concentrations are virtually age-consistent. Table 8 shows the results of linear regression analysis for urinary bile acids concentrations by age and sex. Significance sex differences appeared in DCA with higher concentrations in female probands. A significant correlation between bile acids concentrations and age was detected for GCA, which correlated negatively with age.

These significant correlations agreed with the pictures presented by the scatterplots of DCA and GCA in Figure 8.

	p-value	
	Age	**Sex**
CA	0.611	0.930
GCA	**0.048**	0.414
TCA	0.186	0.711
TCDCA	0.137	0.472
DCA	0.729	**0.022**
GDCA	0.550	0.276
GCDCA-S	0.050	0.179
GDCA-S	0.997	0.741
LCA-S	0.738	0.508
GLCA-S	0.601	0.658
TLCA-S	0.351	0.345
Total	0.455	0.897

Table 8. Linear regression analysis: Urinary bile acids concentrations [nmol/L] in healthy children by age and sex.
Significant results appeared for GCA regarding age as well as for DCA regarding sex.

Concentrations of the six most abundant bile acids (CA, GCA, GCDCA-S, GDCA-S, GLCA-S and TLCA-S) are also shown as percentile curves in Figure 17 for visualization of age-dependent reference values.

Creatinine-related Bile Acids Concentrations [nmol BA/mmol Crea]

Table 9 shows the reference values in healthy children for urinary bile acids concentration per urinary creatinine concentration [nmol BA/mmol Crea].

The six most abundant bile acids were GCDCA-S (median: 73.8 nmol BA/mmol Crea), followed by GLCA-S (52.1 nmol BA/mmol Crea), GDCA-S (45.9 nmol BA/mmol Crea), TLCA-S (24.2 nmol BA/mmol Crea), CA (21.1. nmol BA/mmol Crea) and GCA (14.5 nmol BA/mmol Crea).

	Mean ± SD	**.05**	**.10**	**.25**	**.50**	**.75**	**.90**	**.95**
CA ($^{77}/_{80}$)	32.6 ± 35.6	2.5	3.5	6.3	21.1	44.9	87.2	116.3
GCA ($^{79}/_{80}$)	21.0 ± 18.7	5.0	6.1	9.7	14.5	26.7	40.9	54.5
TCA ($^{40}/_{80}$)	1.4 ± 1.8	0.0	0.0	0.0	0.5	2.2	3.7	4.4
CDCA ($^{3}/_{80}$)	0.1 ± 0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GCDCA ($^{0}/_{80}$)	0.0 ± 0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
TCDCA ($^{19}/_{80}$)	0.6 ± 1.3	0.0	0.0	0.0	0.0	0.0	2.7	3.5
DCA ($^{28}/_{80}$)	1.4 ± 2.8	0.0	0.0	0.0	0.0	2.4	4.0	5.3
GDCA ($^{31}/_{80}$)	1.5 ± 2.6	0.0	0.0	0.0	0.0	2.3	4.6	5.0
TDCA ($^{0}/_{80}$)	0.0 ± 0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
LCA ($^{0}/_{80}$)	0.0 ± 0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GLCA ($^{0}/_{80}$)	0.0 ± 0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
TLCA ($^{0}/_{80}$)	0.0 ± 0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
UDCA ($^{1}/_{80}$)	0.03 ± 0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GCDCA-S ($^{80}/_{80}$)	106.7 ± 85.0	25.3	30.5	49.2	73.8	152.9	192.4	230.3
GDCA-S ($^{75}/_{80}$)	66.2 ± 70.9	0.0	5.8	19.4	45.9	83.6	165.1	173.1
LCA-S ($^{12}/_{80}$)	0.5 ± 1.8	0.0	0.0	0.0	0.0	0.0	1.8	2.8
GLCA-S ($^{77}/_{80}$)	81.7 ± 80.9	4.2	9.4	23.6	52.1	115.0	214.2	249.6
TLCA-S ($^{78}/_{80}$)	32.7 ± 29.2	3.1	5.3	10.5	24.2	50.8	78.0	91.9
Total ($^{80}/_{80}$)	346.6 ± 217.3	107.5	130.3	189.0	262.5	455.5	689.1	724.7

Table 9. Creatinine-related urinary bile acids concentrations [nmol BA/mmol Crea] in healthy children (DONALD study, N=80). The table lists mean ± standard deviation as well as 7 different percentiles. In brackets after each analyte: number of quantifiable samples (samples > LOQ).

Figure 9 (1/3) Scatterplots of creatinine-related urinary bile acids concentrations [nmol BA/mmol Crea] in healthy children.
Bile acid concentration per creatinine concentration is plotted against the children's age. CDCA, GCDCA, TDCA, LCA, GLCA, TLCA and UDCA were left out, since each of these analytes delivered less than 4 results beyond the limit of quantification.
Pink dots: female; blue dots: male, black line: linear regression trendline.


DCA
Creatinine-related Concentration [nmol BA/mmol Crea]
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
5
10
15
20
Age [years]
GDCA
Creatinine-related Concentration [nmol BA/mmol Crea]
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
5
10
15
20
Age [years]
GCDCA-S
Creatinine-related Concentration [nmol BA/mmol Crea]
450.0
400.0
350.0
300.0
250.0
200.0
150.0
100.0
50.0
0.0
0
5
10
15
20
Age [years]
GDCA-S
Creatinine-related Concentration [nmol BA/mmol Crea]
350.0
300.0
250.0
200.0
150.0
100.0
50.0
0.0
0
5
10
15
20
Age [years]

Figure 9 (2/3)


LCA-S
Creatinine-related Concentration [nmol BA/mmol Crea]
Age [years]
GLCA-S
Creatinine-related Concentration [nmol BA/mmol Crea]
Age [years]
TLCA-S
Creatinine-related Concentration [nmol BA/mmol Crea]
Age [years]
Total 18 Bile Acids
Creatinine-related Concentration [nmol BA/mmol Crea]
Age [years]

Figure 9 (3/3)

For visualization of potential age and sex associations, Figure 9 depicts scatterplots, plotting the creatinine-related urinary bile acids concentration against the probands' age and discriminating between male (blue dots) and female (pink dots) probands. Overall, creatinine-related urinary bile acids concentrations seem to decrease with age in case of most analytes. The results of the respective linear regression analysis are listed in Table 10.
Equivalent to the concentration in nmol/L, the creatinine-related bile acid concentration also showed significantly higher results for DCA in females. Compared to concentration in nmol/L, the creatinine-relation led to a higher number of significantly age-correlated analytes, with five individual analytes as well as the total sum of the 18 analytes correlating negatively with age. These statistical findings coincided with the respective scatterplots. Urinary creatinine concentration showed a significant positive correlation with age.

	p-value	
	Age	**Sex**
CA	**0.039**	0.958
GCA	**<0.001**	0.554
TCA	**0.033**	0.848
TCDCA	0.085	0.410
DCA	0.895	**0.010**
GDCA	0.309	0.160
GCDCA-S	**<0.001**	0.318
GDCA-S	0.431	0.534
LCA-S	0.714	0.485
GLCA-S	0.144	0.605
TLCA-S	**0.027**	0.294
Total	**<0.001**	0.501
Creatinine	**<0.001**	0.467

Table 10. Linear regression analysis: Creatinine-related urinary bile acids concentrations [nmol BA/mmol Crea] in healthy children by age and sex. Significant results appeared for DCA regarding sex as well as for CA, GCA, TCA, GCDCA-S, TLCA-S and the sum of the 18 bile acids (Total) regarding age. Creatinine also correlated with age.

The creatinine-related concentrations of the six most abundant bile acids (CA, GCA, GCDCA-S, GDCA-S, GLCA-S and TLCA-S) are also shown as percentile curves in Figure 18 for visualization of age-dependent reference values.

Daily urinary Bile Acids Excretions [nmol/d]

Table 11 shows the reference values in healthy children for daily urinary bile acids excretions [nmol/d]. The median excretion rates of the six most abundant bile acids amounted to 437.5 nmol/d (GCDCA-S), 327.7 nmol/d (GLCA-S), 222.9 nmol/d (GDCA-S), 129.6 nmol/d (TLCA-S), 95.4 nmol/d (CA) or 88.9 nmol/d (GCA), respectively.

	Mean ± SD	**.05**	**.10**	**.25**	**.50**	**.75**	**.90**	**.95**
CA ($^{77}/_{80}$)	173.3 ± 213.5	16.2	25.6	37.8	95.4	199.0	403.6	529.0
GCA ($^{79}/_{80}$)	102.8 ± 71.4	33.7	39.8	55.5	88.9	123.1	178.8	238.5
TCA ($^{40}/_{80}$)	6.5 ± 7.9	0.0	0.0	0.0	2.8	11.6	17.1	23.5
CDCA ($^{3}/_{80}$)	0.7 ± 3.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GCDCA ($^{0}/_{80}$)	0.0 ± 0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
TCDCA ($^{19}/_{80}$)	2.7 ± 5.9	0.0	0.0	0.0	0.0	0.0	10.0	18.3
DCA ($^{28}/_{80}$)	7.6 ± 14.6	0.0	0.0	0.0	0.0	9.7	23.3	40.2
GDCA ($^{31}/_{80}$)	7.1 ± 11.8	0.0	0.0	0.0	0.0	10.5	25.9	31.6
TDCA ($^{0}/_{80}$)	0.0 ± 0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
LCA ($^{0}/_{80}$)	0.0 ± 0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GLCA ($^{0}/_{80}$)	0.0 ± 0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
TLCA ($^{0}/_{80}$)	0.0 ± 0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
UDCA ($^{1}/_{80}$)	0.1 ± 0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GCDCA-S ($^{80}/_{80}$)	548.5 ± 378.6	171.8	202.8	280.9	437.5	693.9	1036.9	1159.5
GDCA-S ($^{75}/_{80}$)	385.9 ± 492.4	0.0	28.9	90.5	222.9	498.0	834.8	1058.6
LCA-S ($^{12}/_{80}$)	2.8 ± 8.8	0.0	0.0	0.0	0.0	0.0	9.9	14.6
GLCA-S ($^{77}/_{80}$)	440.2 ± 429.5	18.5	29.4	131.2	327.7	611.6	927.9	1269.0
TLCA-S ($^{78}/_{80}$)	181.7 ± 190.0	14.9	25.4	58.4	129.6	243.7	338.2	445.0
Total ($^{80}/_{80}$)	1859.9 ± 1143.4	659.9	759.7	1033.3	1666.0	2395.7	3328.8	4245.9

Table 11. Daily urinary bile acids excretions [nmol/d] in healthy children (DONALD study, N=80). The table lists mean ± standard deviation as well as 7 different percentiles. In brackets after each analyte: number of quantifiable samples (samples > LOQ).

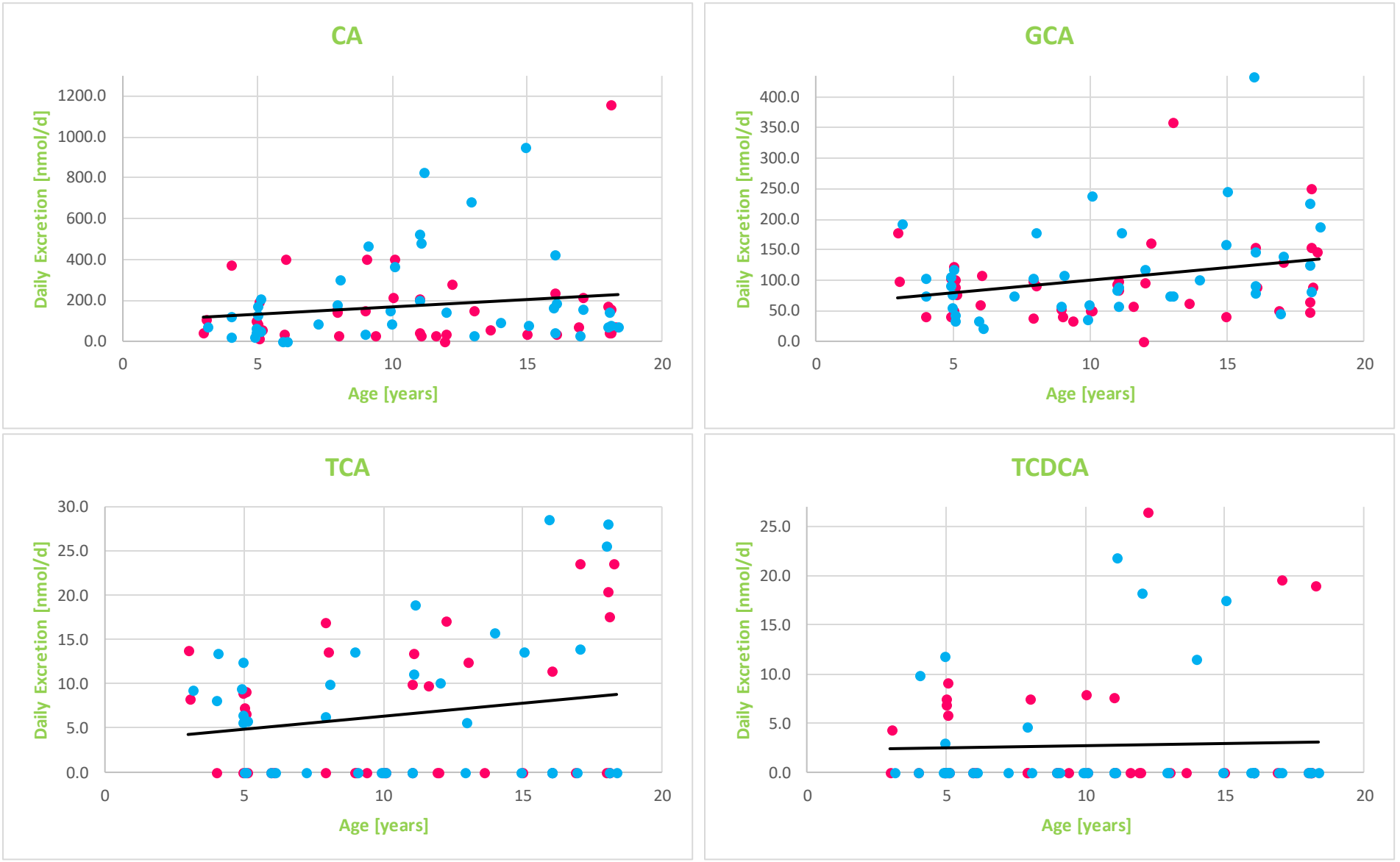


Figure 10 (1/3) Scatterplots of daily urinary bile acids excretions [nmol/d] in healthy children. Daily bile acid excretion is plotted against the children's age. CDCA, GCDCA, TDCA, LCA, GLCA, TLCA and UDCA were left out, since each of these analytes delivered less than 4 results beyond the limit of quantification. Pink dots: female; blue dots: male, black line: linear regression trendline.

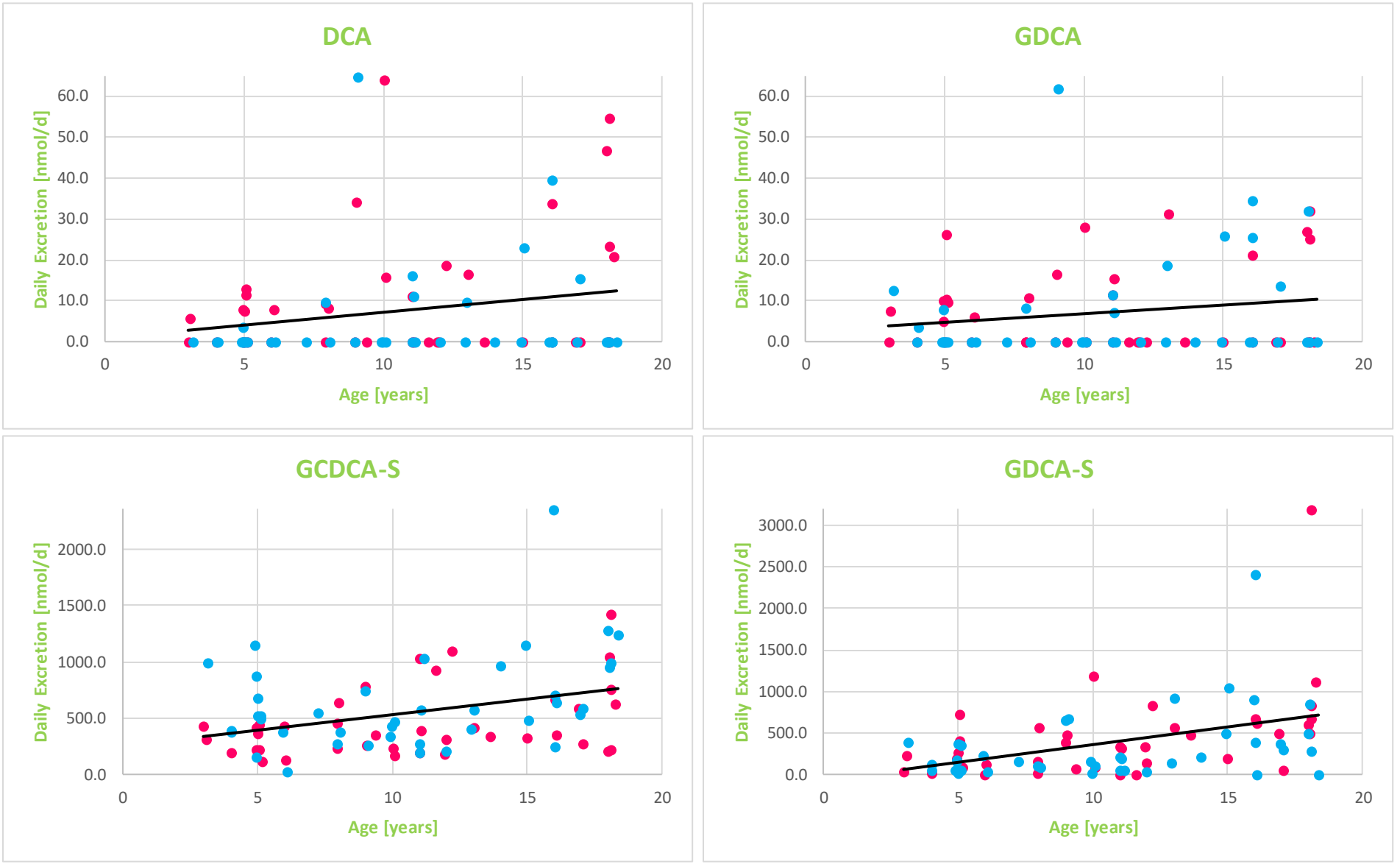

DCA
Daily Excretion [nmol/d]
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0
5
10
15
20
Age [years]
GDCA
Daily Excretion [nmol/d]
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0
5
10
15
20
Age [years]
GCDCA-S
Daily Excretion [nmol/d]
2000.0
1500.0
1000.0
500.0
0.0
0
5
10
15
20
Age [years]
GDCA-S
Daily Excretion [nmol/d]
3000.0
2500.0
2000.0
1500.0
1000.0
500.0
0.0
0
5
10
15
20
Age [years]

Figure 10 (2/3)


LCA-S
Daily Excretion [nmol/d]
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0
5
10
15
20
Age [years]
GLCA-S
Daily Excretion [nmol/d]
2000.0
1500.0
1000.0
500.0
0.0
0
5
10
15
20
Age [years]
TLCA-S
Daily Excretion [nmol/d]
1200.0
1000.0
800.0
600.0
400.0
200.0
0.0
0
5
10
15
20
Age [years]
Total 18 Bile Acids
Daily Excretion [nmol/d]
6000.0
5000.0
4000.0
3000.0
2000.0
1000.0
0.0
0
5
10
15
20
Age [years]

Figure 10 (3/3)

Figure 10 shows scatterplots to demonstrate potential age and sex correlations. The daily urinary bile acids excretions were plotted against the probands' age. Male (blue dots) and female (pink dots) probands can be differentiated by color. In general, the scatterplots and the respective linear regression lines show the tendency of increased daily bile acids excretions with age. The results of the linear regression analysis (Table 12) show a significant age trend for GCDCA-S and the sum of the 18 bile acids (Total). Compared to the two previous units, daily urinary bile acids excretion likewise showed significantly higher levels of DCA in females. Additionally, significantly higher amounts of GCDCA-S in males were observed. 24-Hour urine volume also showed a significant positive age trend.

	p-value	
	Age	**Sex**
CA	0.140	0.687
GCA	0.159	0.158
TCA	0.567	0.719
TCDCA	0.282	0.471
DCA	0.382	**0.019**
GDCA	0.945	0.285
GCDCA-S	**0.002**	**0.044**
GDCA-S	0.143	0.795
LCA-S	0.907	0.514
GLCA-S	0.244	0.744
TLCA-S	0.234	0.390
Total	**<0.001**	0.603
Urine Volume	**<0.001**	0.513

Table 12. Linear regression analysis: Urinary bile acids daily excretions [nmol/d] in healthy children by age and sex. Significant results appeared for GCDCA-S and the sum of the 18 bile acids (Total) regarding age as well as for DCA and GCDCA-S regarding sex. 24-Hour urine volume also correlated with age.

The six most abundant bile acids' (CA, GCA, GCDCA-S, GDCA-S, GLCA-S and TLCA-S) daily excretion rates are also shown as percentile curves in Figure 19 to illustrate age-dependent reference values.

Percentage Amidation and Sulfation of urinary Bile Acids in healthy Children

Several of the 18 included analytes were derivatives of the same underlying class of bile acids – differing in state of amidation or sulfation, respectively.

In the following, the proportions of the different amidation and sulfation states within one class of bile acids were investigated.

In case of amidation, this was done for the bile acid classes that were measured in all three amidation states (unamidated, glycine-amidated, taurine-amidated) - which applies for cholic acid (unamidated: CA; glycine-amidated: GCA, taurine-amidated: TCA), chenodeoxycholic acid (CDCA; GCDCA; TCDCA), deoxycholic acid (DCA; GDCA; TDCA), lithocholic acid (LCA; GLCA; TLCA) as well as for lithocholic acid sulfate (LCA-S; GLCA-S; TLCA-S).

Sulfation percentages were likewise calculated for bile acids that were measured in their unsulfated form as well as in their sulfated form. This includes GCDCA and GCDCA-S, GDCA and GDCA-S along with all three forms of lithocholic acid (LCA, GLCA, TLCA and LCA-S, GLCA-S, TLCA-S).

The respective amidation and sulfation percentages were calculated for each sample individually using the data in nmol/d. Subsequently, the mean ± SD and percentiles (0.05, 0.10, 0.25, 0.50, 0.75, 0.90, 0.95) were formed. The results for healthy children are listed in Tables 13 and 14.

In cholic acid and deoxycholic acid, unamidated and glycine-amidated forms prevailed to almost equal shares, while taurine-amidation played a minor role (TCA) or was non-existent (TDCA). In chenodeoxycholic acid, on the other hand, taurine-amidation accounted for the largest share, followed by unamidated forms. Glycine-amidated forms did not appear. Unsulfated lithocholic acid derivatives appeared in a non-quantifiable amount – regardless of their amidation state (LCA, GLCA and TLCA were below the LOQ in all samples). Eventually, lithocholic acid sulfate was dominated by amidated forms, mainly glycine-amidation and to a lower extent taurine-amidation, while unamidated LCA-S was rarely found (Table 13).

Regarding percentage sulfation, urinary bile acids excretion was vastly led by sulfated forms. In healthy children, GCDCA, LCA, GLCA and TLCA were only found as sulfates (100%), while GDCA appeared in sulfated form to a mean percentage of 98.4% (Table 14).

Amidation	Mean ± SD	.05	.10	.25	.50	.75	.90	.95
	[%]							
Cholic Acid (n=79)								
Unamidated (CA)	50.0 ± 23.9	16.6	22.0	29.0	49.7	69.6	83.2	88.3
Glycine (GCA)	47.0 ± 22.4	11.7	15.4	28.8	46.4	66.9	72.1	76.1
Taurine (TCA)	3.0 ± 4.0	0.0	0.0	0.0	1.8	4.8	8.1	10.5
Chenodeoxycholic Acid (n=22)								
Unamidated (CDCA)	13.6 ± 35.1	0.0	0.0	0.0	0.0	0.0	90.0	100.0
Glycine (GCDCA)	0.0 ± 0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Taurine (TCDCA)	86.4 ± 35.1	0.0	10.0	100.0	100.0	100.0	100.0	100.0
Deoxycholic Acid (n=36)								
Unamidated (DCA)	46.8 ± 31.7	0.0	0.0	30.9	50.3	61.9	100.0	100.0
Glycine (GDCA)	53.2 ± 31.7	0.0	0.0	38.1	49.7	69.1	100.0	100.0
Taurine (TDCA)	0.0 ± 0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Lithocholic Acid (n=0)								
Unamidated (LCA)	NA							
Glycine (GLCA)	NA							
Taurine (TLCA)	NA							
Lithocholic Acid Sulfate (n=78)								
Unamidated (LCA-S)	0.3 ± 1.1	0.0	0.0	0.0	0.0	0.0	1.3	1.8
Glycine (GLCA-S)	67.3 ± 12.7	46.1	50.7	62.6	69.3	75.6	79.7	82.6
Taurine (TLCA-S)	32.4 ± 13.1	16.9	19.1	24.3	30.4	37.3	49.3	53.9

Table 13. Percentage amidation of urinary bile acids in healthy children. For the bile acid classes that were measured in their unamidated as well as their glycine- and taurine-amidated form, percentage shares of each form were calculated for each sample. The mean ± SD and different percentiles were formed. For calculation, the nanomolar daily excretions [nmol/d] were used.
n = number of samples > LOQ; SD = standard deviation.

Sulfation	**Mean ± SD**	**.05**	**.10**	**.25**	**.50**	**.75**	**.90**	**.95**
	[%]							
Glycochenodeoxycholic Acid (n=80)								
Unsulfated (GCDCA)	0.0 ± 0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Sulfated (GCDCA-S)	100 ± 0.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Glycodeoxycholic Acid (n=75)								
Unsulfated (GDCA)	1.6 ± 2.3	0.0	0.0	0.0	0.0	3.3	4.7	6.1
Sulfated (GDCA-S)	98.4 ± 2.3	93.9	95.3	96.7	100.0	100.0	100.0	100.0
Total Lithocholic Acid (n=78)								
Unsulfated (LCA, GLCA, TLCA)	0.0 ± 0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Sulfated (LCA-S, GLCA-S, TLCA-S)	100.0 ± 0.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

Table 14. Percentage sulfation of urinary bile acids in healthy children. For the bile acids that were measured in their unsulfated as well as their sulfated form, percentage shares of each form were calculated for each sample. The mean ± SD and different percentiles were formed. For calculation, the nanomolar daily excretions [nmol/d] were used.
n = number of samples > LOQ; SD = standard deviation.

3.4.3 Urinary Bile Acids in obese Children

In addition to the healthy control group, we also analyzed urinary bile acids in samples of 237 obese children from the MULTIOMICS group. The results are also presented in the three units nanomolar concentration [nmol/L], creatinine-related concentration [nmol BA/mmol Crea] and daily excretion [nmol/d].

Urinary Bile Acids Concentrations [nmol/L]

The major share of the measured urinary bile acids in obese children was constituted by the sulfated ones and furthermore CA and GCA as the most abundant unsulfated bile acids. These analytes achieved the highest median concentrations and the fewest results below the limit of quantification. They appeared in the following order, starting with the highest median concentration: GCDCA-S (median: 360 nmol/L) > GLCA-S (345 nmol/L) > GDCA-S (324 nmol/L) > CA (175 nmol/L) > GCA (92 nmol/L) > TLCA-S (80 nmol/L).

In contrast to the other sulfated bile acids, LCA-S showed rather low urinary concentration in obese children.

All results for LCA, GLCA and TLCA in obese children's urine lay below the limit of quantification. GCDCA (90% of samples < LOQ) and TDCA (99% of samples < LOQ) were also not quantifiable in most MULTIOMICS samples.

Table 15 shows the detailed results for urinary bile acids concentrations [nmol/L] in obese children.

	Mean ± SD	.05	.10	.25	.50	.75	.90	.95
CA ($^{225}/_{237}$)	367.0 ± 588.7	12.9	29.7	70.6	174.6	420.4	810.5	1292.6
GCA ($^{233}/_{237}$)	135.7 ± 201.7	21.1	26.3	50.3	92.4	151.3	237.0	335.4
TCA ($^{59}/_{237}$)	5.8 ± 14.4	0.0	0.0	0.0	0.0	0.0	17.5	23.0
CDCA ($^{73}/_{237}$)	8.4 ± 15.0	0.0	0.0	0.0	0.0	17.6	27.0	37.5
GCDCA ($^{23}/_{237}$)	7.2 ± 26.8	0.0	0.0	0.0	0.0	0.0	0.0	55.6
TCDCA ($^{43}/_{237}$)	4.9 ± 15.1	0.0	0.0	0.0	0.0	0.0	17.6	26.7
DCA ($^{153}/_{237}$)	29.7 ± 34.7	0.0	0.0	0.0	21.7	41.3	72.2	107.5
GDCA ($^{151}/_{237}$)	21.7 ± 43.6	0.0	0.0	0.0	16.0	27.9	42.7	56.4
TDCA ($^{3}/_{237}$)	0.2 ± 1.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0
LCA ($^{0}/_{237}$)	0.0 ± 0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GLCA ($^{0}/_{237}$)	0.0 ± 0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
TLCA ($^{0}/_{237}$)	0.0 ± 0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
UDCA ($^{58}/_{237}$)	11.0 ± 31.0	0.0	0.0	0.0	0.0	0.0	32.9	48.0
GCDCA-S ($^{237}/_{237}$)	483.1 ± 379.5	87.6	117.3	193.1	360.2	661.0	1090.7	1297.8
GDCA-S ($^{229}/_{237}$)	484.8 ± 480.4	25.4	83.6	163.4	323.9	647.9	1047.4	1618.4
LCA-S ($^{34}/_{237}$)	3.0 ± 8.0	0.0	0.0	0.0	0.0	0.0	16.7	22.3
GLCA-S ($^{231}/_{237}$)	429.8 ± 384.8	24.7	67.8	149.7	344.8	580.7	928.6	1225.9
TLCA-S ($^{226}/_{237}$)	127.7 ± 142.6	10.8	18.9	37.3	79.8	169.3	279.8	388.5
Total ($^{237}/_{237}$)	2120.0 ± 1415.0	493.3	705.2	1119.3	1771.6	2895.7	3917.6	4663.7

Table 15. Urinary bile acids concentrations in obese children [nmol/L] (MULTIOMICS study, N=237). The table lists mean ± standard deviation as well as 7 different percentiles. In brackets after each analyte: number of quantifiable samples (samples > LOQ).

Figure 11 (1/4). Scatterplots of urinary bile acids concentrations [nmol/L] in obese children. Bile acid concentration is plotted against the children's age. TDCA, LCA, GLCA and TLCA were left out, since each of these analytes delivered less than 4 results beyond the limit of quantification. Pink dots: female; blue dots: male, black line: linear regression trendline.

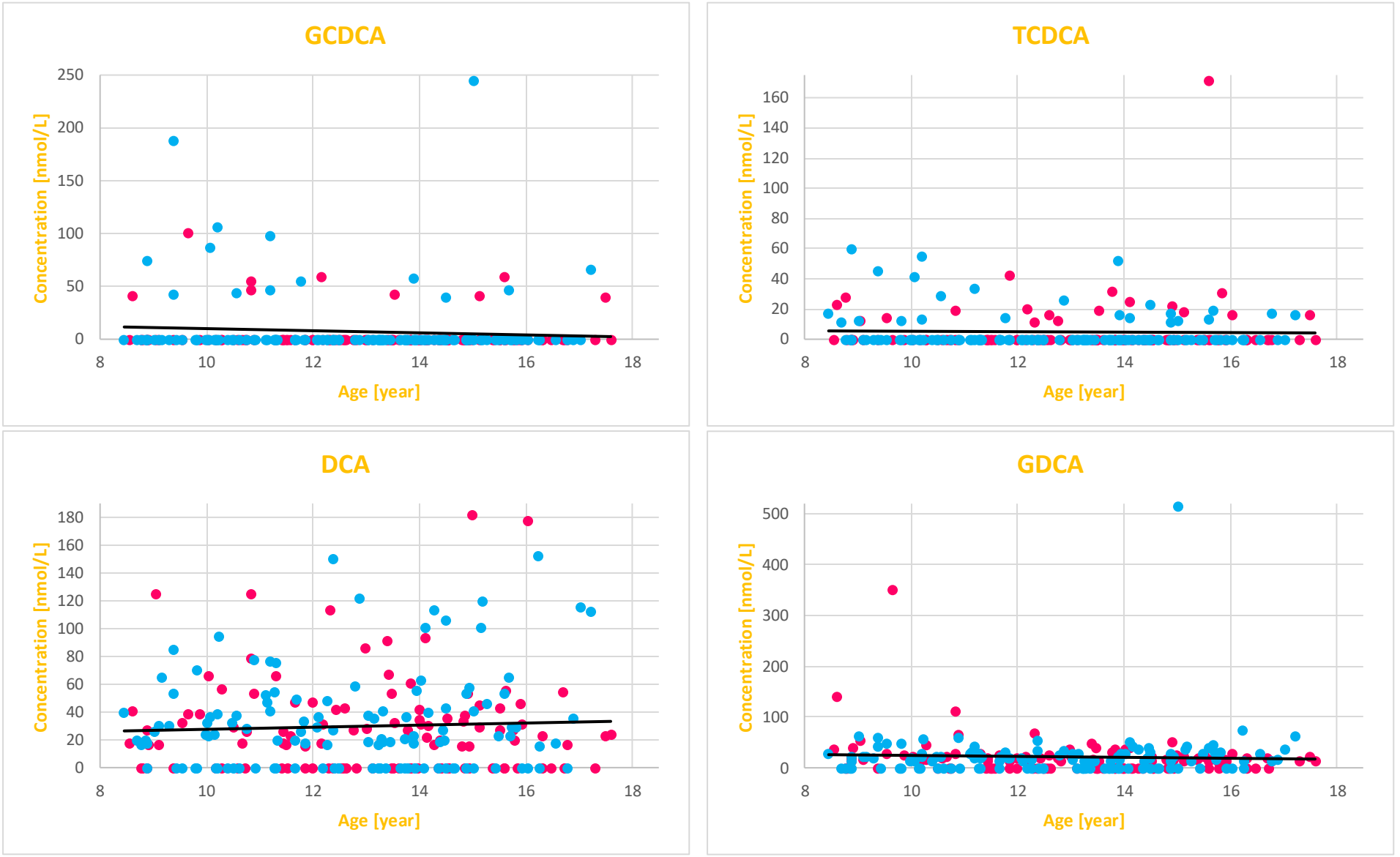

GCDCA
Concentration [nmol/L]
Age [year]
TCDCA
Concentration [nmol/L]
Age [year]
DCA
Concentration [nmol/L]
Age [year]
GDCA
Concentration [nmol/L]
Age [year]

Figure 11 (2/4)

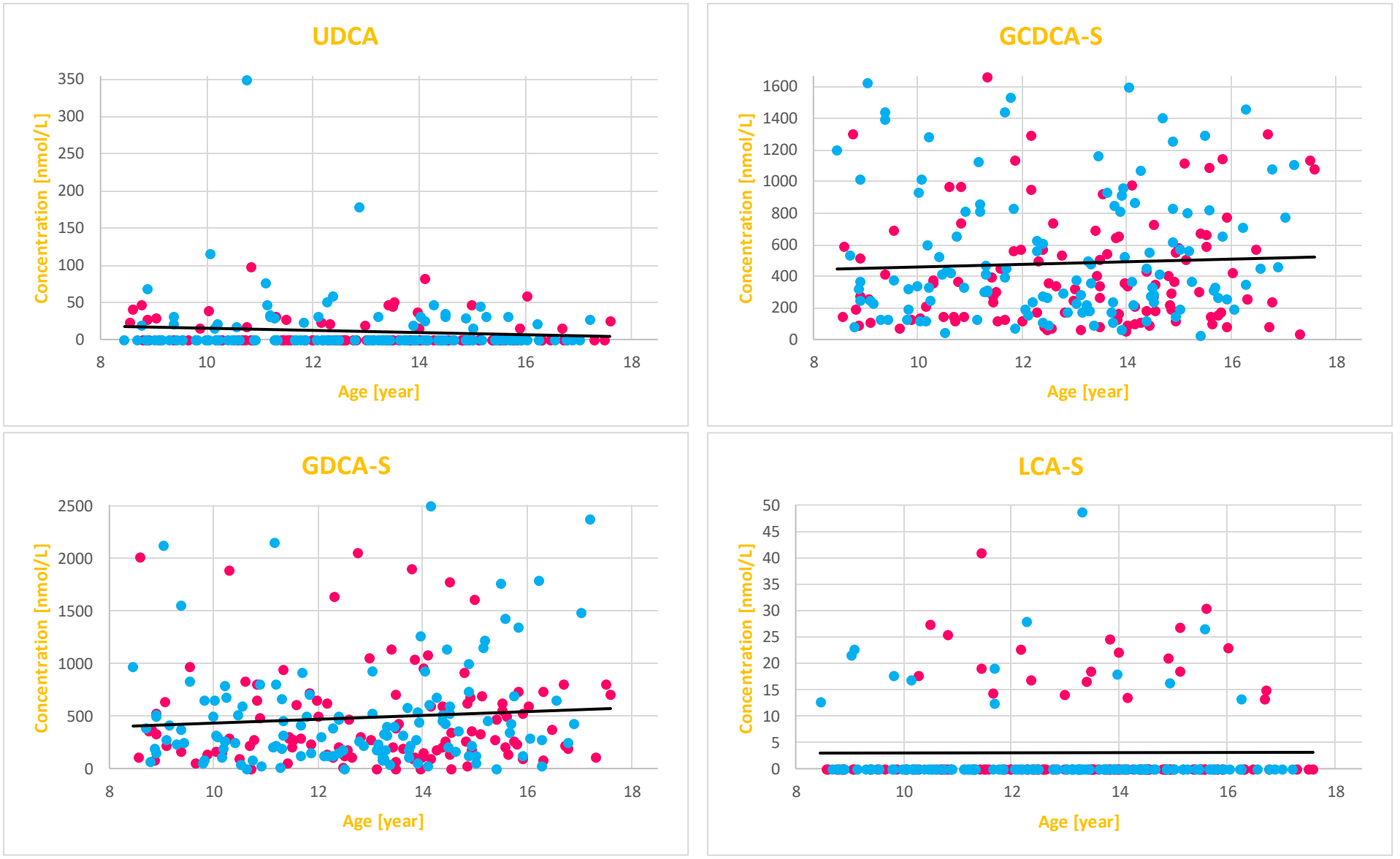
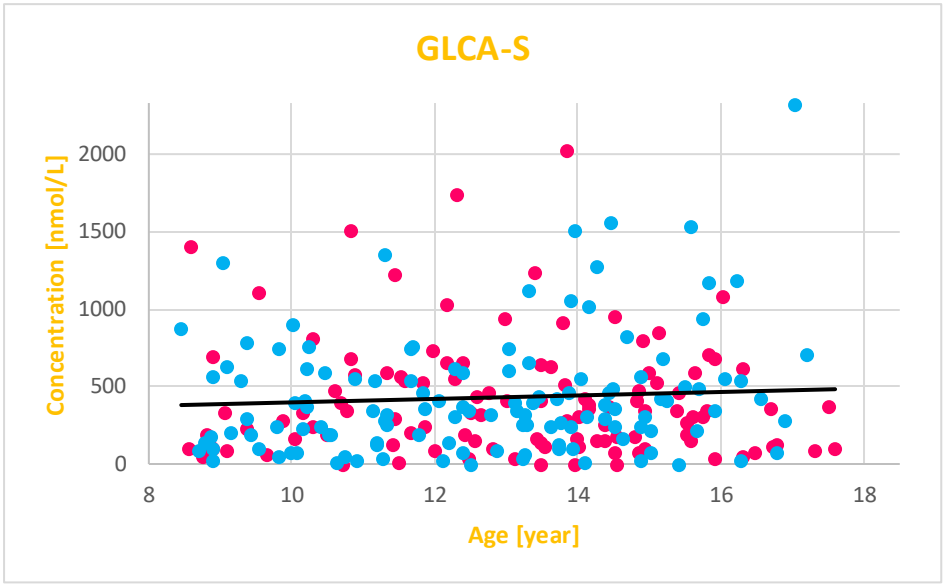
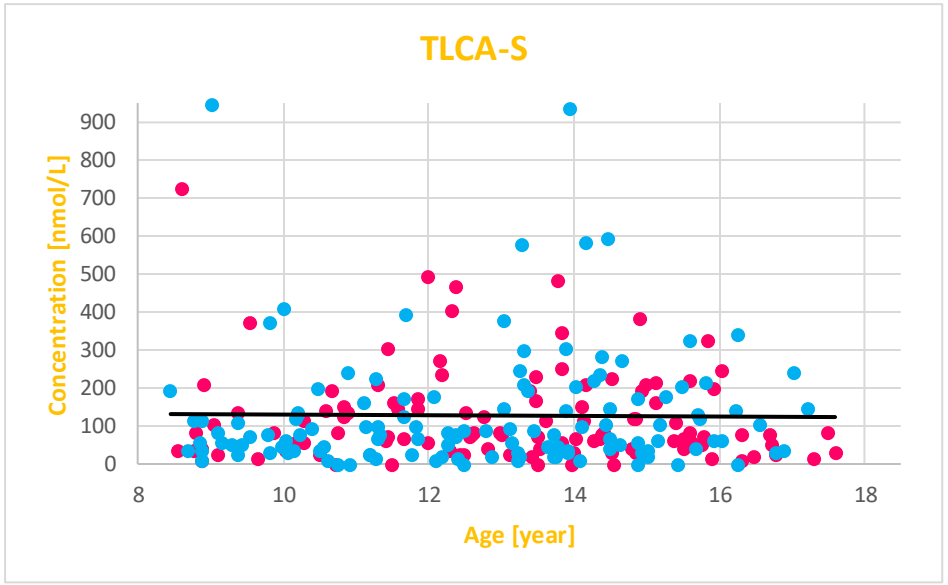

UDCA
Concentration [nmol/L]
Age [year]
GCDCA-S
Concentration [nmol/L]
Age [year]
GDCA-S
Concentration [nmol/L]
Age [year]
LCA-S
Concentration [nmol/L]
Age [year]

Figure 11 (3/4)


GLCA-S
Concentration [nmol/L]
2000
1500
1000
500
0
8
10
12
14
16
18
Age [year]


TLCA-S
Concentration [nmol/L]
900
800
700
600
500
400
300
200
100
0
8
10
12
14
16
18
Age [year]

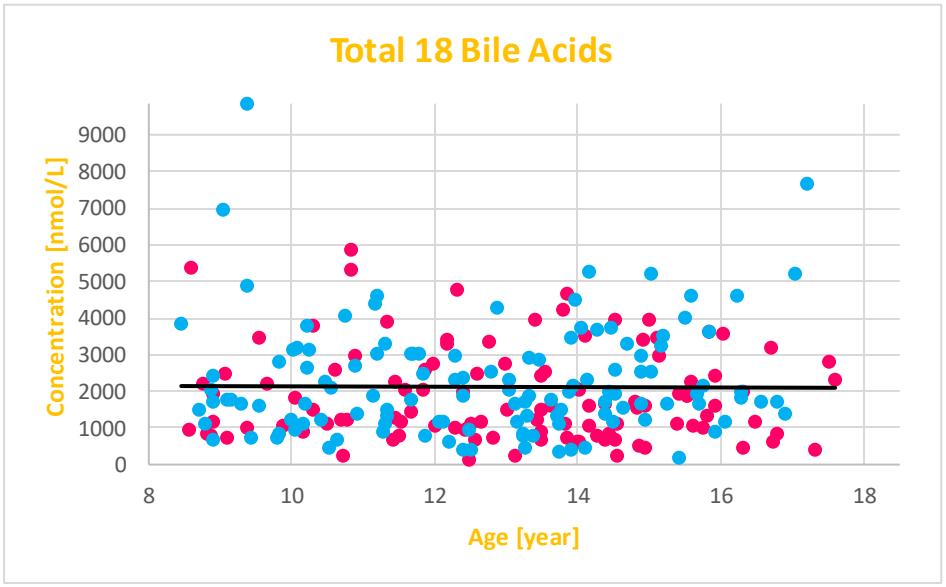

Total 18 Bile Acids
Concentration [nmol/L]
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
8
10
12
14
16
18
Age [year]

Figure 11 (4/4)

Figure 11 shows the obese children's urinary bile acids concentration with respect to age and sex in form of scatterplots. Table 16 delivers the respective test results to investigate potential significant age or sex differences. These test results show a significant negative correlation between GCA concentration in obese children and age. Sex differences, on the other hand, were significant for CA, GCDCA-S and the total sum of the 18 bile acids (Total) – each of which showing higher concentrations in male probands.

	p-value	
	Age	**Sex**
CA	0.308	**0.031**
GCA	**0.022**	0.510
TCA	0.808	0.676
CDCA	0.838	0.199
GCDCA	0.213	0.415
TCDCA	0.428	0.745
DCA	0.640	0.360
GDCA	0.901	0.615
UDCA	0.122	0.499
GCDCA-S	0.265	**0.019**
GDCA-S	0.510	0.440
LCA-S	0.941	0.067
GLCA-S	0.715	0.466
TLCA-S	0.862	0.830
Total	0.898	**0.031**

Table 16. Linear regression analysis: Urinary bile acids concentrations [nmol/L] in obese children by age and sex. 237 observations. Significant results regarding sex differences appeared for CA, GCDCA-S as well as for the total sum of the 18 bile acids, while a significant age correlation was shown for GCA.

Creatinine-related Bile Acids Concentrations [nmol BA/mmol Crea]

Table 17 shows the detailed data for creatinine-related urinary bile acids concentration [nmol BA/mmol Crea] in obese children. Due to the lack of creatinine data, 12 samples had to be removed from the study group, resulting in N=225.

The six most abundant bile acids appeared in the following order of median creatinine-related concentration: GCDCA-S (48.5 nmol BA/mmol Crea), GDCA-S (42.5 nmol BA/mmol Crea), GLCA-S (40.7 nmol BA/mmol Crea), CA (25.1 nmol BA/mmol Crea), TLCA-S (11.5 nmol BA/mmol Crea), GCA (11.2 nmol BA/mmol Crea).

	Mean ± SD	.05	.10	.25	.50	.75	.90	.95
CA ($^{214}/_{225}$)	45.5 ± 64.9	1.9	4.0	9.8	25.1	54.0	97.5	154.1
GCA ($^{222}/_{225}$)	16.8 ± 24.7	3.5	4.6	7.7	11.2	18.6	29.8	37.8
TCA ($^{55}/_{225}$)	0.5 ± 1.2	0.0	0.0	0.0	0.0	0.0	1.8	2.5
CDCA ($^{68}/_{225}$)	1.0 ± 1.9	0.0	0.0	0.0	0.0	1.5	3.2	4.6
GCDCA ($^{22}/_{225}$)	0.6 ± 2.3	0.0	0.0	0.0	0.0	0.0	0.0	4.6
TCDCA ($^{43}/_{225}$)	0.4 ± 1.1	0.0	0.0	0.0	0.0	0.0	1.7	2.7
DCA ($^{145}/_{225}$)	3.5 ± 4.0	0.0	0.0	0.0	2.8	5.4	8.8	10.5
GDCA ($^{141}/_{225}$)	2.8 ± 5.4	0.0	0.0	0.0	1.9	3.6	5.8	7.6
TDCA ($^{3}/_{225}$)	0.02 ± 0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
LCA ($^{0}/_{225}$)	0.0 ± 0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GLCA ($^{0}/_{225}$)	0.0 ± 0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
TLCA ($^{0}/_{225}$)	0.0 ± 0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
UDCA ($^{56}/_{225}$)	1.3 ± 4.4	0.0	0.0	0.0	0.0	0.0	3.8	5.1
GCDCA-S ($^{225}/_{225}$)	65.1 ± 140.0	15.2	21.8	29.1	48.5	71.3	107.4	138.7
GDCA-S ($^{217}/_{225}$)	67.7 ± 129.3	5.8	12.3	22.3	42.5	72.4	140.0	189.1
LCA-S ($^{29}/_{225}$)	0.4 ± 1.2	0.0	0.0	0.0	0.0	0.0	1.7	2.6
GLCA-S ($^{219}/_{225}$)	61.1 ± 83.5	3.6	6.4	21.4	40.7	74.9	130.7	166.5
TLCA-S ($^{215}/_{225}$)	17.5 ± 26.2	1.2	2.2	5.1	11.5	20.3	35.1	43.8
Total ($^{225}/_{225}$)	284.2 ± 385.5	93.0	112.5	153.5	221.1	302.4	479.2	538.7

Table 17. Creatinine-related urinary bile acids concentrations in obese children [nmol BA/mmol Crea] (MULTIOMICS study, N=225). The table lists mean ± standard deviation as well as 7 different percentiles. In brackets after each analyte: number of quantifiable samples (samples > LOQ).

Figure 12 (1/4). Scatterplots of creatinine-related urinary bile acids concentrations [nmol BA/mmol Crea] in obese children. Bile acid concentration is plotted against the children's age. TDCA, LCA, GLCA and TLCA were left out, since each of these analytes delivered less than 4 results beyond the limit of quantification. Pink dots: female; blue dots: male, black line: linear regression trendline.


GCDCA
Creatinine-related Concentration [nmol BA/mmol Crea]
Age [year]
TCDCA
Creatinine-related Concentration [nmol BA/mmol Crea]
Age [year]
DCA
Creatinine-related Concentration [nmol BA/mmol Crea]
Age [year]
GDCA
Creatinine-related Concentration [nmol BA/mmol Crea]
Age [year]

Figure 12 (2/4)

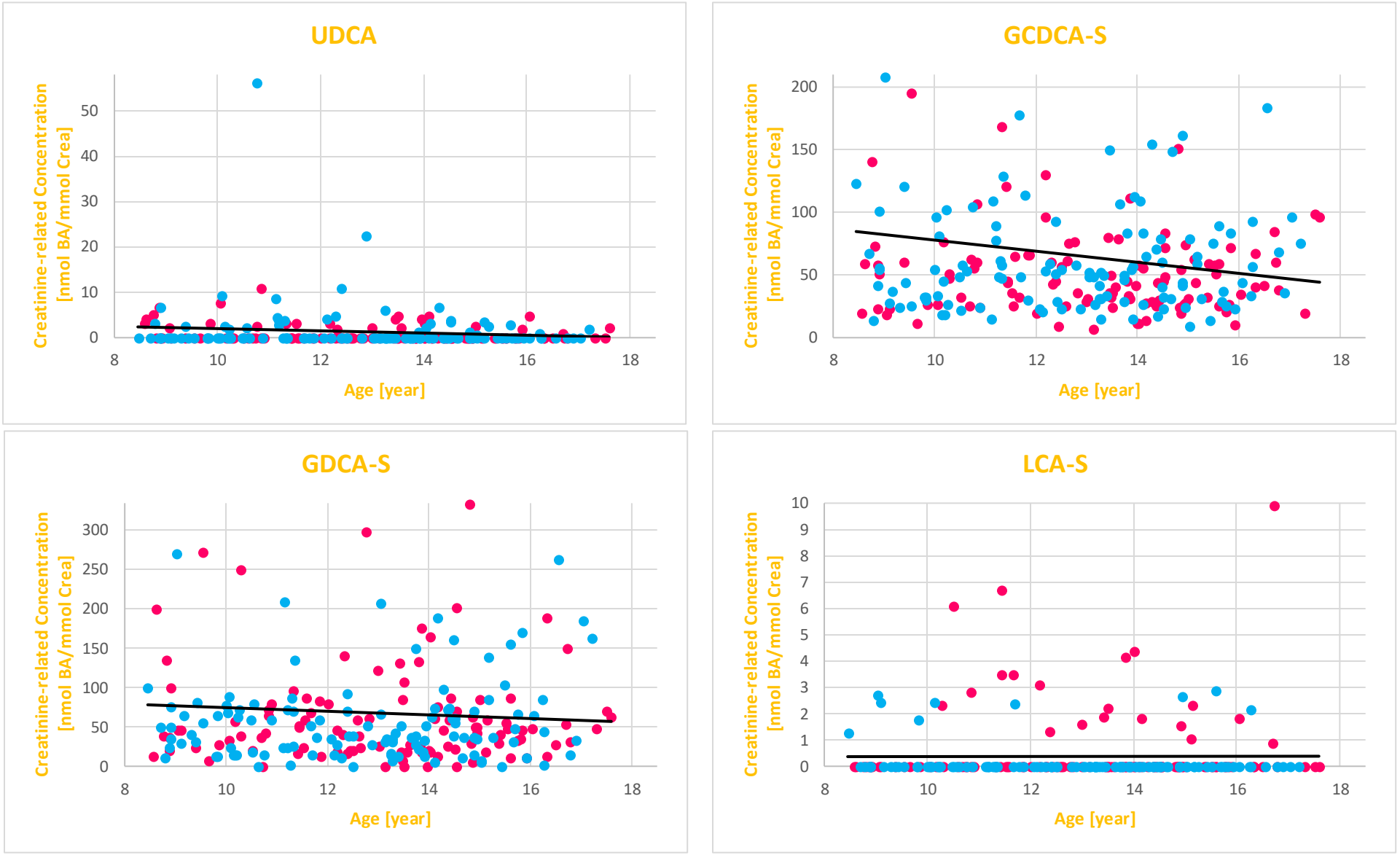
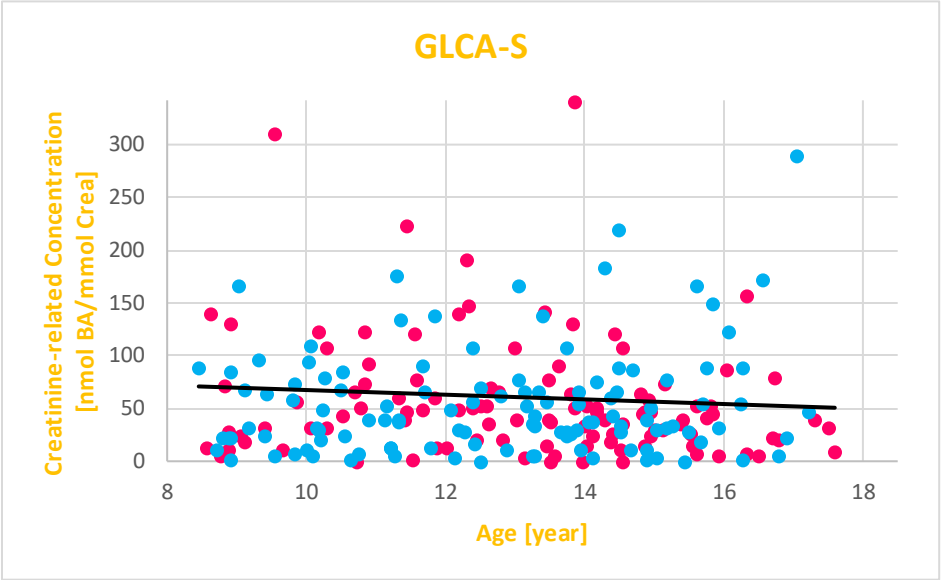
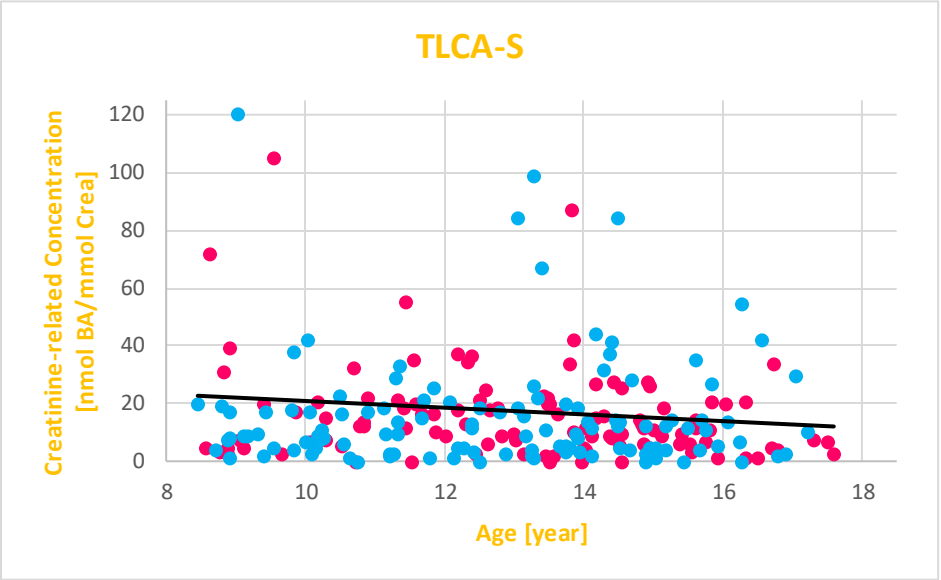

UDCA
Creatinine-related Concentration [nmol BA/mmol Crea]
0
10
20
30
40
50
8
10
12
14
16
18
Age [year]
GCDCA-S
Creatinine-related Concentration [nmol BA/mmol Crea]
0
50
100
150
200
8
10
12
14
16
18
Age [year]
GDCA-S
Creatinine-related Concentration [nmol BA/mmol Crea]
0
50
100
150
200
250
300
8
10
12
14
16
18
Age [year]
LCA-S
Creatinine-related Concentration [nmol BA/mmol Crea]
0
1
2
3
4
5
6
7
8
9
10
8
10
12
14
16
18
Age [year]

Figure 12 (3/4)





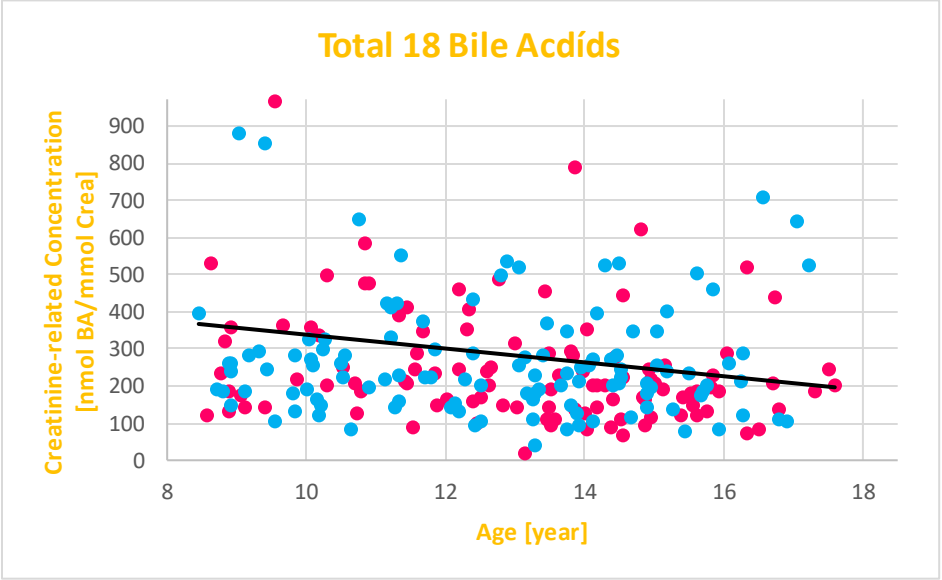


Figure 12 (4/4)

Note to Figure 12: For reasons of clarity and comprehensibility, single outliers were excluded from the plots.

In Figure 12, the creatinine-related urinary bile acids concentration in obese children is plotted against their age. Male and female probands are separated by using blue or pink dots, respectively. The results of linear regression analysis by age and sex are listed in Table 18. LCA-S showed significantly higher results in female probands. A significant negative age correlation was ascertained for CA, GCA, and the sum of the 18 analytes (Total). Urinary creatinine concentration significantly increased with age.

	p-value	
	Age	**Sex**
CA	**0.024**	0.129
GCA	**<0.001**	0.519
TCA	0.367	0.580
CDCA	0.864	0.346
GCDCA	0.199	0.421
TCDCA	0.211	0.601
DCA	0.259	0.531
GDCA	0.345	0.998
UDCA	0.063	0.571
GCDCA-S	0.631	0.294
GDCA-S	0.861	0.888
LCA-S	0.689	**0.012**
GLCA-S	0.516	0.875
TLCA-S	0.202	0.379
Total	**0.017**	0.560
Creatinine	**0.006**	0.098

Table 18. Linear regression analysis: Creatinine-related urinary bile acids concentrations [nmol BA/mmol Crea] in obese children by age and sex. 225 observations. Significant results regarding sex differences appeared for LCA-S. CA, GCA and the sum of the 18 bile acids (Total) significantly correlated with age. Creatinine concentration was also age-correlated.

Daily urinary Bile Acids Excretions [nmol/d]

Table 19 shows the data for daily urinary bile acids excretion [nmol/d] in obese children. Due to missing information about the 24-hour urine volume of one sample, the group size amounts to N=236.

The highest median daily excretion was measured for GCDCA-S (428.8 nmol/d), followed by GDCA-S (380.8 nmol/d), GLCA-S (376.0 nmol/d), CA (224.7 nmol/d), GCA (104.4 nmol/d) and TLCA-S (100.7 nmol/d).

	Mean ± SD	.05	.10	.25	.50	.75	.90	.95
CA ($^{224}/_{236}$)	398.6 ± 579.2	4.7	33.1	76.1	224.7	489.5	846.5	1407.4
GCA ($^{232}/_{236}$)	135.6 ± 179.1	34.1	42.2	61.4	104.4	155.0	225.3	325.0
TCA ($^{59}/_{236}$)	4.7 ± 10.3	0.0	0.0	0.0	0.0	0.0	17.0	23.7
CDCA ($^{72}/_{236}$)	9.0 ± 17.7	0.0	0.0	0.0	0.0	15.5	30.4	42.6
GCDCA ($^{23}/_{236}$)	5.1 ± 20.9	0.0	0.0	0.0	0.0	0.0	0.0	38.2
TCDCA ($^{43}/_{236}$)	3.6 ± 9.4	0.0	0.0	0.0	0.0	0.0	14.8	21.8
DCA ($^{152}/_{236}$)	32.9 ± 42.2	0.0	0.0	0.0	23.1	46.4	79.0	107.4
GDCA ($^{150}/_{236}$)	22.1 ± 40.8	0.0	0.0	0.0	15.5	30.0	50.9	59.7
TDCA ($^{3}/_{236}$)	0.1 ± 1.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0
LCA ($^{0}/_{236}$)	0.0 ± 0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GLCA ($^{0}/_{236}$)	0.0 ± 0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
TLCA ($^{0}/_{236}$)	0.0 ± 0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
UDCA ($^{58}/_{236}$)	11.2 ± 43.5	0.0	0.0	0.0	0.0	0.0	30.9	45.3
GCDCA-S ($^{236}/_{236}$)	511.5 ± 349.7	109.5	154.2	247.5	428.8	695.4	985.2	1237.6
GDCA-S ($^{228}/_{236}$)	553.7 ± 573.1	38.4	86.4	185.3	380.8	683.2	1216.2	1800.1
LCA-S ($^{33}/_{236}$)	3.8 ± 11.8	0.0	0.0	0.0	0.0	0.0	17.2	25.4
GLCA-S ($^{230}/_{236}$)	517.5 ± 515.3	26.4	62.2	161.1	376.0	719.7	1100.7	1431.3
TLCA-S ($^{225}/_{236}$)	153.7 ± 197.2	9.4	17.2	41.2	100.7	190.8	302.1	497.5
Total ($^{236}/_{236}$)	2363.0 ± 1539.1	678.0	785.9	1204.0	2053.5	3146.1	4207.0	5520.1

Table 19. Daily urinary bile acids excretions in obese children [nmol/d] (MULTIOMICS study, N=236). The table lists mean ± standard deviation as well as 7 different percentiles. In brackets after each analyte: number of quantifiable samples (samples > LOQ).

Figure 13 (1/4). Scatterplots of daily urinary bile acids excretions [nmol/d] in obese children. Bile acid excretion is plotted against the children's age. TDCA, LCA, GLCA and TLCA were left out, since each of these analytes delivered less than 4 results beyond the limit of quantification. Pink dots: female; blue dots: male, black line: linear regression trendline.

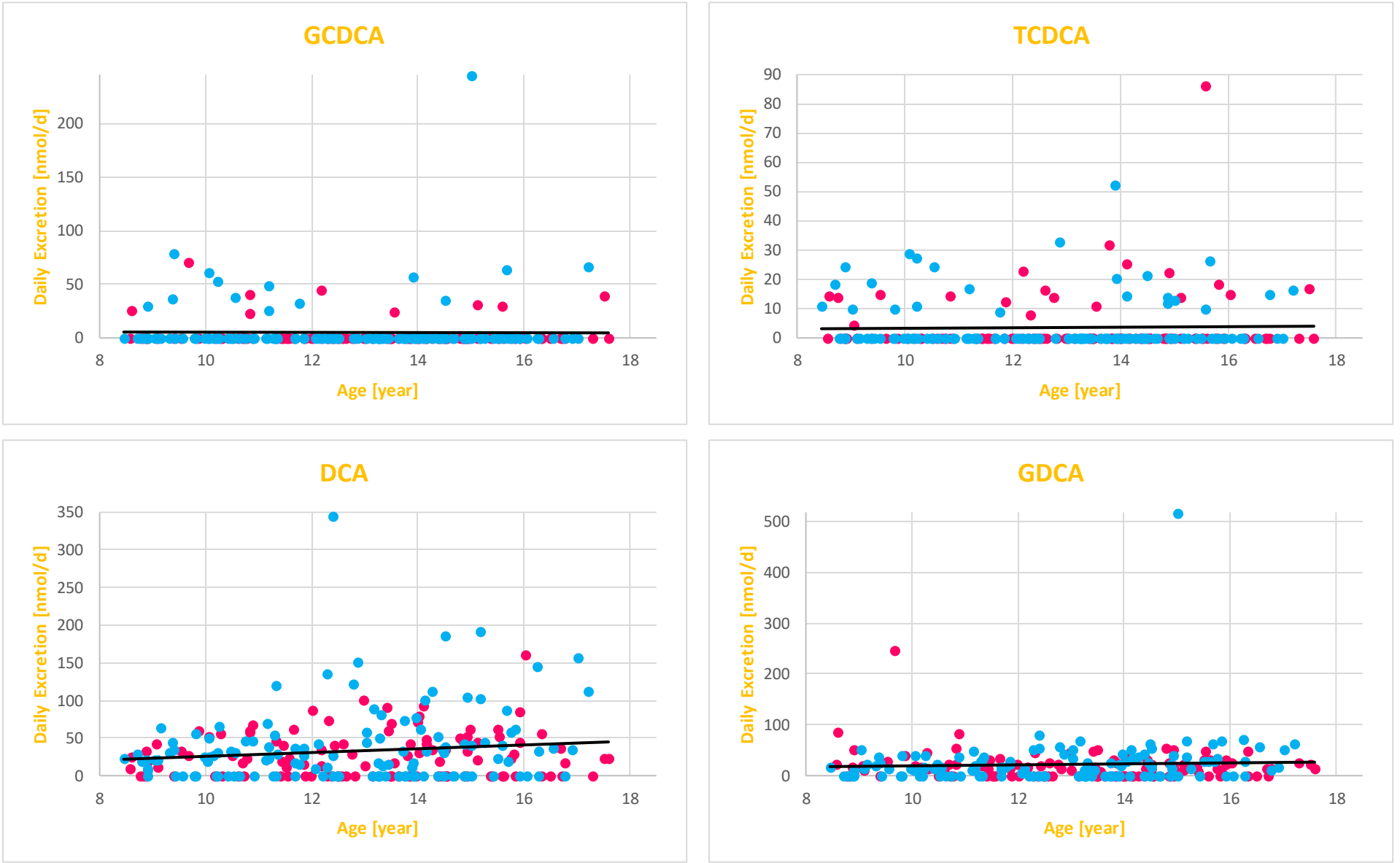

GCDCA
Daily Excretion [nmol/d]
0
50
100
150
200
8
10
12
14
16
18
Age [year]
TCDCA
Daily Excretion [nmol/d]
0
10
20
30
40
50
60
70
80
90
8
10
12
14
16
18
Age [year]
DCA
Daily Excretion [nmol/d]
0
50
100
150
200
250
300
350
8
10
12
14
16
18
Age [year]
GDCA
Daily Excretion [nmol/d]
0
100
200
300
400
500
8
10
12
14
16
18
Age [year]

Figure 13 (2/4)

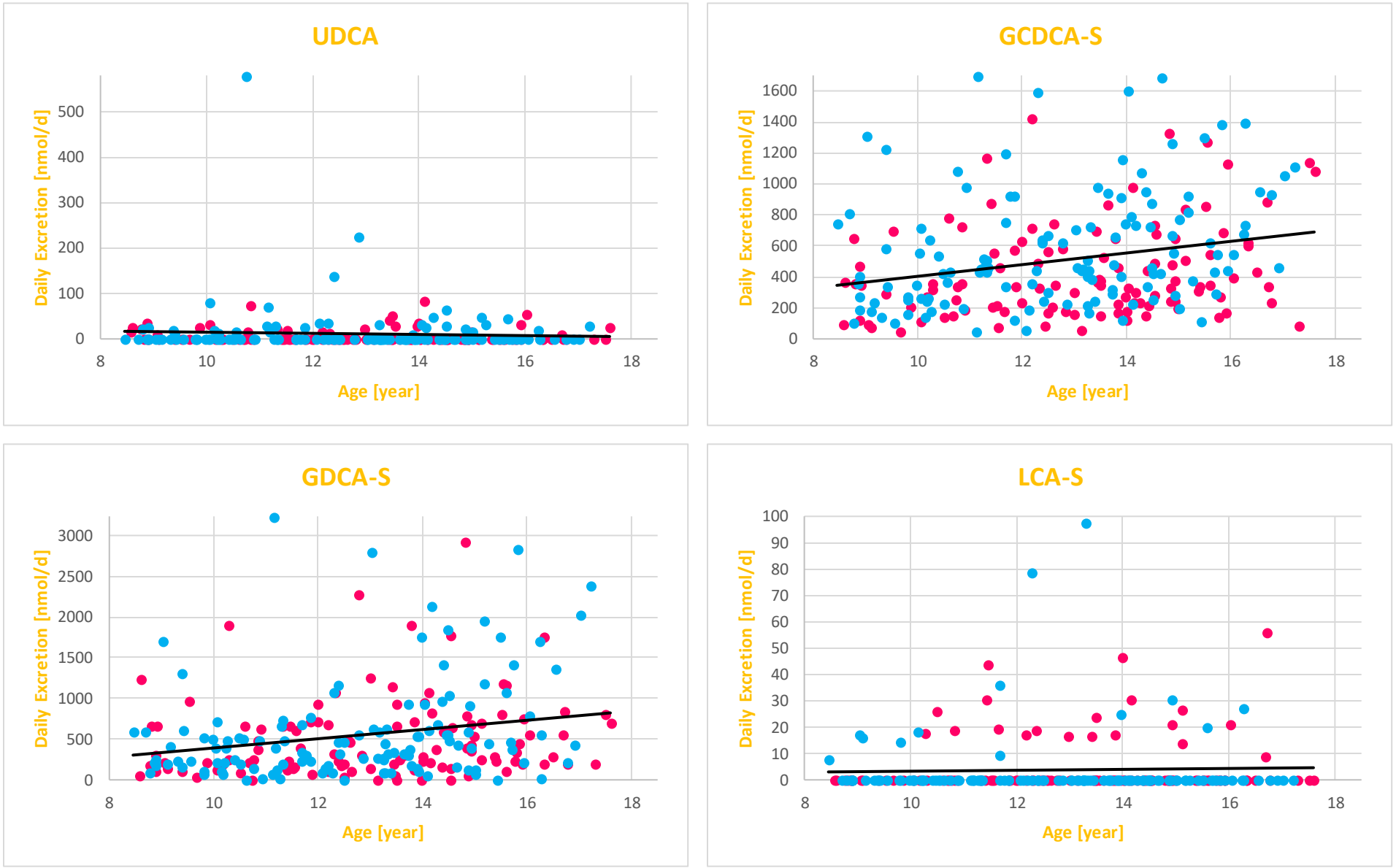
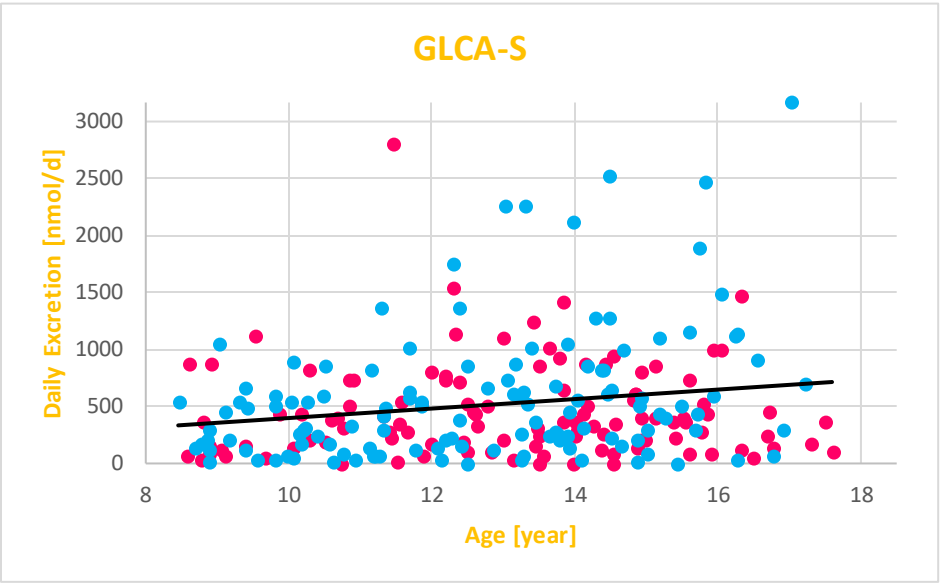
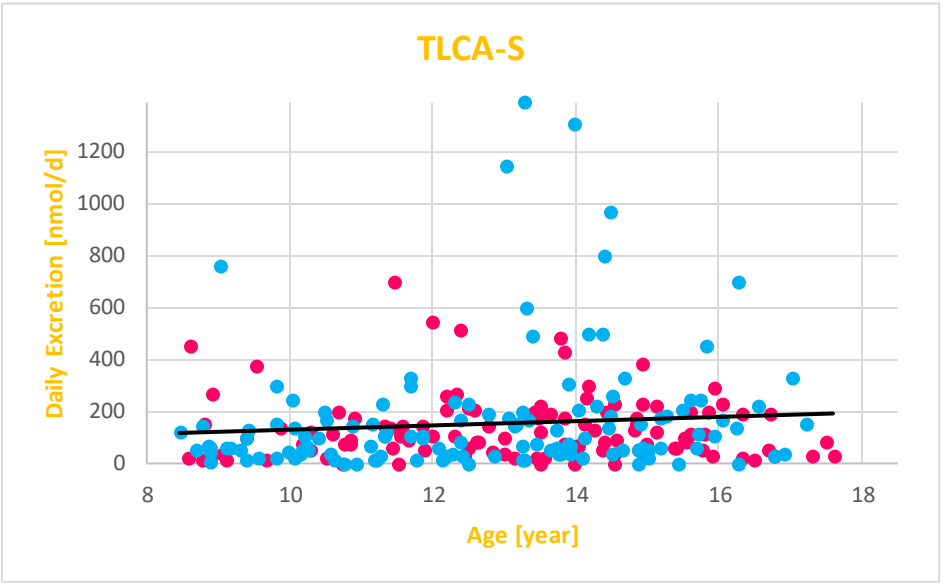

UDCA
Daily Excretion [nmol/d]
0
100
200
300
400
500
8
10
12
14
16
18
Age [year]
GCDCA-S
Daily Excretion [nmol/d]
0
200
400
600
800
1000
1200
1400
1600
8
10
12
14
16
18
Age [year]
GDCA-S
Daily Excretion [nmol/d]
0
500
1000
1500
2000
2500
3000
8
10
12
14
16
18
Age [year]
LCA-S
Daily Excretion [nmol/d]
0
10
20
30
40
50
60
70
80
90
100
8
10
12
14
16
18
Age [year]

Figure 13 (3/4)


GLCA-S
Daily Excretion [nmol/d]
3000
2500
2000
1500
1000
500
0
8
10
12
14
16
18
Age [year]


TLCA-S
Daily Excretion [nmol/d]
1200
1000
800
600
400
200
0
8
10
12
14
16
18
Age [year]

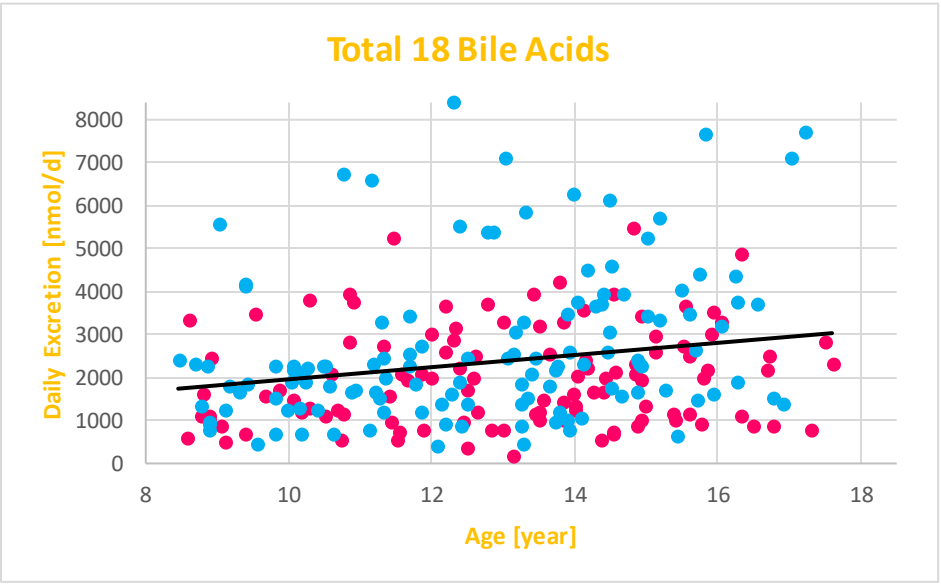

Total 18 Bile Acids
Daily Excretion [nmol/d]
8000
7000
6000
5000
4000
3000
2000
1000
0
8
10
12
14
16
18
Age [year]

Figure 13 (4/4)

Figure 13 depicts scatterplots, plotting the daily urinary bile acids excretion against the probands' age and discriminating between male (blue dots) and female (pink dots) probands. For most analytes, the scatterplots and the respective linear regression lines tend to show an increase of daily bile acids excretion with age. The results of the linear regression analysis (Table 20) show a significant positive age correlation for GCDCA-S and the sum of the 18 bile acids (Total). A sex correlation proved also significant for GCDCA-S and Total, as well as for CA - all of them being excreted in higher amounts by male probands. 24-Hour urine volume also showed a significant positive age trend.

	p-value	
	Age	**Sex**
CA	0.955	**0.012**
GCA	0.585	0.209
TCA	0.912	0.582
CDCA	0.728	0.144
GCDCA	0.294	0.395
TCDCA	0.599	0.692
DCA	0.983	0.284
GDCA	0.633	0.502
UDCA	0.193	0.460
GCDCA-S	**<0.001**	**<0.001**
GDCA-S	0.054	0.274
LCA-S	0.933	0.100
GLCA-S	0.103	0.267
TLCA-S	0.271	0.911
Total	**<0.001**	**0.001**
Volume	**<0.001**	0.173

Table 20. Linear regression analysis: Urinary bile acids daily excretions [nmol/d] in obese children by age and sex. 236 observations. CA showed a significant sex correlation. GCDCA-S and the sum of the 18 analytes (Total) significantly correlated with age and sex. The 24-hour urine volume also showed an age correlation.

Percentage Amidation and Sulfation of urinary Bile Acids in obese Children

Concordant with the data of the healthy study group, percentages of amidation and sulfation were also formed for the obese study group. Included were those analytes that were measured as derivatives of the same underlying type of bile acid – differing in state of amidation or sulfation, respectively.

Regarding amidation, this are cholic acid (CA, GCA, TCA), chenodeoxycholic acid (CDCA, GCDCA, TCDCA), deoxycholic acid (DCA, GDCA, TDCA) and lithocholic acid (LCA, GLCA, TLCA) as well as lithocholic acid sulfate (LCA-S, GLCA-S, TLCA-S).

Concerning percentage sulfation, the three lithocholic acid derivatives (LCA/GLCA/TLCA; LCA-S/GLCA-S/TLCA-S) as well as glycochenodeoxycholic acid (GCDCA; GCDCA-S) and glycodeoxycholic acid (GDCA; GDCA-S) were looked into.

The respective percentages were calculated for each sample individually using the data in nmol/d. Subsequently, the mean ± standard deviation as well as percentiles were formed. The results are listed in Table 21 + 22.

For cholic acid, chenodeoxycholic acid and deoxycholic acid, the unamidated derivatives made up the bulk. In cholic acid and deoxycholic acid, the unamidated derivatives were followed by the glycine-amidated forms. In chenodeoxycholic acid, the taurine-amidated form landed second place. The lithocholic acid sulfates showed the following order: glycine-amidated > taurine-amidated > unamidated.

Unsulfated lithocholic acid derivatives appeared in a non-quantifiable amount – regardless of their amidation state (LCA, GLCA and TLCA were below the LOQ in all samples). As a logical consequence, the lithocholic acid derivatives appeared to 100% in sulfated form. Likewise, the vast majority of glycochenodeoxycholic acid and glycodeoxycholic acid was present as sulfates (mean percentage sulfation 98,9% or 95,7%, respectively).

Amidation	Mean ± SD	.05	.10	.25	.50	.75	.90	.95
	[%]							
Cholic Acid (n=234)								
Unamidated (CA)	61.3 ± 25.1	12.8	25.3	44.9	68.8	81.0	88.2	91.2
Glycine (GCA)	37.5 ± 24.4	8.7	11.7	18.7	31.0	53.5	72.8	84.0
Taurine (TCA)	1.2 ± 3.0	0.0	0.0	0.0	0.0	0.4	4.6	6.8
Chenodeoxycholic Acid (n=93)								
Unamidated (CDCA)	60.7 ± 42.5	0.0	0.0	19.8	71.2	100.0	100.0	100.0
Glycine (GCDCA)	14.5 ± 26.4	0.0	0.0	0.0	0.0	0.0	59.7	64.9
Taurine (TCDCA)	24.9 ± 35.7	0.0	0.0	0.0	0.0	37.5	100.0	100.0
Deoxycholic Acid (n=173)								
Unamidated (DCA)	57.3 ± 28.0	0.0	0.0	48.2	60.5	72.4	100.0	100.0
Glycine (GDCA)	42.6 ± 27.9	0.0	0.0	27.6	39.0	51.8	100.0	100.0
Taurine (TDCA)	0.1 ± 1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Lithocholic Acid (n=0)								
Unamidated (LCA)	NA							
Glycine (GLCA)	NA							
Taurine (TLCA)	NA							
Lithocholic Acid Sulfate (n=230)								
Unamidated (LCA-S)	0.5 ± 1.5	0.0	0.0	0.0	0.0	0.0	1.5	2.8
Glycine (GLCA-S)	77.1 ± 10.1	59.1	63.3	71.4	78.6	83.9	87.8	90.4
Taurine (TLCA-S)	22.5 ± 10.2	9.3	11.4	15.7	20.8	28.1	36.7	40.8

Table 21. Percentage amidation of urinary bile acids in obese children. For the bile acids that were measured in their unamidated as well as their glycine- and taurine-amidated form, percentage shares of each form were calculated for each sample. The mean + SD and different percentiles were formed. For calculation, the nanomolar daily excretion [nmol/d] was used.
n = number of samples > LOQ; SD = standard deviation

Sulfation	**Mean ± SD**	**.05**	**.10**	**.25**	**.50**	**.75**	**.90**	**.95**
	[%]							
Glycochenodeoxycholic Acid (n=236)								
Unsulfated (GCDCA)	1.1 ± 5.6	0.0	0.0	0.0	0.0	0.0	0.0	6.0
Sulfated (GCDCA-S)	98.9 ± 5.6	94.0	100.0	100.0	100.0	100.0	100.0	100.0
Glycodeoxycholic Acid (n=228)								
Unsulfated (GDCA)	4.3 ± 8.5	0.0	0.0	0.0	3.0	5.3	9.1	12.0
Sulfated (GDCA-S)	95.7 ± 8.5	88.0	90.9	94.7	97.0	100.0	100.0	100.0
Total Lithocholic Acid (n=230)								
Unsulfated (LCA, GLCA, TLCA)	0.0 ± 0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Sulfated (LCA-S, GLCA-S, TLCA-S)	100.0 ± 0.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

Table 22. Percentage sulfation of urinary bile acids in obese children. For the bile acids that were measured in their unsulfated as well as their sulfated form, percentage shares of each form were calculated for each sample. The mean + SD and different percentiles were formed. For calculation, the nanomolar daily excretion [nmol/d] was used.
n = number of samples > LOQ; SD = standard deviation

3.4.4 Comparison - Urinary Bile Acids in healthy Children versus obese Children

After measuring and analyzing urinary bile acids in 80 healthy and 237 obese children, the results within these two study groups were compared with each other.

The most abundant analytes were the same six bile acids in both study groups: The sulfates GCDCA-S, GDCA-S, GLCA-S and TLCA-S as well as the unsulfated primary bile acids CA and GCA.

In both groups, all results for LCA, GLCA and TLCA lay below the limit of quantification. While GCDCA and TDCA were also not quantifiable in the healthy group, the obese group delivered a few quantifiable results for these two bile acids.

The two study groups were compared in concentration [nmol/L], creatinine-related concentration [nmol BA/mmol Crea] as well as in daily excretion [nmol/d].

Urinary Bile Acids Concentrations [nmol/L]

Sulfated bile acids accounted for the major share of the measured urinary bile acids in both study groups. In healthy as well as in obese samples, CA and GCA were the most abundant unsulfated bile acids. However, the order of abundances differed: In healthy children, GCDCA-S was followed by GLCA-S, GDCA-S, TLCA-S, CA and GCA in descending order of median concentration. In obese children, on the other hand, TLCA-S was outrun by CA and GCA, resulting in the following order: GCDCA-S > GLCA-S > GDCA-S > CA > GCA > TLCA-S.

Testing for potential significant group differences, linear regression analysis was done. The resulting p-values are displayed in Table 23. Urinary concentrations of GCDCA-S and TLCA-S were significantly higher in healthy children. For GDCA-S, GLCA-S, CA and GCA, no significant differences in concentration between healthy and obese children were observed. In both groups, LCA-S represents an outlying analyte within the sulfated bile acids, since its urinary concentration was rather low and more than 80% of the samples did not exceed the limit of quantification. Hence, the performance of urinary LCA-S in obese children did not significantly change in comparison to healthy children.

As mentioned above, GCDCA and TDCA were only quantifiable in obese children. For GCDCA, 23 samples ended up being above the limit of quantification in obese children. Even with only 23 out of 237 samples over the limit of quantification, GCDCA was significantly more abundant ($p = 0.003$, Table 23) in obese children's urine than in healthy children's urine. The same applies for CDCA (73/237 samples > LOQ, $p < 0.001$, Table 23) and UDCA (58/237 samples > LOQ, $p < 0.001$, Table 23). To sum up, CDCA, GCDCA and UDCA were

not quantifiable on a regular basis in healthy children as well as in obese children, but in obese children they still appeared more frequently, resulting in significantly higher concentrations.

TDCA only exceeded the limit of quantification in three obese samples, resulting in no significant differences compared to the healthy group. Therefore, it seemed not reasonable to use it in further calculations and it was treated equally to the bile acids with no workable results (LCA, GLCA and TLCA).

	p-value		
	Age	**Sex**	**Study Group**
CA	0.290	**0.046**	0.162
GCA	**0.004**	0.314	0.597
TCA	0.201	0.611	**<0.001**
CDCA	0.963	0.176	**<0.001**
GCDCA	0.354	0.375	**0.003**
TCDCA	0.084	0.902	0.637
DCA	0.901	0.802	**<0.001**
GDCA	0.563	0.897	**<0.001**
UDCA	0.168	0.539	**<0.001**
GCDCA-S	0.528	**0.010**	**<0.001**
GDCA-S	0.717	0.694	0.208
LCA-S	0.786	0.220	0.893
GLCA-S	0.782	0.766	0.855
TLCA-S	0.415	0.506	**0.005**
Total	0.598	0.075	0.245

Table 23. Linear regression analysis: Urinary bile acids concentrations [nmol/L] in healthy and obese children by age, sex and study group. 317 observations. GCDCA-S, TLCA-S and TCA were significantly higher in healthy children. CDCA, GCDCA, DCA, GDCA and UDCA delivered significantly higher results in obese children's urine.

DCA and GDCA delivered significantly higher results in obese children's urine. But still, the concentrations presented on a relatively low level. There were also less samples below the limit of quantification in the obese study group.
TCA acted vice versa, since its concentration was significantly lower in obese children compared to healthy ones and also less samples of the MULTIOMICS group reached the limit of quantification.

Further visualization of the group comparison can be found in the boxplots of Figure 14. The boxplots are concordant with the test results in Table 23, since bile acids with significant results in the linear regression analysis show also markedly different boxplots comparing healthy (green boxes) with obese (orange boxes).

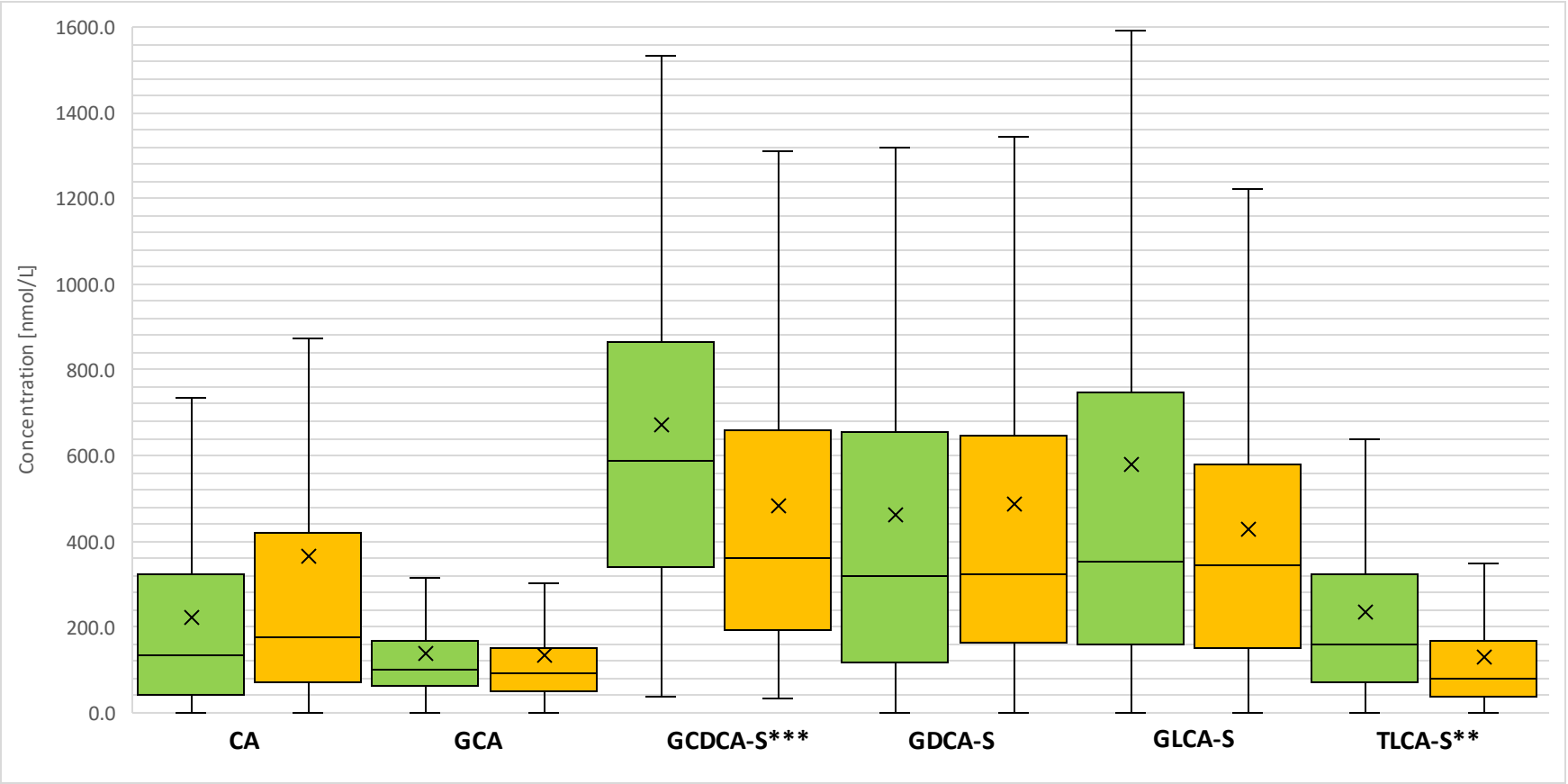


Figure 14 (1/2). Boxplots for group comparisons [nmol/L]. Concordant to the results of the linear regression analysis, significant group differences are visible for GCDCA-S and TLCA-S, especially when looking at the horizontal median line and the × for mean concentration. × = mean, horizontal line = median, lower end of box = first quartile, upper end of box = third quartile, span = minimum to maximum, outliers were excluded for reasons of clarity and comprehensibility. Green = healthy, orange = obese.
**p-value < 0.05; **p-value < 0.01; ***p-value < 0.001*

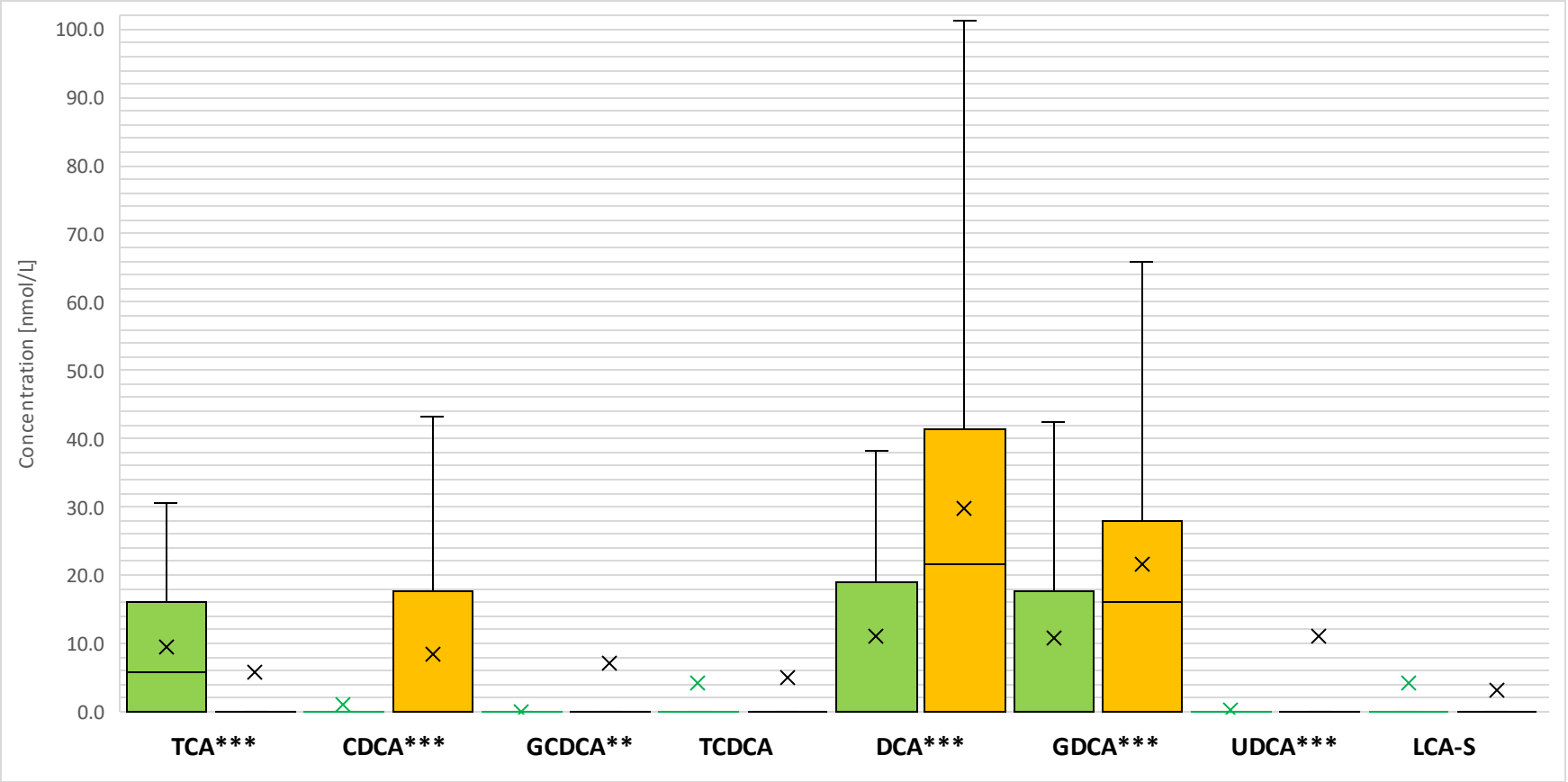


Figure 14 (2/2). Boxplots for group comparisons [nmol/L]. Concordant to the results of the linear regression analysis, significant group differences are visible for TCA, CDCA, GCDCA, DCA, GDCA and UDCA, especially when looking at × for mean concentration.
× = mean, horizontal line = median, lower end of box = first quartile, upper end of box = third quartile, span = minimum to maximum, outliers were excluded for reasons of clarity and comprehensibility. Green = healthy, orange = obese.
**p-value < 0.05; **p-value < 0.01; ***p-value < 0.001*

Creatinine-related Bile Acids Concentrations [nmol BA/mmol Crea]

The order of median abundances for healthy children's urine stays the same in creatinine-relation [nmol BA/mmol Crea] compared to plain concentration [nmol/L]: GCDCA-S > GLCA-S > GDCA-S > TLCA-S > CA > GCA. In obese children, however, the order changed when comparing the two units: GCDCA-S > GDCA-S > GLCA-S > CA > TLCA-S > GCA [nmol BA/mmol Crea] compared to GCDCA-S > GLCA-S > GDCA-S > CA > GCA > TLCA-S [nmol/L].

Regarding the six most abundant bile acids, there were significant group differences for GCDCA-S and TLCA-S. Equally to nmol/L, both of them appeared in higher creatinine-related concentration in healthy children. The same applies for TCA when looking at the less abundant bile acids. Also concordant to concentration in nmol/L, CDCA, GCDCA, DCA, GDCA and UDCA delivered significantly higher creatinine-related concentrations in obese children's urine. The remaining bile acids as well as the creatinine concentration itself did not significantly differ between the study groups.

Figure 15 illustrates the group comparison in form of boxplots.

	p-value		
	Age	**Sex**	**Study Group**
CA	**0.003**	0.165	0.067
GCA	**<0.001**	0.814	0.401
TCA	**0.012**	0.608	**<0.001**
CDCA	0.829	0.297	**<0.001**
GCDCA	0.344	0.389	**0.003**
TCDCA	**0.024**	0.966	0.579
DCA	0.490	0.536	**<0.001**
GDCA	0.160	0.479	**<0.001**
UDCA	0.111	0.617	**<0.001**
GCDCA-S	**<0.001**	0.205	**<0.001**
GDCA-S	0.403	0.611	0.327
LCA-S	0.613	0.088	0.700
GLCA-S	0.098	0.655	0.465
TLCA-S	**0.014**	0.195	**<0.001**
Total	**<0.001**	0.894	0.057
Creatinine	**<0.001**	0.068	0.608

Table 24. Linear regression analysis: Creatinine-related urinary bile acids concentrations [nmol BA/mmol Crea] in healthy and obese children by age, sex and study group. 305 observations. TCA, GCDCA-S and TLCA-S appeared in higher creatinine-related concentration in healthy children, while CDCA, GCDCA, DCA, GDCA and UDCA delivered significantly higher results in the obese group.

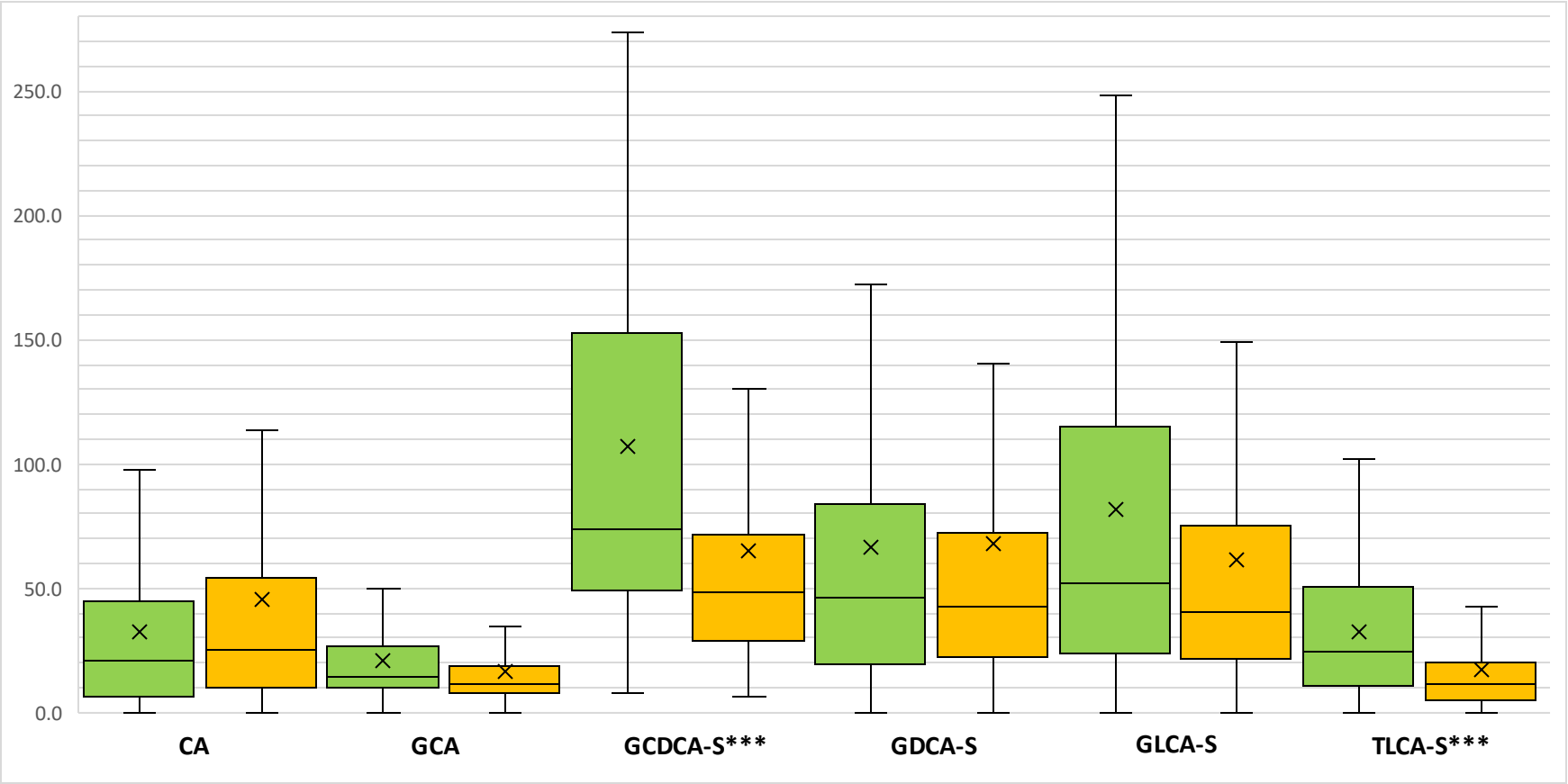


Figure 15 (1/2). Boxplots for group comparisons [nmol BA/mmol Crea]. Concordant to the results of the linear regression analysis, significant group differences are visible for GCDCA-S and TLCA-S, especially when looking at the horizontal median line and the × for mean. × = mean, horizontal line = median, lower end of box = first quartile, upper end of box = third quartile, span = minimum to maximum, outliers were excluded for reasons of clarity and comprehensibility. Green = healthy, orange = obese.
**p-value < 0.05; **p-value < 0.01; ***p-value < 0.001*

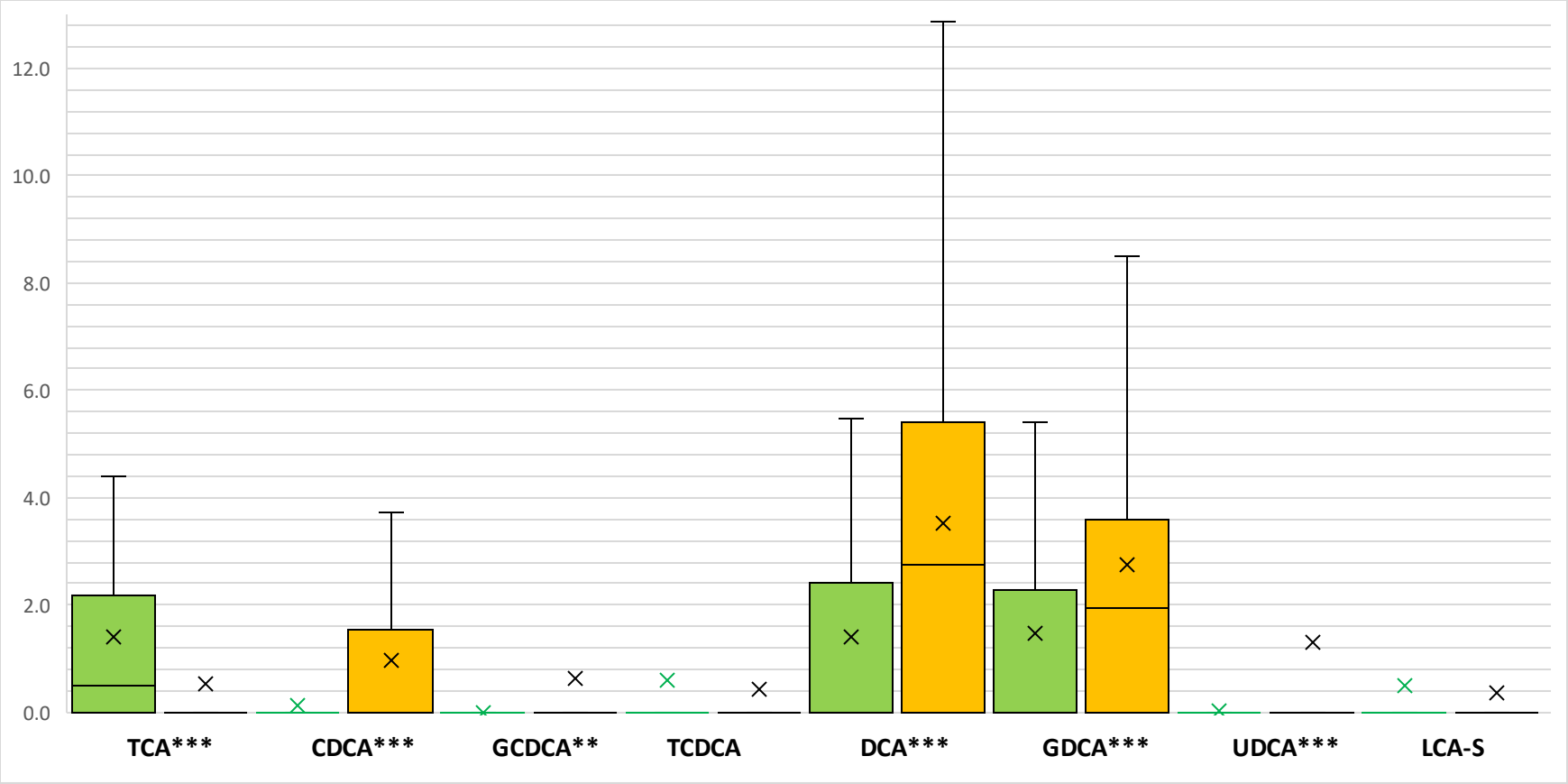


Figure 15 (2/2). Boxplots for group comparisons [nmol BA/mmol Crea]. Concordant to the results of the linear regression analysis, significant group differences are visible for TCA, CDCA, GCDCA, DCA, GDCA and UDCA, especially when looking at × for mean.
× = mean, horizontal line = median, lower end of box = first quartile, upper end of box = third quartile, span = minimum to maximum, outliers were excluded for reasons of clarity and comprehensibility. Green = healthy, orange = obese.
**p-value < 0.05; **p-value < 0.01; ***p-value < 0.001*

Daily urinary Bile Acids Excretions [nmol/d]

The Order of abundance in the DONALD group when looking at the median daily excretion [nmol/d] is the same order as in median concentration [nmol/L] and median creatinine-related concentration [nmol BA/mmol Crea]: GCDCA-S > GLCA-S > GDCA-S > TLCA-S > CA > GCA. However, the MULTIOMICS group presented in the following order: GCDCA-S > GDCA-S > GLCA-S > CA > GCA > TLCA-S. (in nmol/L: GCDCA-S > GLCA-S > GDCA-S > CA > GCA > TLCA-S; in nmol BA/mmol Crea: GCDCA-S > GDCA-S > GLCA-S > CA > TLCA-S > GCA).

While the first two units have shown significant group differences for the same eight analytes, nmol/d showed a slightly different pattern. Unlike the first two units, the daily excretion delivered no significant group differences for TLCA-S. On the other hand, CA and GDCA-S only displayed significant group differences in daily excretion.

While CA, CDCA, GCDCA, DCA, GDCA, UDCA and GDCA-S were higher in obesity, TCA and GCDCA-S appeared higher in health. GCDCA-S still delivered a significant p-value (Table 25), even though the group differences are not that obvious when looking at the boxplots (Figure 16).

	p-value		
	Age	**Sex**	**Study Group**
CA	0.353	**0.012**	**0.040**
GCA	0.542	0.067	0.437
TCA	0.665	0.507	**<0.001**
CDCA	0.876	0.129	**<0.001**
GCDCA	0.426	0.354	**0.003**
TCDCA	0.224	0.990	0.755
DCA	0.598	0.965	**<0.001**
GDCA	0.747	0.945	**<0.001**
UDCA	0.236	0.471	**<0.001**
GCDCA-S	**<0.001**	**<0.001**	**0.016**
GDCA-S	**0.014**	0.472	**0.038**
LCA-S	0.920	0.262	0.912
GLCA-S	**0.049**	0.470	0.480
TLCA-S	0.128	0.765	0.063
Total	**<0.001**	**0.001**	0.296
Volume	**<0.001**	0.101	**0.003**

Table 25. Linear regression analysis: Daily urinary bile acids excretions [nmol/d] in healthy and obese children by age, sex and study group. 316 observations. Significant results appeared for CA, TCA, CDCA, GCDCA, DCA, GDCA, UDCA, GCDCA-S and GDCA-S.

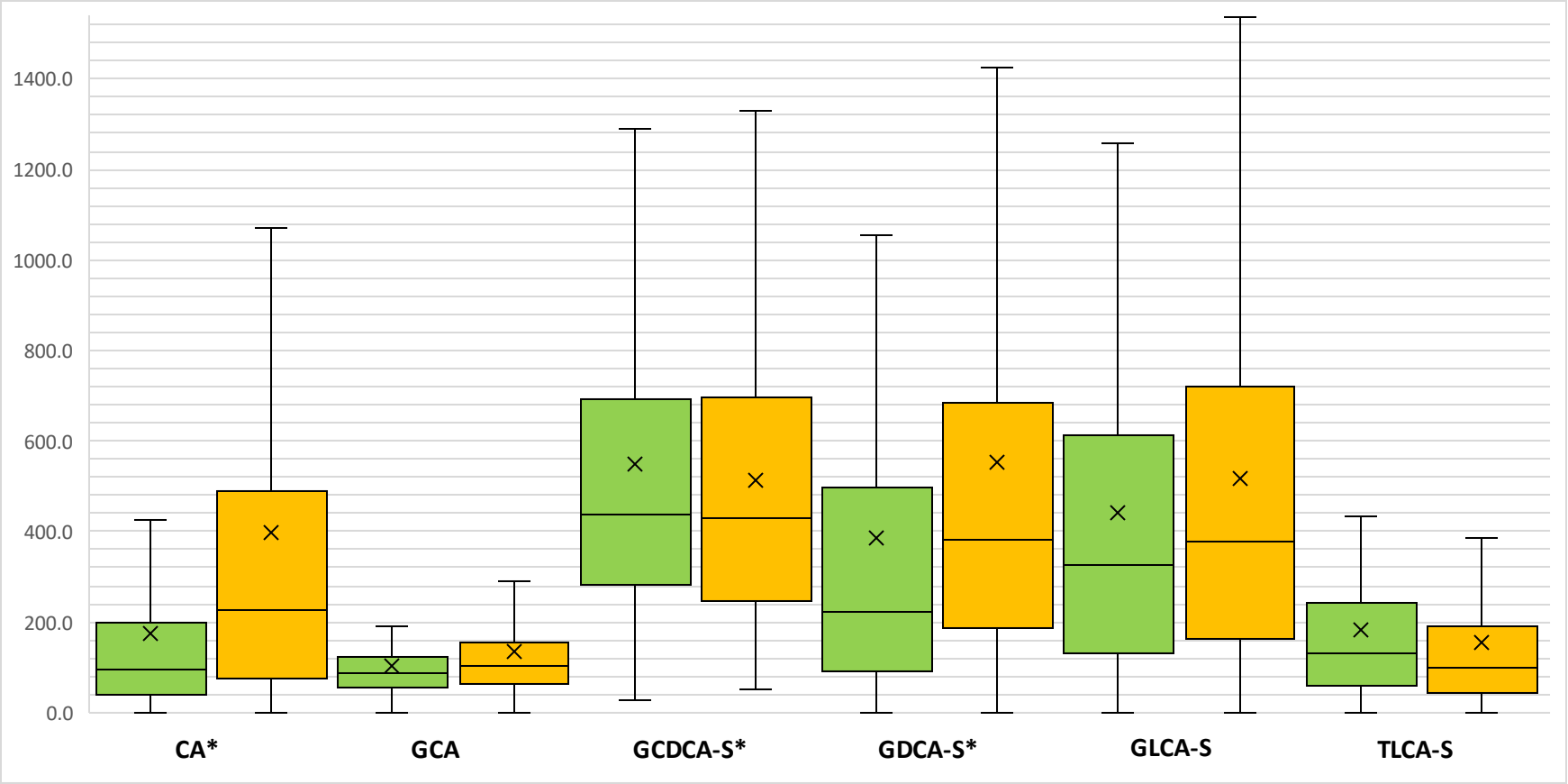


Figure 16 (1/2). Boxplots for group comparisons [nmol/d]. Concordant to the results of the linear regression analysis, significant group differences are visible for CA, GCDCA-S and GDCA-S, especially when looking at the horizontal median line and the × for mean.
× = mean, horizontal line = median, lower end of box = first quartile, upper end of box = third quartile, span = minimum to maximum, outliers were excluded for reasons of clarity and comprehensibility. Green = healthy, orange = obese.
**p-value < 0.05; **p-value < 0.01; ***p-value < 0.001*

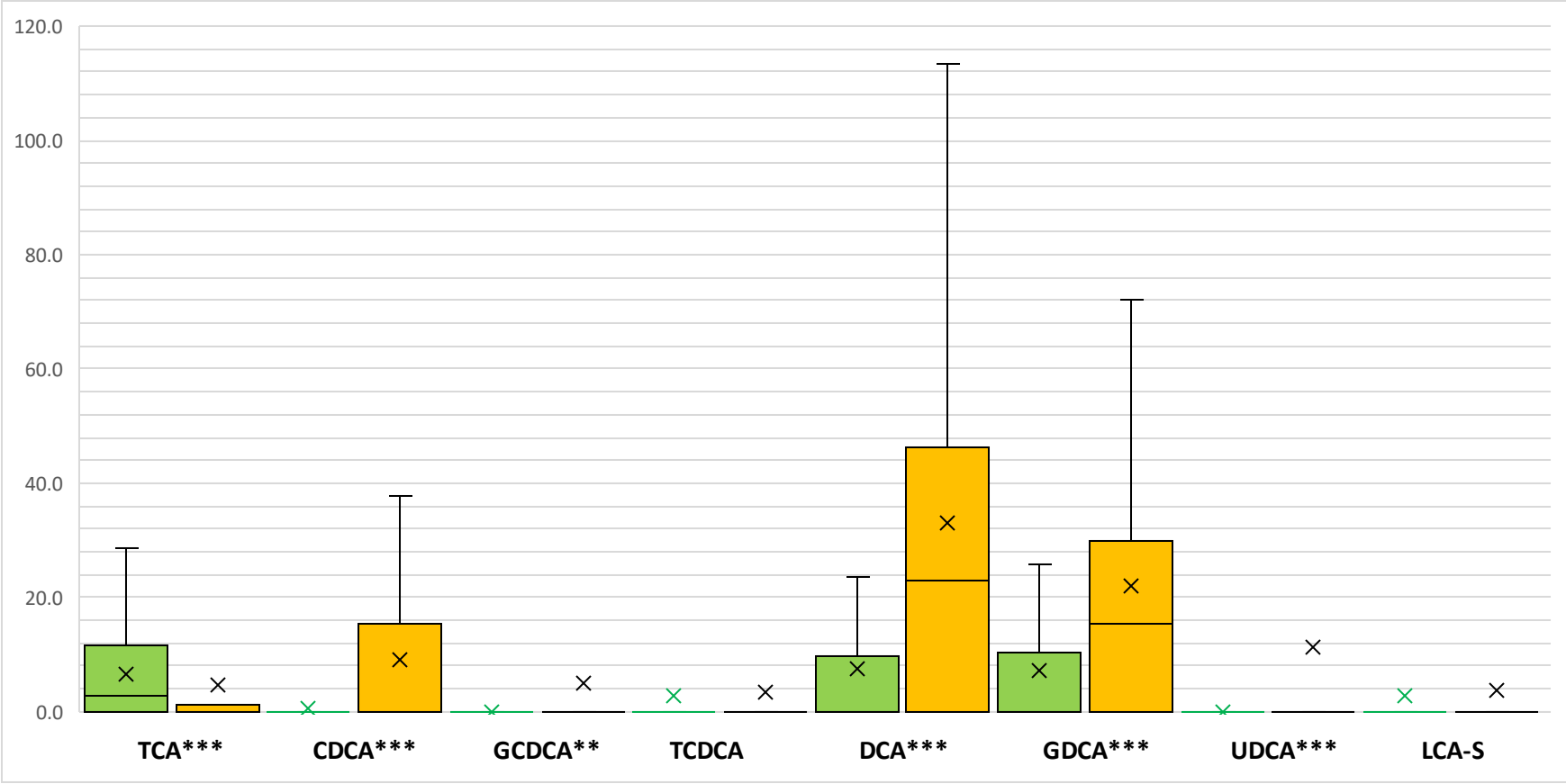


Figure 16 (2/2). Boxplots for group comparisons [nmol/d]. Concordant to the results of the linear regression analysis, significant group differences are visible for TCA, CDCA, GCDCA, DCA, GDCA and UDCA, especially when looking at × for mean.
× = mean, horizontal line = median, lower end of box = first quartile, upper end of box = third quartile, span = minimum to maximum, outliers were excluded for reasons of clarity and comprehensibility. Green = healthy, orange = obese.
**p-value < 0.05; **p-value < 0.01; ***p-value < 0.001*

Percentile Curves for the six most abundant urinary Bile Acids

For further investigation of potential age-correlation and group differences, we created percentile curves for the six most abundant bile acids in our study, namely CA, GCA, GCDCA-S, GDCA-S, GLCA-S and TLCA-S. (Figures 17-19)

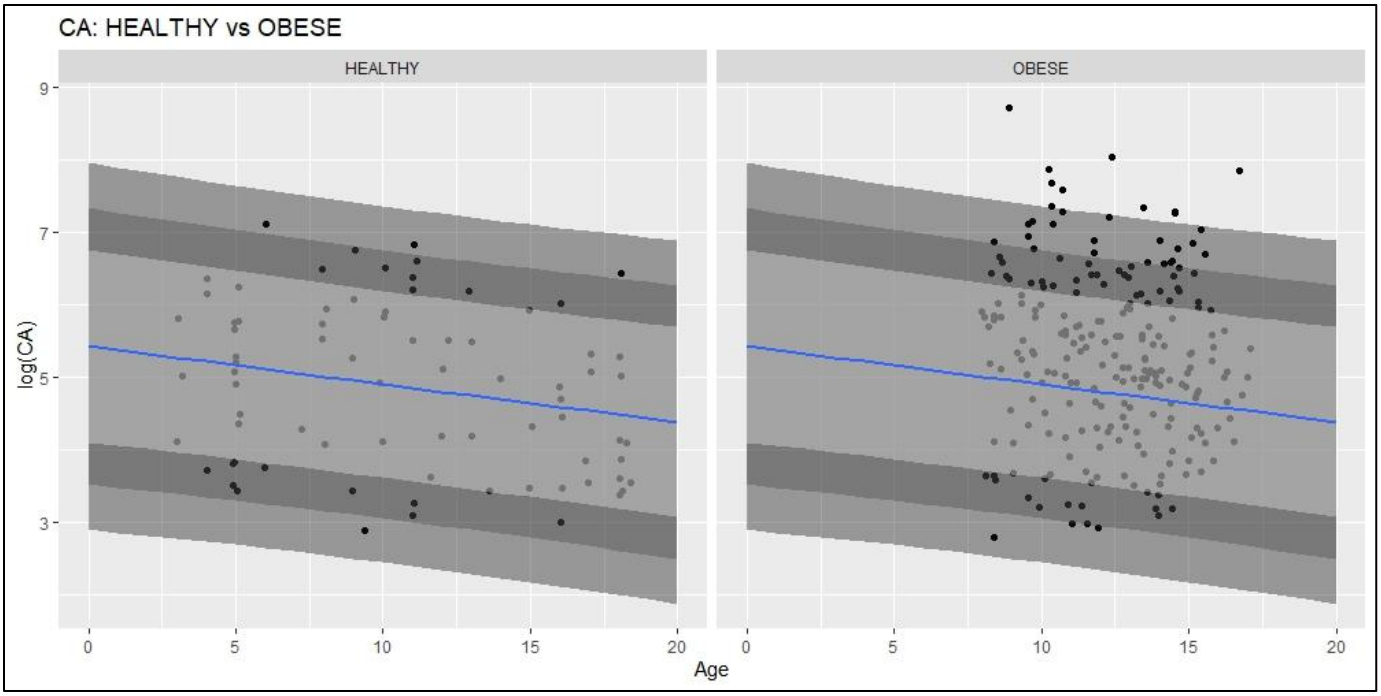
The different shade transitions of background hatching from bottom to top represent the percentiles P3, P10, P25, P75, P90 and P97. The blue line in the middle marks the median (P50). The course of the curves shows the variations of these percentiles with age. The percentile curves were created by using the healthy DONALD study group as a reference. For calculation of the curves, the natural logarithm of the bile acids results was used in sake of emulating a normal distribution. For clarity reasons, the samples with results below the limit of quantification were excluded from the calculation.

The left image for each bile acid shows the percentile curves with the healthy results put into it as scatter dots. In a second step, we used the same (healthy) percentile curves and put the obese results as scatter dots into the image (right image) to depict potential group differences. Since the y-axis is adapted to said normal-logarithmic transformation, one has to take this into account when reading off results from the graph. When reading off a result from the y-axis, it has to be used as the exponent of "*e*" (Euler's number) to calculate for the actual bile acid concentration, creatinine-related concentration or daily excretion, respectively:

Calculation: From percentile graph to actual bile acid result

$$e^{(result\ from\ y-axis)} = actual\ bile\ acid\ result\ \left[\frac{nmol}{L}\right] or \left[\frac{nmol\ BA}{mmol\ Crea}\right] or \left[\frac{nmol}{d}\right]$$

e = Euler's number



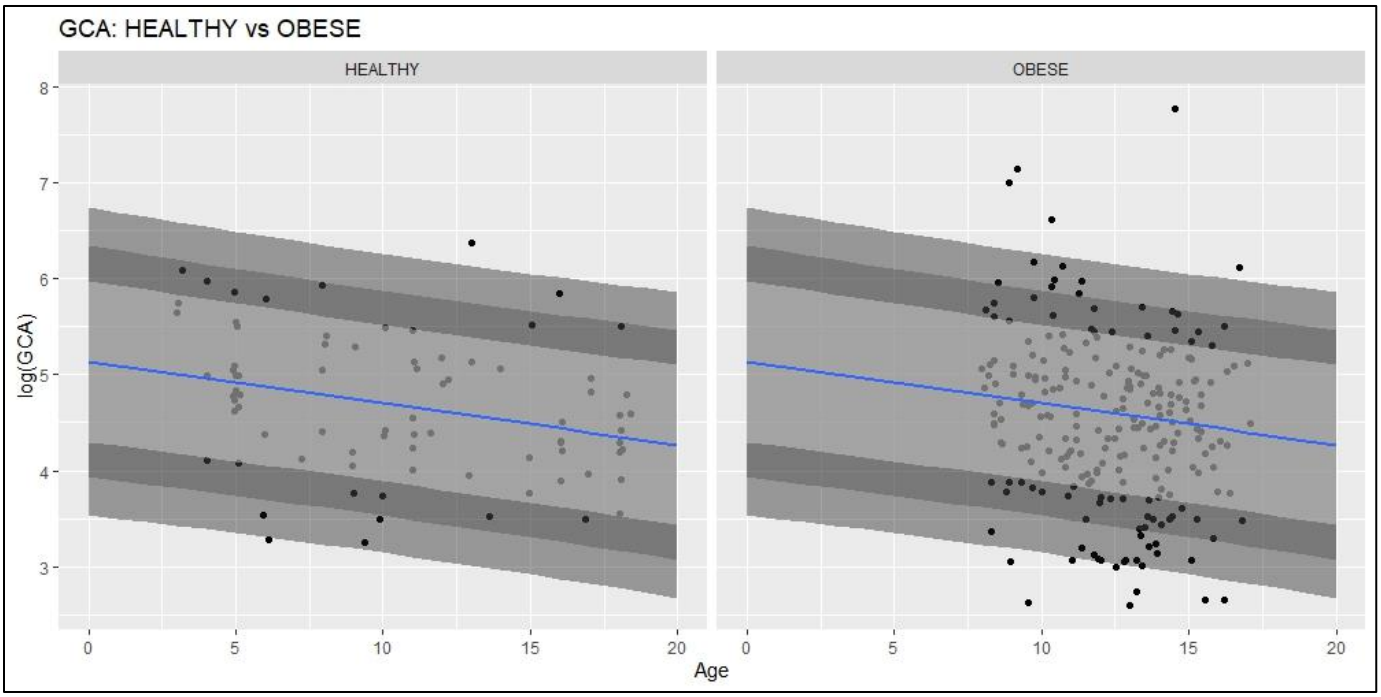
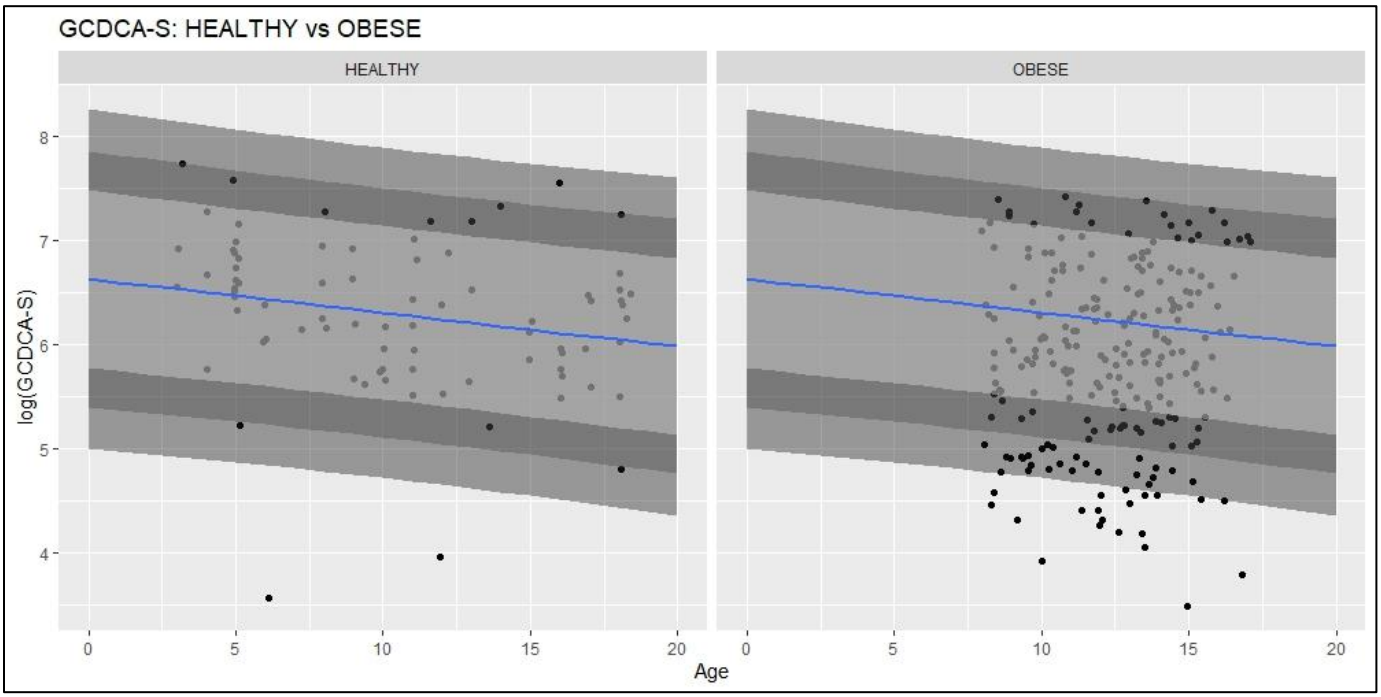


Figure 17 (1/3). Percentile curves for urinary bile acids concentration [nmol/L] of our study's six most abundant bile acids.
The different shade transitions of background hatching from bottom to top represent the percentiles P3, P10, P25, P75, P90 and P97. The blue line in the middle marks the median (P50). For calculation of the curves, the natural logarithm of the bile acids results was used in sake of emulating a normal distribution. For clarity reasons, the samples with results below the limit of quantification were excluded from the calculation.


GCDCA-S: HEALTHY vs OBESE
HEALTHY
OBESE
log(GCDCA-S)
8
7
6
5
4
0
5
10
15
20
Age

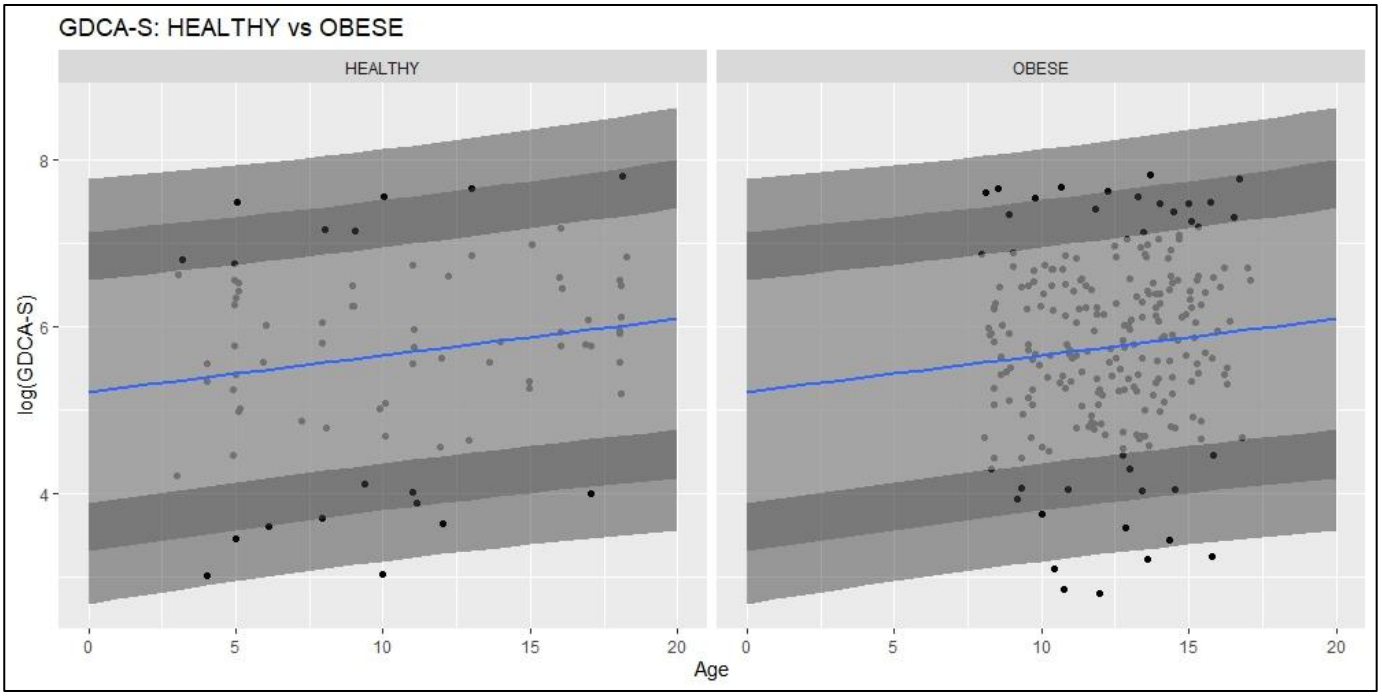
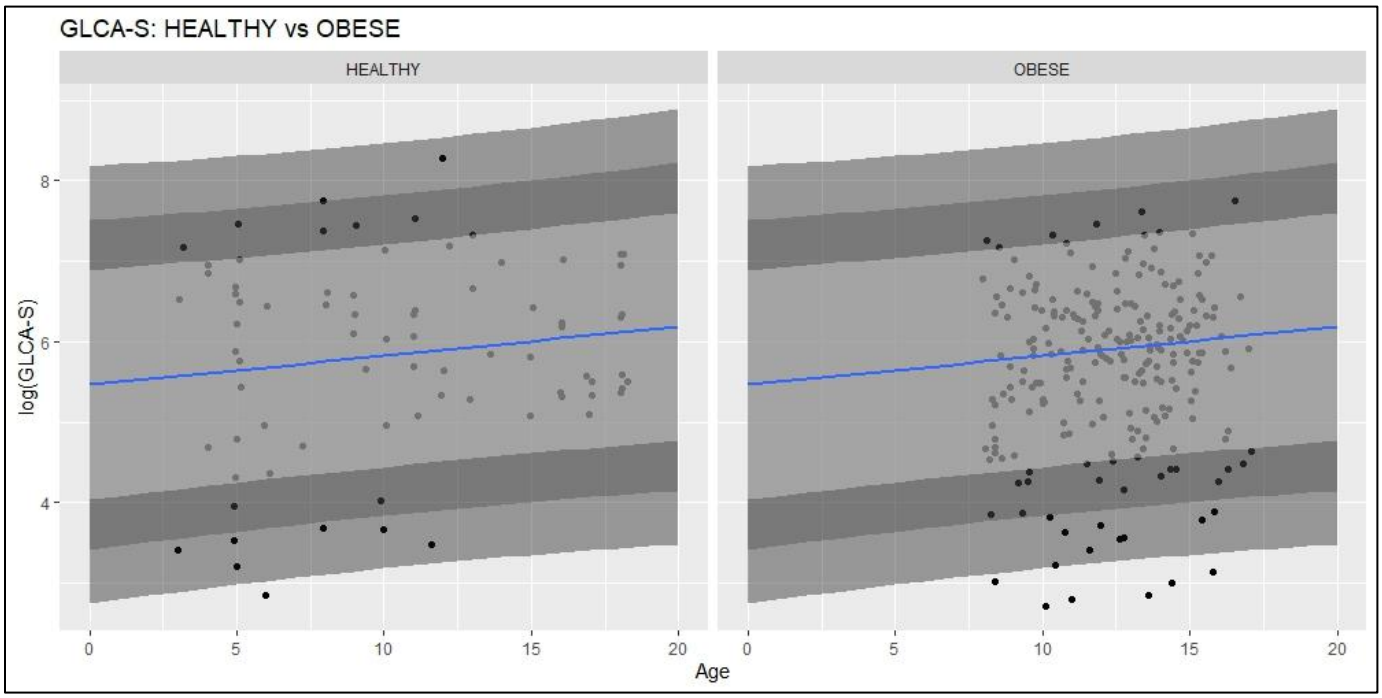
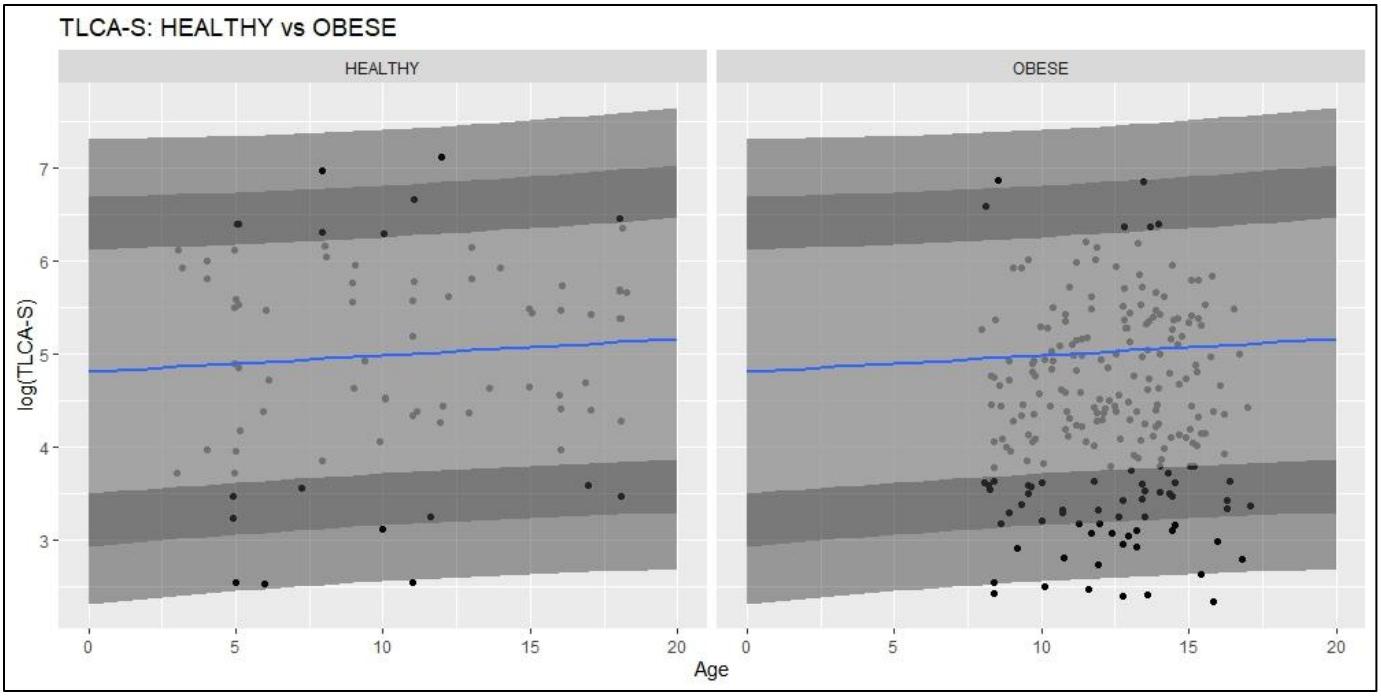

GDCA-S: HEALTHY vs OBESE
HEALTHY
OBESE
log(GDCA-S)
8
6
4
0
5
10
15
20
Age

Figure 17 (2/3)

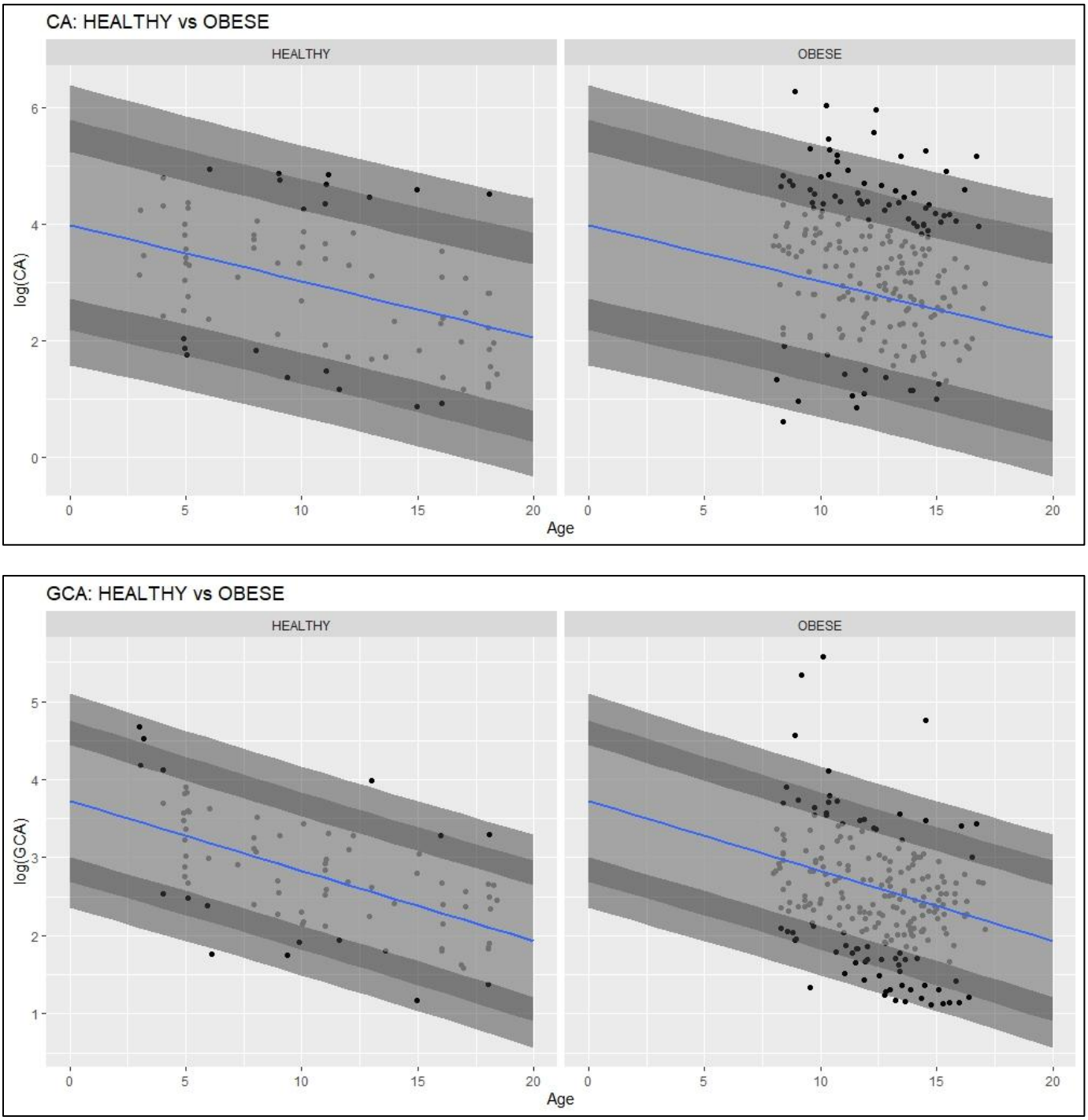

GLCA-S: HEALTHY vs OBESE
HEALTHY
OBESE
log(GLCA-S)
8
6
4
0
5
10
15
20
Age


TLCA-S: HEALTHY vs OBESE
HEALTHY
OBESE
log(TLCA-S)
7
6
5
4
3
0
5
10
15
20
Age

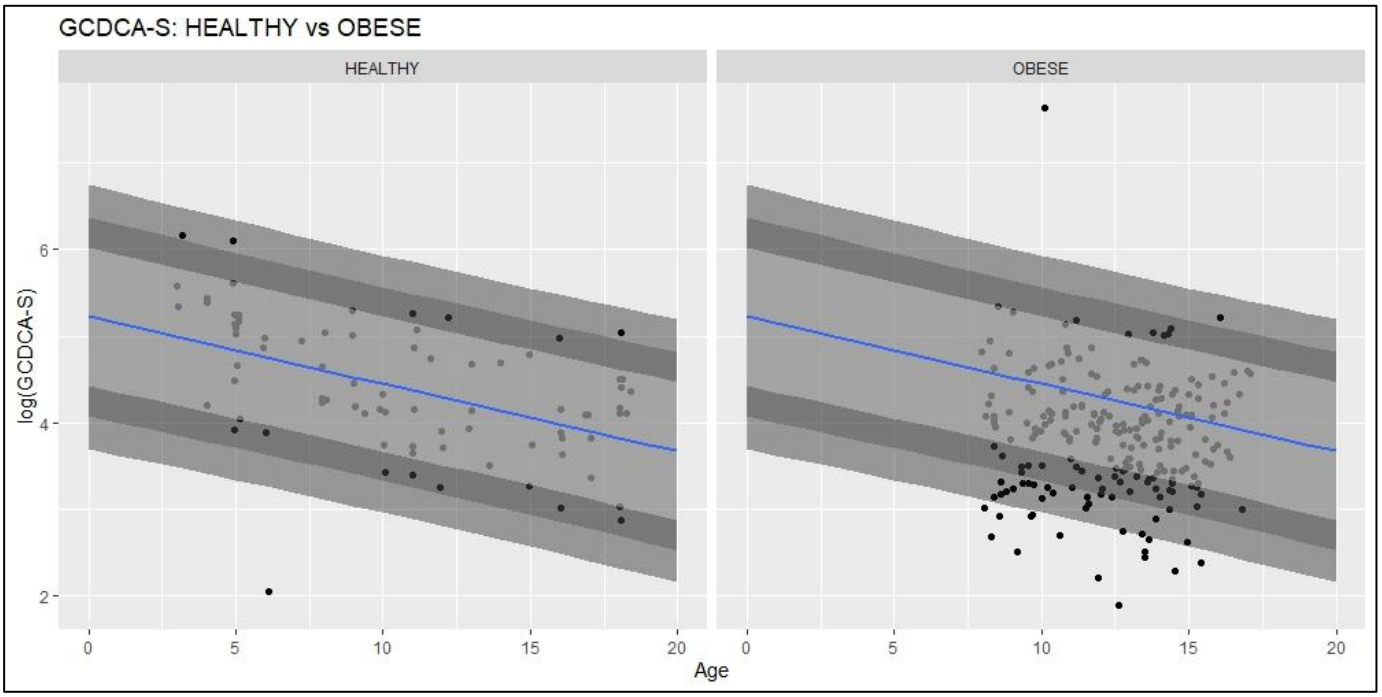
F*igure 17 (3/3)*

Regarding the healthy group and consequently the created percentile curves, a statistically significant age correlation in nmol/L only appeared for GCA with a decrease of bile acids concentration with age, which is reflected in the respective percentile graph. However, the percentile curves for the remaining five depicted bile acids still show some age-related variations even though they are not statistically significant.

Significant group differences in nmol/L appeared for GCDCA-S and TLCA-S. Both of them showed higher results in healthy children, which is illustrated by the fact that the respective obese scatter dots (right images) lie in the lower area of the referential percentile curves.



F*igure 18 (1/3). Percentile curves for creatinine-related urinary bile acids concentration [nmol BA/mmol Crea] of our study's six most abundant bile acids.*
The different shade transitions of background hatching from bottom to top represent the percentiles P3, P10, P25, P75, P90 and P97. The blue line in the middle marks the median (P50). For calculation of the curves, the natural logarithm of the bile acids results was used in sake of emulating a normal distribution. For clarity reasons, the samples with results below the limit of quantification were excluded from the calculation.


GCDCA-S: HEALTHY vs OBESE
HEALTHY
OBESE
log(GCDCA-S)
6
4
2
0
5
10
15
20
Age

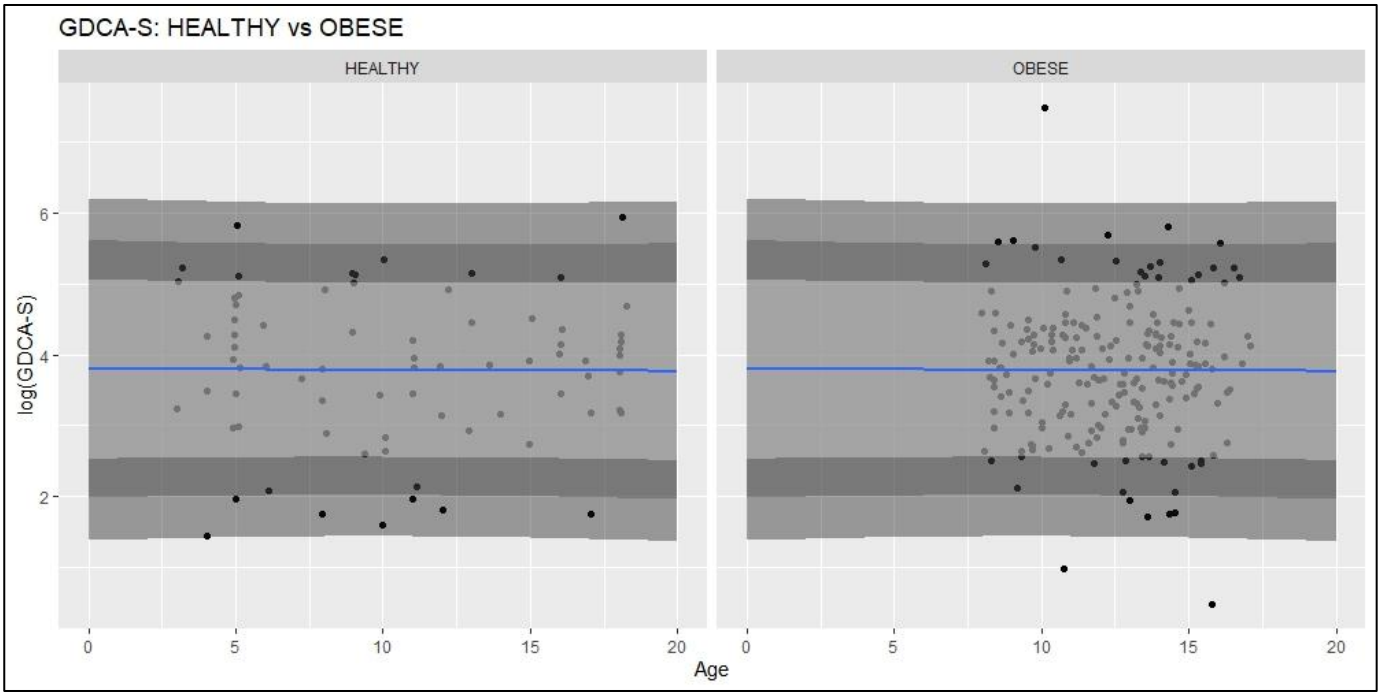

GDCA-S: HEALTHY vs OBESE
HEALTHY
OBESE
log(GDCA-S)
6
4
2
0
5
10
15
20
Age

Figure 18 (2/3)

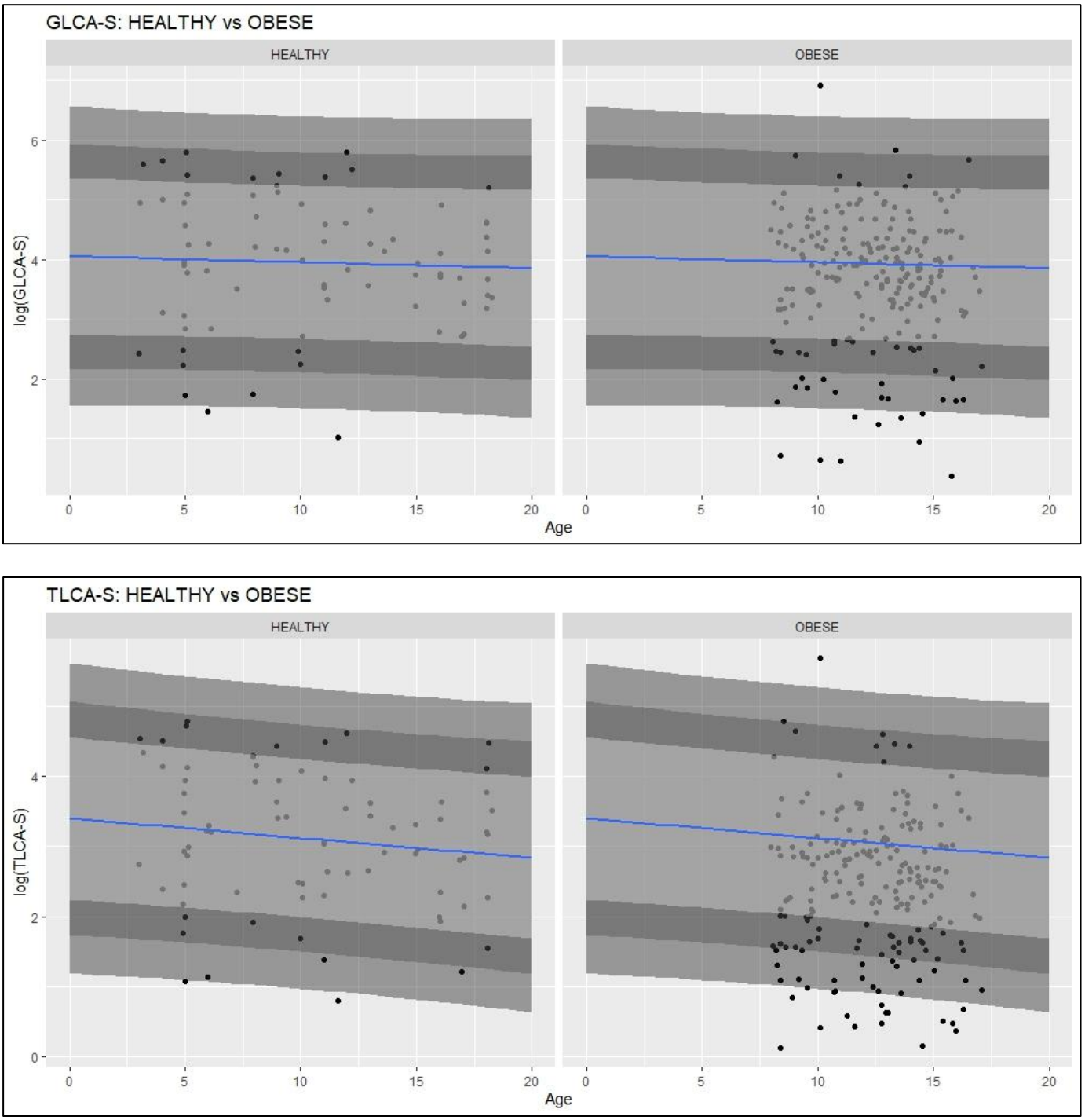
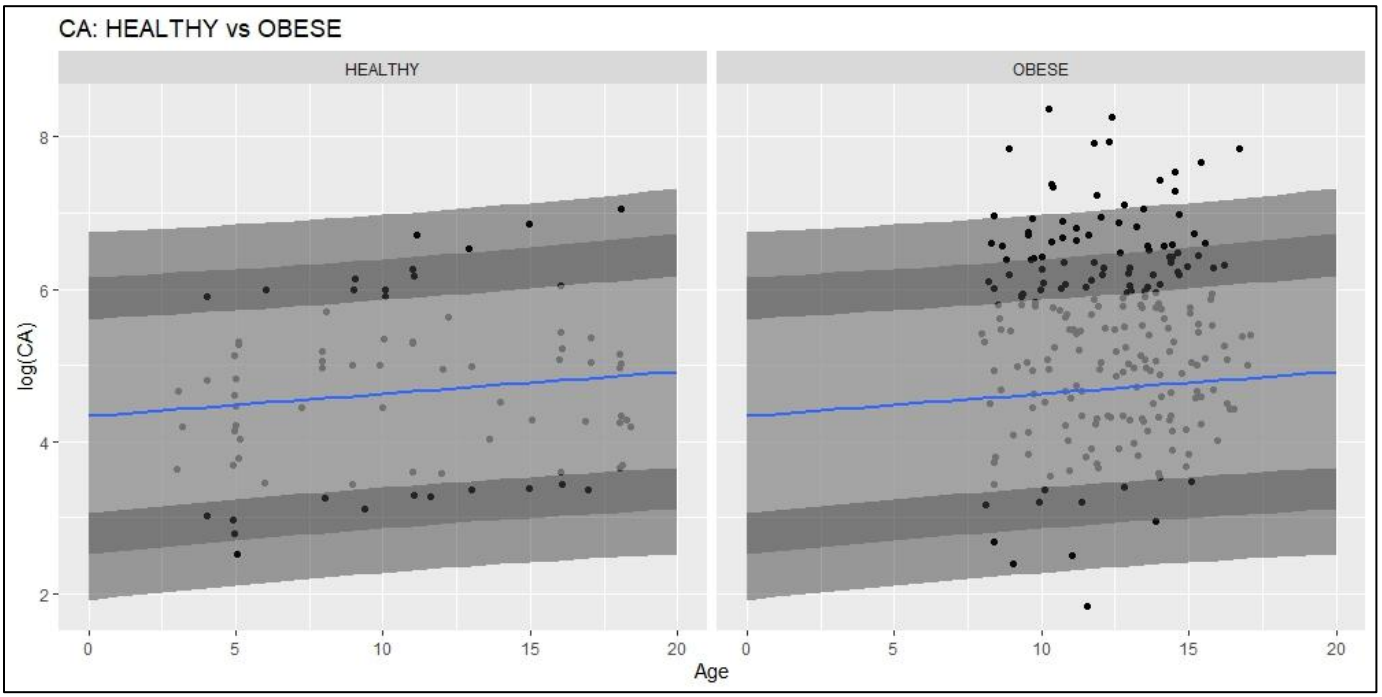

GLCA-S: HEALTHY vs OBESE
HEALTHY
OBESE
log(GLCA-S)
6
4
2
0
5
10
15
20
Age
TLCA-S: HEALTHY vs OBESE
HEALTHY
OBESE
log(TLCA-S)
4
2
0
0
5
10
15
20
Age

Figure 18 (3/3)

For creatinine-related bile acids concentration in the healthy group, significant negative age correlations appeared for CA, GCA, GCDCA-S and TLCA-S. Concordant with that, the respective percentile curves also show a distinct decline with age, while the curves for GDCA-S and GLCA-S run relatively horizontal.

Significant group differences were shown for GCDCA-S and TLCA-S. Once again, this is clearly visible by the obese scatter dots in the right images laying lower than the referential healthy percentile curves.



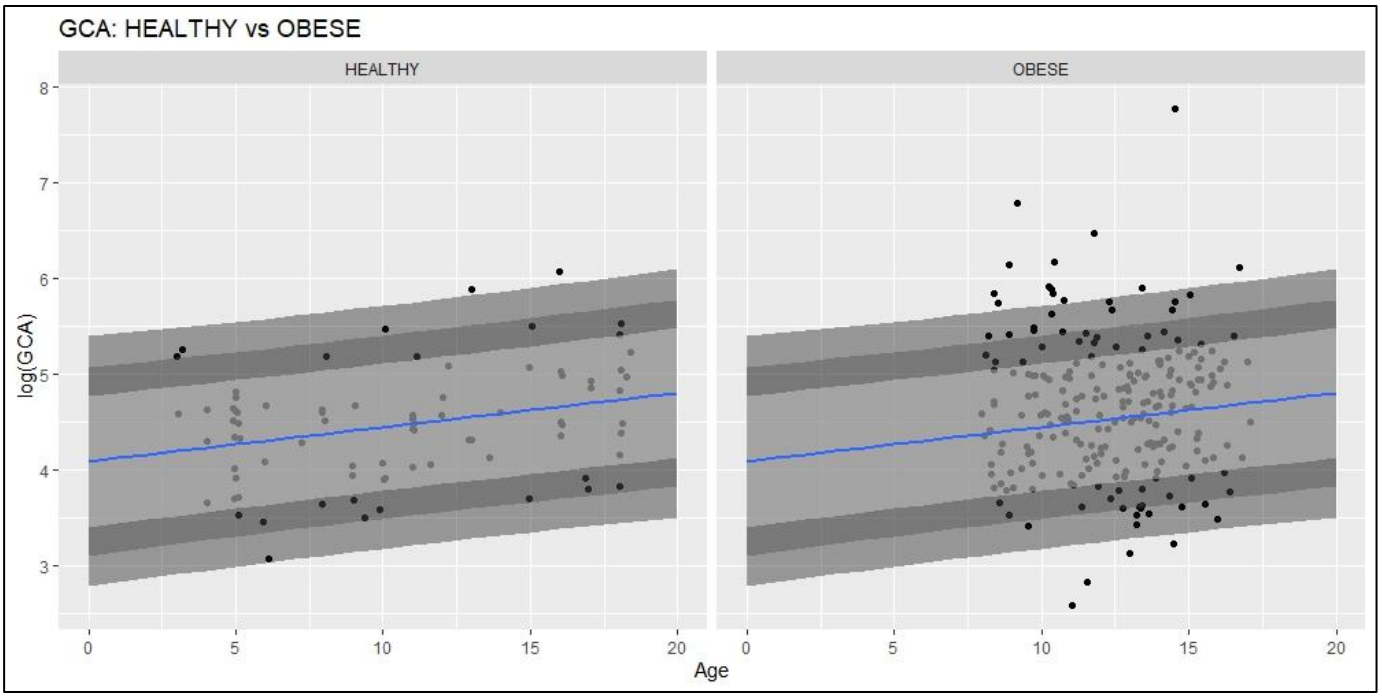
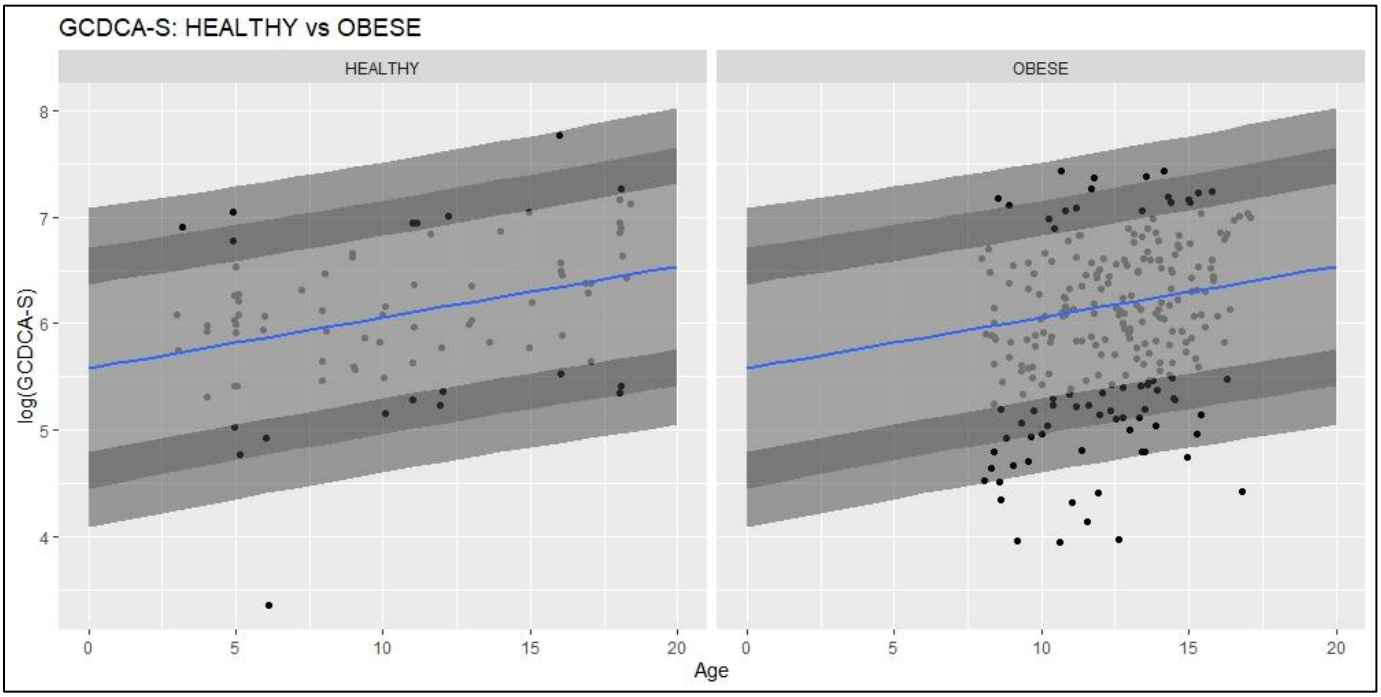


Figure 19 (1/3). Percentile curves for daily urinary bile acids excretion [nmol/d] of our study's six most abundant bile acids.
The different shade transitions of background hatching from bottom to top represent the percentiles P3, P10, P25, P75, P90 and P97. The blue line in the middle marks the median (P50). For calculation of the curves, the natural logarithm of the bile acids results was used in sake of emulating a normal distribution. For clarity reasons, the samples with results below the limit of quantification were excluded from the calculation.


GCDCA-S: HEALTHY vs OBESE
HEALTHY
OBESE
log(GCDCA-S)
Age

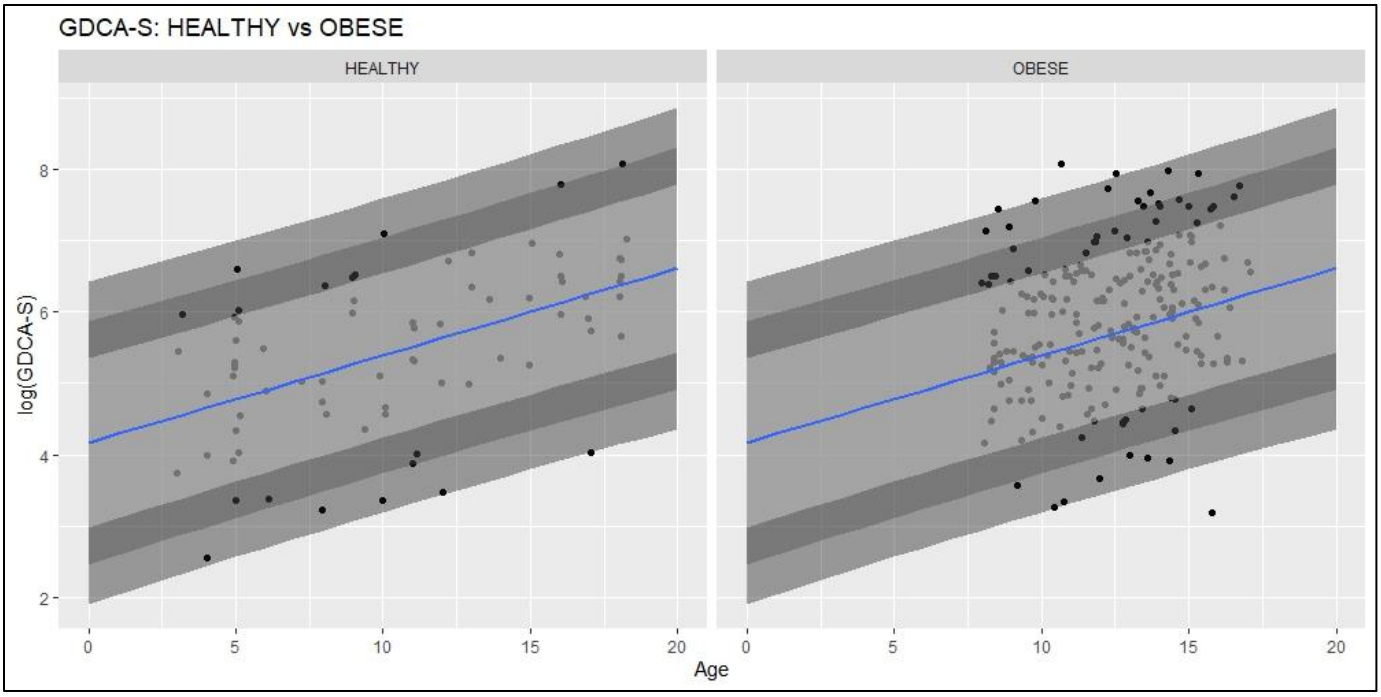
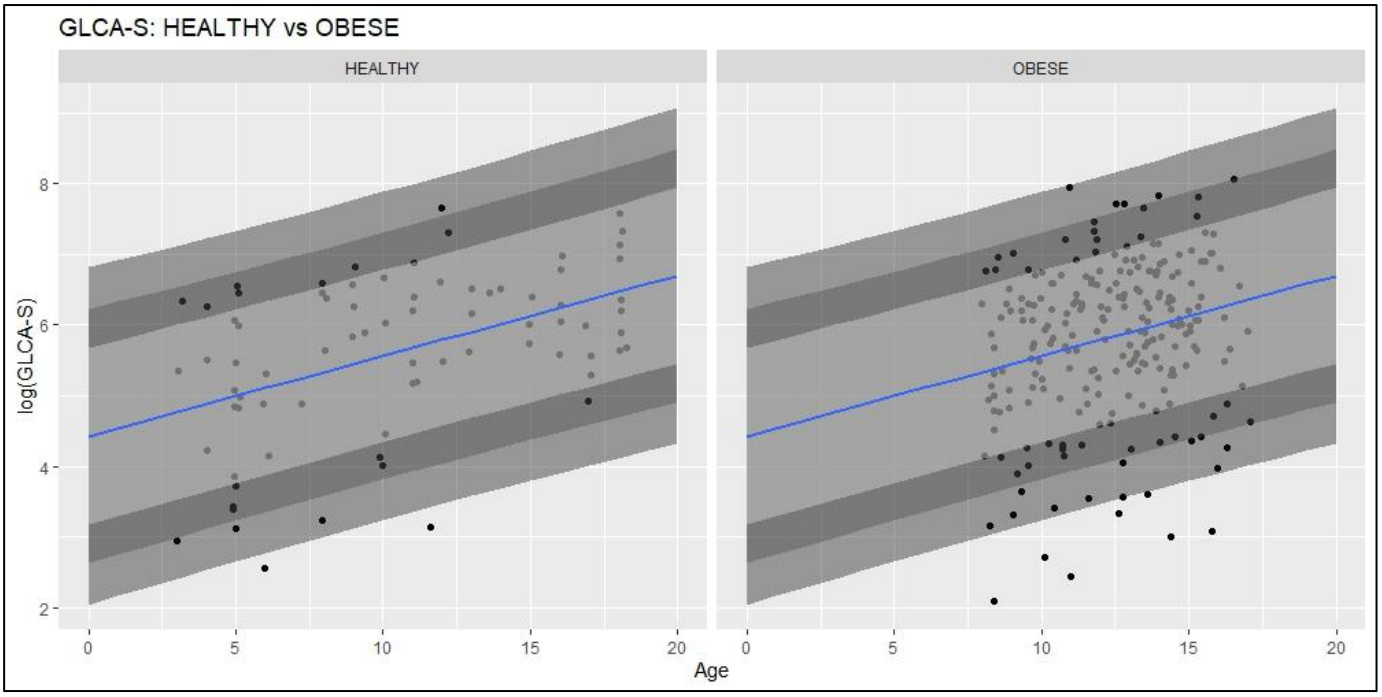

GDCA-S: HEALTHY vs OBESE
HEALTHY
OBESE
log(GDCA-S)
Age

Figure 19 (2/3)


GLCA-S: HEALTHY vs OBESE
HEALTHY
OBESE
log(GLCA-S)
8
6
4
2
0
5
10
15
20
Age

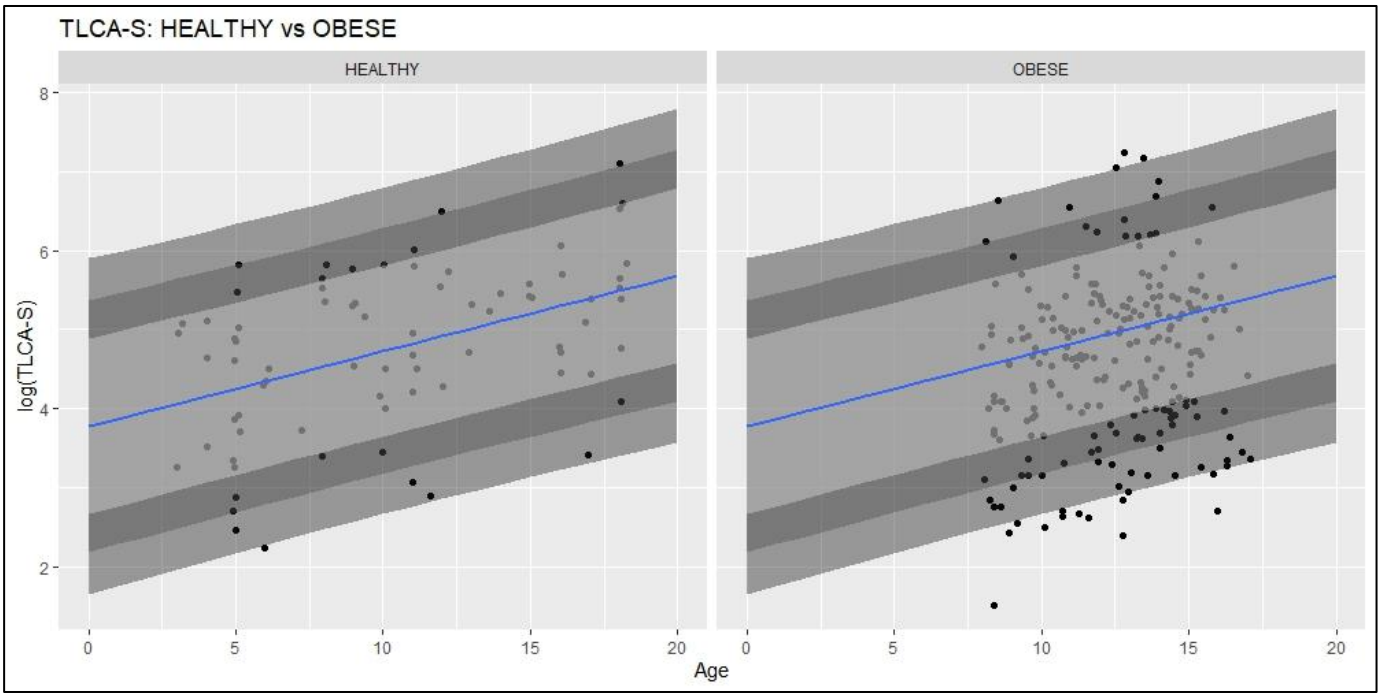

TLCA-S: HEALTHY vs OBESE
HEALTHY
OBESE
log(TLCA-S)
8
6
4
2
0
5
10
15
20
Age

Figure 19 (3/3)

In the healthy group's daily urinary bile acids excretion, significant age correlations were only spotted for GCDCA-S, whose percentile curves visibly rise with age. However, the other five depicted bile acids also show percentile curves increasing with age, even though their regression analysis results were not statistically significant.

Statistically significant group differences resulted for CA, GCDCA-S and GDCA-S. While daily excretion of CA and GDCA-S was higher in obese children, GCDCA-S was excreted comparatively more abundantly by healthy children. Figure 19 visualizes this in the respective right images for each of said bile acids. Regarding CA and GDCA-S, the obese scatter dots lie relatively higher compared to the referential percentile curves, whereas the obese dots for GCDCA-S lie lower.

Amidation and Sulfation Percentage

Comparing health and obesity, a comparison of the above mentioned amidation and sulfation percentages within both study groups was also done.

In both groups, unsulfated lithocholic acid derivatives appeared in a non-quantifiable amount – regardless of their amidation state (LCA, GLCA and TLCA were below the LOQ in all samples). These three derivatives appeared in both groups to 100% in their sulfated forms (LCA-S, GLCA-S, TLCA-S). However, the sulfation percentages of glycochenodeoxycholic acid and glycodeoxycholic acid were slightly lower in obese children (see Table 26). For glycodeoxycholic acid this group difference could be proved statistically significant (see Table 27).

	HEALTHY	**OBESE**
GCDCA / GCDCA-S	0.0 ± 0.0 / 100.0 ± 0.0	1.1 ± 5.6 / 98.9 ± 5.6
GDCA / GDCA-S	1.6 ± 2.3 / 98.4 ± 2.3	4.3 ± 8.5 / 95.7 ± 8.5

Table 26. Juxtaposition of healthy and obese sulfation percentages (mean ± SD) for glycochenodeoxycholic acid and glycodeoxycholic acid.

	p-value		
	Age	**Sex**	**Study Group**
GCDCA (Sulfation Percentage)	0.559	0.624	0.070
GDCA (Sulfation Percentage)	0.151	0.547	**0.003**

Table 27. Linear regression analysis for group differences regarding sulfation percentage. The sulfation percentage for GDCA was significantly lower in the obese group.

Figure 20 delivers visualization of the amidation states' distribution within both study groups. Looking at the amidation states in both groups, cholic acid was mainly divided into its unamidated (CA) and glycine-amidated form (GCA). In obesity, the unamidated form made up more than 50%. Taurine-amidation (TCA) only appeared to a small extent in both groups. A similar case applied for deoxycholic acid, where taurine-amidation (TDCA) played an even smaller role in both groups. In lithocholic acid sulfate, however, the prevailing glycine-amidation (GLCA-S) was followed by taurine-amidation (TLCA-S), while the unamidated form (LCA-S) hardly occurred. The most visual group differences appeared for chenodeoxycholic acid: In healthy children, glycine-amidation (GCDCA) did not occur at all and taurine-amidation (TCDCA) represented the vast majority, followed by the unamidated form (CDCA). In obesity on the other hand, the unamidated form made up the lion's share, followed by TCDCA. Unlike in healthy children, GCDCA also contributed about 14% in the obese group.

Significant group differences in amidation appeared for cholic acid, chenodeoxycholic acid and lithocholic acid sulfate (Table 28).

		p-value		
		Age	**Sex**	**Study Group**
Cholic Acid	**CA**	0.751	0.074	**<0.001**
	GCA	0.650	0.084	**0.005**
	TCA	0.366	0.372	**<0.001**
Chenodeoxy-cholic Acid	**CDCA**	0.146	0.454	**<0.001**
	GCDCA	0.351	0.563	**0.009**
	TCDCA	0.289	0.209	**<0.001**
Deoxycholic Acid	**DCA**	0.287	0.855	0.096
	GDCA	0.308	0.852	0.089
	TDCA	0.124	0.929	0.303
Lithocholic Acid	**LCA-S**	0.712	0.074	0.563
	GLCA-S	0.054	0.075	**<0.001**
	TLCA-S	0.052	0.129	**<0.001**

Table 28. Linear regression analysis for group differences regarding amidation percentage. Significant results appeared for cholic acid, chenodeoxycholic acid and lithocholic acid sulfate.

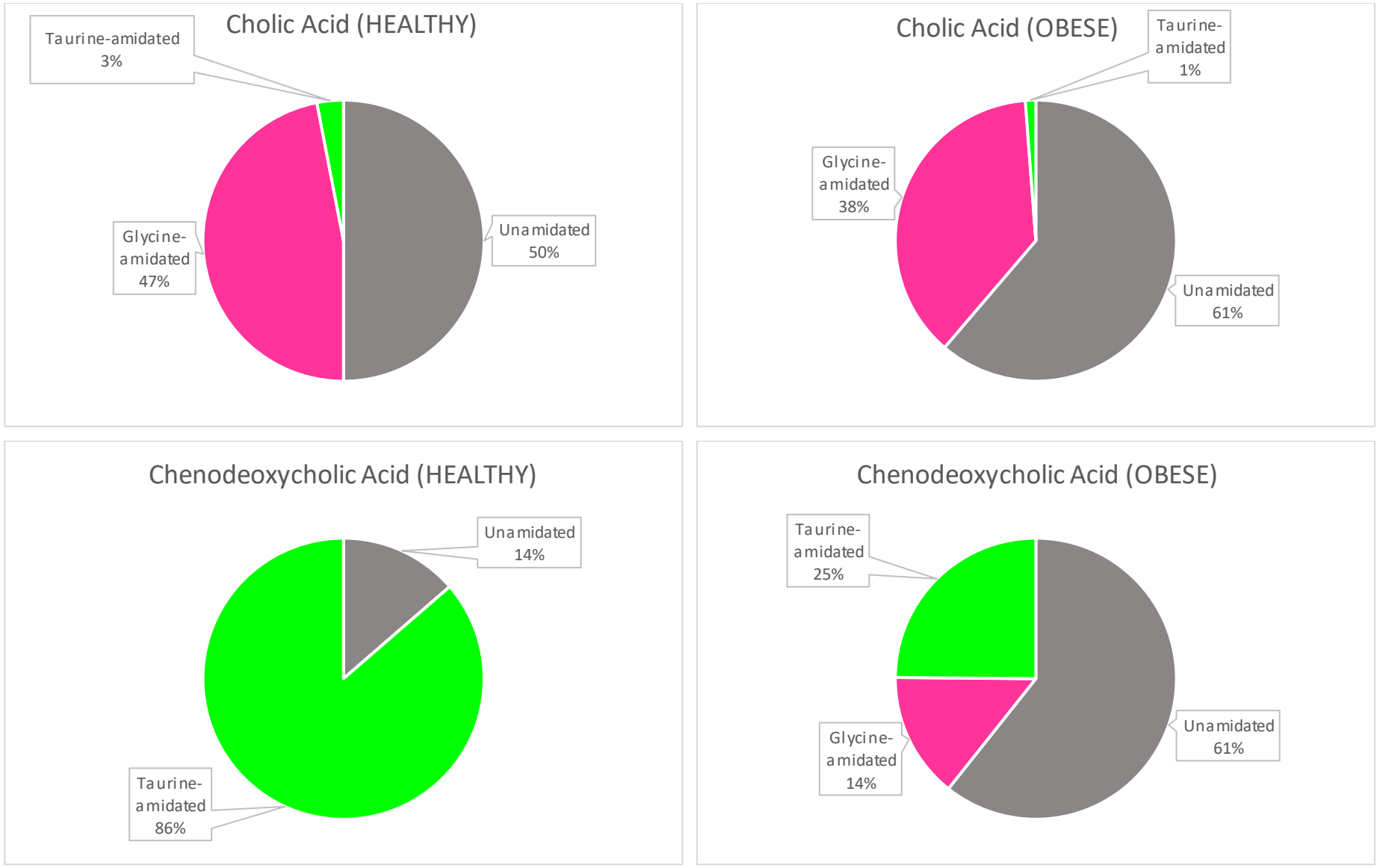


Figure 20 (1/2). Pie charts for visualization of mean bile acids amidation percentages and comparison between healthy (left) and obese (right) group. Grey = unamidated; magenta = Glycine-amidated; green = Taurine-amidated; 0% = small share rounded down to 0%; amidation-forms that did not appear in one group were excluded from the respective chart.

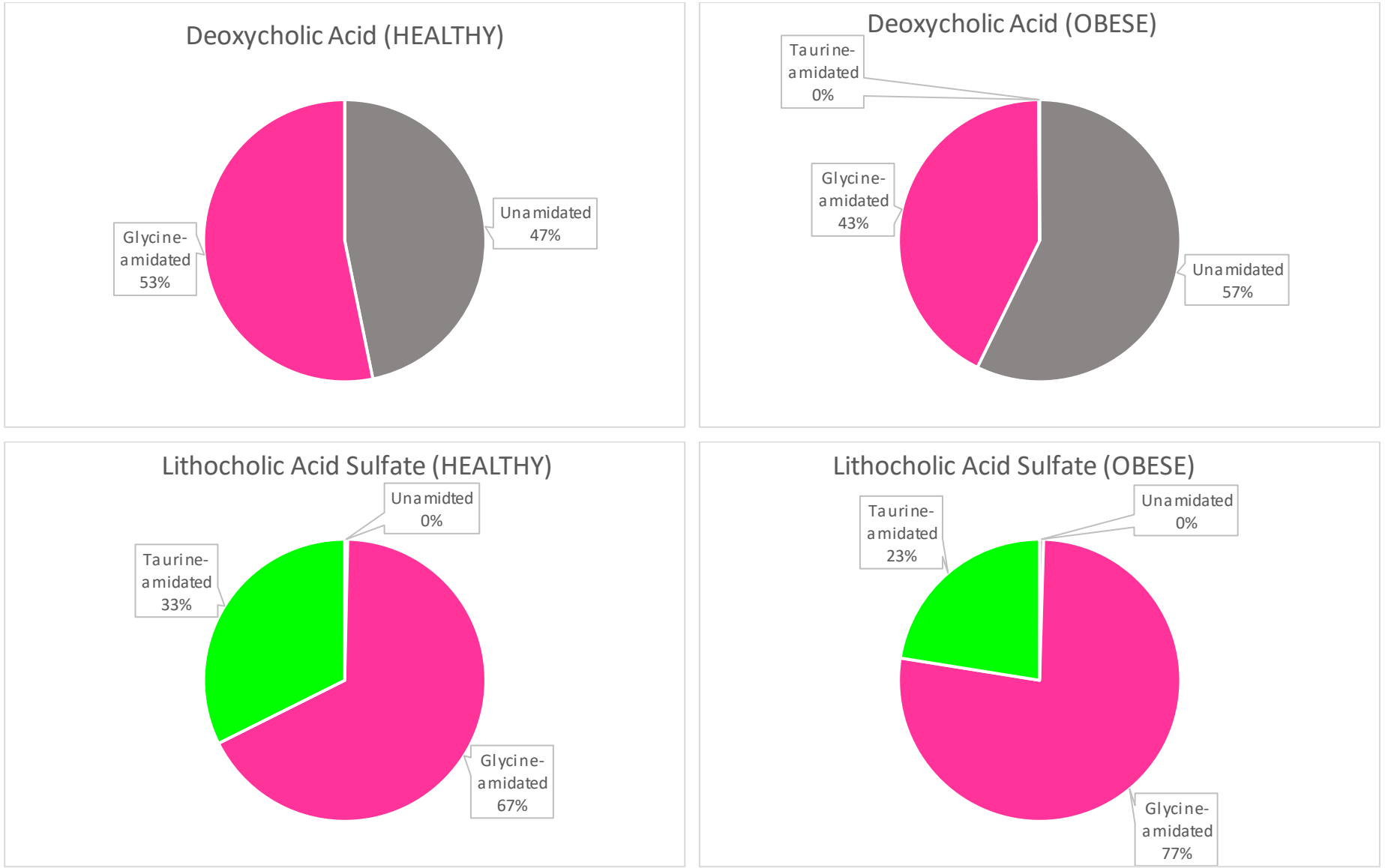


Figure 20 (2/2). Pie charts for visualization of mean bile acids amidation percentages and comparison between healthy (left) and obese (right) group. Grey = unamidated; magenta = Glycine-amidated; green = Taurine-amidated; 0% = small share rounded down to 0%; amidation-forms that did not appear in one group were excluded from the respective chart.

3.4.5 Nutrition and urinary Bile Acids

In cooperation with the DONALD study group in Dortmund, we looked into possible correlations between urinary bile acids concentrations and nutrition of the 80 healthy subjects. Within the reference group, many analytes lay below the limit of quantification in the majority of the 80 samples. Therefore, it seemed reasonable to only examine potential nutritional influences on bile acids that were quantifiable in more than 50% of the reference group (> 40 quantifiable samples). This includes the following six analytes: CA, GCA, GCDCA-S, GDCA-S, GLCA-S and TLCA-S.

Since the DONALD study captures its probands' daily dietary intake during the time of sample collection, we were able to correlate each of these six urinary bile acids with dietary fat, protein and carbohydrate intake, representing the major nutrients. The calculations were done using the nutrient density approach introduced by Willet et al. (Willett et al., 1997) to account for possible interferences caused by total energy intake.

The detailed results of the regression analyses are depicted in Table 29. The only bile acid showing significant correlations with nutritional factors ($p < 0.05$) is GCDCA-S. This analyte correlated positively with fat and protein intake and negatively with carbohydrate intake. This means that probands with higher fat intake or higher protein intake, respectively, showed significant higher urinary concentrations of GCDCA-S, whereas higher carbohydrate intake was associated with significant lower concentrations of GCDCA-S in the probands' urine. We could not substantiate a significant correlation between any of the other five examined analytes and fat, protein or carbohydrate intake, respectively.

	CA (n = 73)	GCA (n = 75)	GCDCA-S (n = 76)	GDCA-S (n = 71)	GLCA-S (n = 73)	TLCA-S (n = 74)
Dietary fat intake[a]	–1.86 (–10.08, 6.35)	1.42 (–1.30, 4.15)	13.99 (0.79, 27.19)**	13.92 (–5.00, 32.84)	–1.52 (–18.18, 15.13)	–0.63 (–6.38, 5.11)
Total energy intake	0.14 (–0.04, 0.33)	0.06 (–0.002, 0.12)*	0.19 (–0.10, 0.48)	0.06 (–0.31, 0.44)	–0.36 (–0.69, –0.02)**	–0.28 (–0.41, –0.16)***
Urinary creatinine	–22.95 (–54.76, 8.87)	4.55 (–5.98, 15.07)	44.84 (–5.91, 95.59)*	–6.46 (–77.51, 64.59)	41.05 (–21.25, 103.35)	51.93 (27.96, 75.89)***
Age	14.28 (–9.85, 38.41)	–3.32 (–11.30, 4.67)	–19.95 (–58.68, 18.77)	49.13 (–2.41, 100.66)*	31.48 (–13.73, 76.68)	–1.68 (–19.18, 15.82)
Dietary protein intake[a]	–0.19 (–8.76, 8.38)	2.23 (–0.58, 5.03)	14.33 (0.71, 27.94)**	6.43 (–11.50, 24.36)	–1.08 (–16.68, 14.52)	0.05 (–5.94, 6.04)
Total energy intake	0.14 (–0.04, 0.33)	0.06 (0.004, 0.12)**	0.23 (–0.06, 0.53)	0.07 (–0.32, 0.45)	–0.36 (–0.69, –0.02)**	–0.28 (–0.41, –0.16)***
Urinary creatinine	–22.35 (–54.97, 10.27)	2.33 (–8.34, 13.00)	28.81 (–23.34, 28.76)	–12.39 (–85.85, 61.07)	42.03 (–21.53, 105.59)	52.10 (27.80, 76.40)***
Age	13.96 (–10.62, 38.54)	–1.95 (–9.99, 6.09)	–10.63 (–50.01, 28.76)	53.38 (0.54, 106.22)**	30.83 (–14.92, 76.58)	–1.77 (–19.44, 15.91)
Dietary carbohydrate intake[a]	0.46 (–2.66, 3.58)	–0.58 (–1.61, 0.45)	–5.47 (–10.45, –0.48)**	–4.85 (–11.94, 2.24)	0.27 (–5.95, 6.50)	0.13 (–2.05, 2.30)
Total energy intake	0.14 (–0.04, 0.33)	0.06 (–0.001, 0.12)*	0.20 (–0.09, 0.49)	0.07 (–0.31, 0.45)	–0.36 (–0.69, –0.02)**	–0.28 (–0.41, –0.16)***
Urinary creatinine	–22.46 (–54.24, 9.33)	4.15 (–6.35, 14.64)	40.64 (–9.92, 91.21)	–10.15 (–81.50, 61.19)	41.31 (–21.09, 103.71)	52.12 (28.23, 76.02)***
Age	13.99 (–10.14, 38.12)	–3.06 (–11.03, 4.91)	–17.48 (–56.10, 21.14)	51.40 (–0.24, 103.04)*	31.28 (–13.92, 76.48)	–1.79 (–19.26, 15.69)

*p < 0.1; **p < 0.05; ***p < 0.001;

[a]Calculated by using the nutrient density approach (nutrient residual model) introduced by Willett et al. (Willett et al., 1997)

Table 29. Regressions between bile acid analytes and energy-adjusted nutrients. The only significant correlations ($p < 0.05$) between bile acids and nutrients are visible for GCDCA-S. This analyte correlates positively with fat and protein intake and negatively with carbohydrate intake.

4 Discussion

The main goals of the present study were threefold: 1) development of a suitable analytical method using state of the art technology, 2) creation of reference values in healthy children, and 3) investigating bile acid excretion in obese children. In a short digression, potential nutritional effects on urinary bile acids were examined.

4.1 Method

The present method was developed for identification and quantification of 18 pre-selected urinary bile acids by targeted liquid chromatography-tandem mass spectrometry (LC-MS/MS). The method validation following the guidance by the United States Food and Drug Administration (FDA) (*Guidance for Industry: Bioanalytical Method Validation*, 2001) achieved excellent results meeting the FDA-default terms of reference. Successful method validation indicates the reliability and reproducibility of the method and the obtained data.

Since gas chromatography-mass spectrometry (GC-MS) delivers the uppermost specificity, it is considered gold standard in steroid analysis. LC-MS/MS, on the other hand, reaches lower specificity due to weaker chromatographic separation and less fragmentation during soft ionization. However, LC-MS/MS has advantages like measuring conjugated steroids as intact molecules (Wudy et al., 2018). This is of great use for bile acids analysis, because most of urinary bile acids appear in conjugated forms such as amides and sulfates and also glucuronides (Alnouti, 2009).

Furthermore, LC-MS/MS can be utilized as a high-throughput method due to its short run times and comparatively uncomplicated sample preparation. In contrast, GC-MS requires cleavage of conjugates and derivatization of samples prior to analysis (Wudy et al., 2018), which is more elaborate and time-consuming.

To compensate for the lack of specificity in LC-MS/MS compared to GC-MS, the present method uses a targeted approach. In this way, the analysis settings can be aligned to pre-selected analytes and it can be assured that clear chromatographic separation and precise mass spectrograms are reached for these analytes. This guarantees for the unambiguous identification and reliable quantification of said analytes. However, a drawback of targeted analyses is the need for chemical standards of the analytes in order to calibrate the method. There often are problems with commercial availability of required standards, especially when

it comes to complex molecules like polyconjugated bile acids. The same applies for the obtainability of suitable deuterated internal standards.

In general, the techniques of GC-MS and LC-MS/MS should be used in a complementary way by utilizing the advantages of each technique when needed (Griffiths and Sjövall, 2010; Wudy et al., 2018). GC-MS probably would be the method of choice for identification of isomeric bile acids in compound biological mixtures as well as for obtaining detailed information about bile acids' structures due to more specific fragmentation patterns by hard ionization. LC-MS/MS on the other hand is able to deliver data on bile acids conjugation (Sjövall et al., 2010).
Since the present method uses a targeted approach to analyze mostly conjugated bile acids, the validation data are hugely satisfying and the uncomplicated sample preparation and short run times are convenient for a potential routine application, it seems very reasonable to choose LC-MS/MS.

During the process of method development, there are plenty of decisions to make regarding the design of sample preparation and the analysis settings.
Although sample preparation for LC-MS/MS is rather uncomplicated compared to other techniques like GC-MS, it still prolongates the duration of an analytical run. Regardless of that, sample preparation is a crucial step within an LC-MS/MS method. The goal of sample preparation is the removal of interfering substances like proteins, salts and lipids (Griffiths and Sjövall, 2010). The necessity of sample preparation is justified due to several benefits. The removal of easily precipitating components (e. g. proteins) prior to chromatography prevents blockage of the LC column and therefore helps to circumvent tremendous increases in pressure during chromatography. Apart from the LC system, also the functionality of the mass spectrometer can be maintained more easily due to "cleaner" samples. As a result, an analytical run with preceded sample preparation tends to be gentler on the materials and the whole LC-MS/MS system. In addition to that, sample preparation can be utilized to turn a compound biological matrix into a more LC-suitable fluid by adjusting its properties and composition. In this fashion, the chromatographic performance can be maximized. Since interfering substances are depleted, while the requested analytes are preserved, sample preparation also leads to an increase in the analyte-to-matrix ratio. Consequently, disruptive matrix effects are diminished and the measurements lead to more reliable results. In summary,

sample preparation not only helps to preserve material and the whole LC-MS/MS system, it also leads to higher validity of the obtained results (Stone, 2018).

After acknowledging sample preparation as an essential part of the method in general, it needs to be determined which steps should be included during sample preparation in detail. For biological matrices containing bile acids in high concentration (e. g. bile), dilution may be an adequate way of sample preparation (Griffiths and Sjövall, 2010), as it lowers the concentration of interfering substances, while the amount of bile acids may still be high enough for reliable analysis. However, when it comes to measurement in urine, the amount of bile acids is expected to be rather low. Consequently, sample preparation should be a little more elaborate. Beginning with protein precipitation seems to be a reasonable approach, since it serves as a quick and uncomplicated first purification of the sample. In the present method this was done by using an aqueous $ZnSO_4$ solution (89 g/L) mixed 1:4 with acetonitrile. The following process of solid-phase extraction adds even more matrix depletion and in addition to that also helps concentrating the analytes (Stone, 2018). In older publications, solid-phase extraction has already been considered gold standard when it comes to extraction of free steroids and their conjugates from aqueous solutions, since it was shown that it enhances the recovery for certain steroids compared to sample treatment without solid-phase extraction (Shackleton, 1986). More recent publications have also confirmed the suitability of solid-phase extraction for urinary steroid analysis by LC-MS/MS. It has been described as the preferable extraction technique, especially in case of direct measurement of conjugated steroids, in which the non-polar side of steroid molecules allows the feasibility of solid-phase extraction (Rodríguez-Morató et al., 2018). More specifically related to bile acids, solid-phase extraction is also one of the most frequently applied techniques and especially C_{18} cartridges have already been used for evaluation purposes (Sjövall et al., 2010). This explains our choice to also use 'reversed-phase' solid phase extraction in form of C_{18} cartridges. The sorbent C_{18} provides for a reliable attachment of unconjugated and conjugated steroids to the cartridge - also during washing with water. The steroids' recovery can be done by eluting them from the cartridge using methanol (Shackleton, 1986). The intermediate washing step with hexane serves to eliminate vastly lipophilic substances from the cartridge.

The last step of sample preparation in the present method comprises the evaporation of the methanolic fraction and the following reconstitution. Due to reconstitution, it can be guaranteed that the analytes within each sample are dissolved in exactly the same defined volume (in the present case 250 µL), ensuring the comparability of the measured concentrations. Furthermore, reconstitution allows for an adjustment of the solvent, turning it

into an LC-suitable fluid (50% methanol, 40% water and 10% ammonia solution (2.5%)). This increases the LC performance, because shape and separation of chromatographic peaks as well as their retention times are influenced by solvent properties like pH and aqueous composition (Stone, 2018).
In summary, the protocol for sample preparation was chosen by analytical expertise in consideration of physicochemical principles underlying steroids - in this case bile acids - and the biological matrix of urine. The fact that method validation achieved gratifying results seems to confirm the capability of the chosen protocol. Another confirmation, which underlines this, is a publication by Humbert et al. (2012). They compared different sample preparation protocols for measurement of urinary bile acids by LC-MS/MS and came to the conclusion that protein precipitation using an acetonitrile-containing solution combined with following solid-phase extraction with C_{18} cartridges delivered the best results (Humbert et al., 2012).

Regarding liquid chromatography, our choice fell on reversed-phase LC (RPLC) performed on a core-shell particle phenyl-hexyl column. RPLC is one of the LC types classically used for steroid analysis and as such is widespread and well-tested in this field (Makin et al., 2010). When it comes to bile acids in particular, RPLC is also considered the better alternative compared to normal-phase LC (NPLC). The chromatographic performance of NPLC in measuring bile acids is limited, since bile acids possess numerous polar groups such as hydroxy groups within the sterane ring, the carboxyl group at C_{24} as well as the polar moieties of glycine, taurine and sulfate in case of conjugated bile acids. Regioisomers, such as CDCA and DCA, are considered inseparable by NPLC (Sjövall et al., 2010).
Several other publications analyzing urinary bile acids used ultra-high-performance liquid chromatography (UHPLC or UPLC) (Bathena et al., 2013; Sarafian et al., 2015; Thakare et al., 2018). UPLC uses columns with smaller particle sizes allowing for even better chromatographic resolution compared to HPLC. Due to the small particle sizes, UPLC requires the application of higher pressures which cannot be carried out on a regular HPLC system (González-Ruiz et al., 2015). Having used HPLC with its “weaker” chromatographic performance marks a potential drawback of our method. However, we tried to overcome this drawback by using a core-shell particle column. The core-shell technology utilizes particles with a dense core and a porous shell. This composition has beneficial effects promoting the chromatographic efficiency by reducing band broadening effects. Furthermore, by increasing column permeability, the setup of core-shell particles allows for high chromatographic flow

without the need for excessive pressure within the LC system. In summary, core-shell technology can be used as an opportunity to improve chromatographic performance within an existing HPLC system, whereas UPLC systems require special ultra-high-pressure equipment. This makes UPLC rather expensive and limits the number of laboratories where it is used. The implementation of a core-shell column within an existing HPLC system seems more economical and is also capable of high-level chromatographic performance, making it a good alternative to UPLC (González-Ruiz et al., 2015).

As mentioned above, the chromatographic separation of regioisomers like CDCA and DCA is only considered feasible using RPLC. More specifically, it should be done using RPLC with a neutral or minimally basic mobile phase. With bile acids being usually present in their anionic form, the pH value of the mobile phase has a major influence on their retention time - and as a consequence on their chromatographic separation (Sjövall et al., 2010). This is why in our protocol mobile phase A was strictly adjusted to pH 7 with 10 mM ammonium acetate. In general, phosphate buffers are commonly used for pH adjustments of mobile phases in RPLC. When it comes to LC-MS, however, the interface between LC and MS (in our case electrospray ionization) requires a more volatile buffer (Sjövall et al., 2010). Therefore, the present method uses ammonium acetate.
The use of acetonitrile in our mobile phase mixtures seemed reasonable, since it is not only suitable for chromatography, but is also considered a good solvent for coupling with ESI-MS (Sjövall et al., 2010).

Our choices of LC column and mobile phases allowed us to reliably identify the 18 targeted bile acids. We could obtain a distinct chromatographic peak for each one of them. Most importantly, our LC method was able to achieve the necessary chromatographic baseline separation for regioisomeric bile acids that were quantified at the same mass transition. This concerns the analytes UDCA, CDCA and DCA (m/z 391.3 → 391.3); GCDCA and GDCA (m/z 448.0 → 448.0); TCDCA and TDCA (m/z 498.2 → 124.0); as well as GCDCA-S and GDCA-S (m/z 263.7 → 97.1). (Figure 6, Table 3)

LC on its own or LC coupled with non-specific detection methods is not considered a sufficient way of measuring and quantifying urinary bile acids. This is due to the low concentration of most individual bile acids in urine on the one hand and due to the variety of

the urinary bile acids pool on the other hand. A trusted detection method in this case is ESI-MS (Sjövall et al., 2010).

In order to couple liquid chromatography to mass spectrometry, soft ionization is necessary (Griffiths and Sjövall, 2010). There are different forms of soft ion sources, atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) being the prevailing ones. While ESI is primarily used for measurement of analytes that are easily ionized or already present as ions, APCI is the technique of choice for less polar analytes (Wudy et al., 2018). Since bile acids belong to the former kind of analytes, the application of ESI in the present method was a logical consequence. A disadvantage of soft ionization forms compared to hard ionization is that they are strongly prone to matrix effects (Wudy et al., 2018). The present method tries to circumvent matrix-adulterated results by doing adequate sample preparation, using deuterated internal standards and preparing calibration curves with chemical standards in a matrix surrogate (charcoal-stripped urine). In this way, matrix effects ought to be reduced to an unavoidable minimum and a satisfactory recovery should be received. Potential influence of the biological matrix urine on measurements has been assessed during method validation and since the FDA-default standards have been met, matrix effects can be considered neglectable in the present study.

Literature states characteristic product ions to be expected when measuring certain bile acids using ESI-MS/MS in the negative mode. Taurine conjugated bile acids tend to produce product ions at m/z 124, 107 or 80, whereas glycine conjugated bile acids typically deliver fragments at m/z 74. At m/z 97, product ions of sulfated bile acids can be anticipated (Griffiths and Sjövall, 2010; Sjövall et al., 2010). Achieving concurrent MS results, we used some of these expected product ions for quantification of our urinary bile acids. Fragmentation to m/z 124 was used for quantification of TCA, TCDCA, TDCA and TLCA. GLCA was quantified using the product ion at m/z 74. Finally, all five sulfated bile acids that were included in our method were quantified with the product ion at m/z 97. This includes GCDCA-S, GDCA-S, LCA-S, GLCA-S and TLCA-S.
Furthermore, most of the mass transitions we used for quantification are concordant with findings from previous publications on profiling bile acids by LC-MS/MS (Huang et al., 2011; Humbert et al., 2012; Sánchez-Guijo et al., 2016; Tagliacozzi et al., 2003; Ye et al., 2007). For several of our targeted bile acids, the best MS results were achieved by quantification using a product ion that equals the precursor ion. For instance, this applies for

all analyzed unconjugated bile acids. The absence of workable fragment ions may be due to the high stability of the bile acids' core structure making them less prone to collision fragmentation when there is no amino acid or sulfate group to be cleaved off. This phenomenon has been described before (Huang et al., 2011; Tagliacozzi et al., 2003; Ye et al., 2007). Measuring the characteristical fragmentation pattern of an analyte – its mass transition – is crucial for the high specificity provided by tandem mass spectrometry. As a consequence, the application of multiple reaction monitoring (MRM) with "pseudo transitions" where the product ion equals the precursor ion certainly lowers the specificity of the method (Griffiths and Sjövall, 2010; Sjövall et al., 2010; Tagliacozzi et al., 2003; Ye et al., 2007). However, this approach still provides higher specificity than single ion monitoring (SIM). For example, interfering substances with the same precursor ion as a particular analyte could undergo fragmentation in the collision cell and therefore not deliver the same product ion in high intensity (Tagliacozzi et al., 2003; Ye et al., 2007).

Transfer to nanomolar units

Preceding further data processing, the results were transferred from the initial unit of ng/mL to nmol/L. Since individual bile acids have different molar masses, it seems more suitable to use amount of substance instead of mass for the sake of better comparability between different individual bile acids. Therefore, nmol/L was used as the basic unit for urinary bile acids concentration.

Another advantage in using molar units is the better comparability of the present results to previous publications, which also mainly presented their results in nmol/L (Alamoudi et al., 2021; Bathena et al., 2015b, 2013; Humbert et al., 2012).

The inspiration for using a creatinine-related unit also originated from previous publications - one of which was using ng BA/mg Crea (Goto et al., 2007), while the other one used nmol BA/mmol Crea (Taylor et al., 2014). Generally speaking, creatinine-relation (also called creatinine-correction) is applied to correct for the proband's hydration state (Jain, 2016). This is helpful especially when working with spot urine, where creatinine-relation can be used to estimate 24-hour excretion without actual 24-hour urine collection (Remer et al., 2002). In case of bile acids, it has been reported that creatinine-related urinary bile acids strongly correlate with fasting plasma bile acids when comparing health and liver disease (Simko et al., 1987).

Another way of correcting for hydration state is working with actual 24-hour urine samples and expressing results as daily excretion rates. Daily excretion is also the way in which most

other urinary steroids' amounts are presented (Remer et al., 2009; Wudy et al., 2007) - albeit usually in µg/d instead of nmol/d.

In summary, all three units ([nmol/L], [nmol BA/mmol Crea], [nmol/d]) seemed to be a reasonable way of presenting the results. Since we aimed at establishing the first reference values for urinary bile acids in healthy children, we wanted to cover a wide range of possible forms of data presentation and therefore we used all three units.

4.2 Results

4.2.1 Study Groups

Urinary bile acids were investigated in 80 healthy children (DONALD group) and 237 obese children (MULTIOMICS group). Apart from sex distribution (f:m = 50:50 in DONALD group vs. 47:53 in MULTIOMICS group), the two groups significantly differed in every aspect. As for the aspects of weight and BMI, these group differences were intentionally chosen, since part of the present investigations consisted in comparing urinary bile acids in healthy and obese children. The differences regarding the other aspects, however, occurred unintentionally and led to difficulties in comparing the two groups. To be emphasized here is certainly the age difference, because age alterations in children consequently cause alterations of the remaining group aspects. When looking into children, the height naturally increases with age. In the same way, the weight in the MULTIOMICS group might not only be higher because of the probands' obesity, but also because of the higher average age in this group. Likewise, the excreted 24-hour urine volume rises with age (Beckford et al., 2020). Furthermore, creatinine excretion is reported to increase with age and is also influenced by height and weight (Remer et al., 2002). Taking this into account, the age difference could possibly explain the other group differences.

In conclusion, comparisons of urinary bile acids excretion between the two groups could be influenced by the apparent age difference itself but also by the resulting differences in height, weight, 24-hour urine volume and creatinine concentration. Especially the units nmol BA/mmol Crea and nmol/d are prone to the group differences in creatinine concentration or 24-hour urine volume, respectively. Consequently, the statistical analysis had to correct for possible age influences. This was done by not only including sex and study group in the linear regression analysis for significant group differences, but also including age.

4.2.2 Urinary Bile Acids in healthy Children

According to literature research, data on urinary bile acids concentrations in children are lacking. The present results of the 18 targeted analytes were therefore compared to previously published urinary bile acids concentrations in healthy adults (Alamoudi et al., 2021; Bathena et al., 2013; Humbert et al., 2012).

Looking at the 18 targeted bile acids, the same six bile acids (GCDCA-S, GDCA-S, GLCA-S, TLCA-S, CA and GCA) showed the highest concentrations - with small changes in order of abundance (Alamoudi et al., 2021; Bathena et al., 2013; Humbert et al., 2012). Only Humbert et al. did not include GCDCA-S and GDCA-S in their method. The general areas in which the results for these six analytes landed are comparable, although for some of them there were deviations in mean concentration of several hundred nmol/L. Especially GCDCA-S and GDCA-S were reported in markedly higher concentration by other publications (see Table 30).

The remaining analytes apart from the six most abundant bile acids showed low level concentrations throughout all studies. Their mean concentration consistently lay below 20 nmol/L and on a regular basis even below 10 nmol/L or 5 nmol/L (only exception: DCA with 22 nmol/L in one study (Alamoudi et al., 2021)).

GCDCA, TDCA, LCA, GLCA and TLCA were not quantifiable in our investigations. Humbert et al. were also not able to detect these five bile acids, along with CDCA, UDCA and LCA-S (Humbert et al., 2012). Bathena et al. and Alamoudi et al. (same method) on the other hand, were able to quantify each of the 18 bile acids – probably due to their method's low limit of quantification (Alamoudi et al., 2021; Bathena et al., 2013).

However, one cannot unreservedly compare these studies with each other. While our study as well as Humbert et al. (Humbert et al., 2012) used aliquots of 24-hour urine samples, Bathena et al. and Alamoudi et al. (Alamoudi et al., 2021; Bathena et al., 2013) used spot urine samples. Furthermore, our study stands out by having analyzed children's urine, while the others were looking into urine of adult subjects (Alamoudi et al., 2021; Bathena et al., 2013; Humbert et al., 2012). These circumstances could potentially influence the results - be it through diurnal variations or ontogenetic developments.

Apart from that, Humbert et al. (Humbert et al., 2012) included a smaller number of subjects, while Bathena et al. (Bathena et al., 2013) and Alamoudi et al. (Alamoudi et al., 2021) examined slightly more healthy probands than we did.

Regardless of the unit in which the results were presented, abundance and quantifiability differed markedly between the 18 individual bile acids. With GCDCA, TDCA, LCA, GLCA and TLCA laying below the limit of quantification in all 80 samples, we were not able to obtain any reference values for 5 of our 18 analytes. Almost the same applies for CDCA (77 samples < LOQ) and UDCA (79 samples < LOQ).
As mentioned in the beginning, especially hydrophobic bile acids are considered more toxic (Bathena et al., 2013; Monte et al., 2009; Perez and Britz, 2009). Having the least hydroxy groups (one OH at position C-3), lithocholic acid is the most hydrophobic of the four unconjugated bile acids included in our method and should therefore be the most toxic. It might be a logical consequence that LCA and its amidated derivatives GLCA and TLCA only appeared in a non-quantifiable amount in healthy children. However, this explanatory approach does not apply for the low amounts of CDCA, GCDCA, TDCA and UDCA. Particularly UDCA, on the contrary, is well known for its beneficial effects (Lazaridis et al., 2001; Perez and Britz, 2009). Due to its 7β-OH position, UDCA is more hydrophilic than CDCA and DCA (Lazaridis et al., 2001) or even CA with its three OH-groups (Perez and Britz, 2009). The reason for the low findings of UDCA in urine of healthy children is possibly just the fact that UDCA is no major bile acid in human organism, which appears in human bile only at low levels (Paumgartner and Beuers, 2002). So, even if higher levels of UDCA would probably not harm the organism, it is just not in human nature.
The low findings of CDCA, GCDCA and TDCA, however, are not that obvious to explain. They all are considered (derivatives of) major human bile acids with hydrophobicity in the medium range. Whereas DCA, for instance, appeared more frequently, although it is of higher hydrophobicity.

In any of the three units in which the results were presented, the sulfated bile acids represented the vast majority of the measured urinary bile acids. This is a fact that was to be expected, since sulfation has been described to increase urinary elimination of bile acids (Alnouti, 2009). According to the present work, with mean results about 98-100%, the percentage share of sulfated bile acids compared to their unsulfated equivalents was even higher than the previously reported 37-87% (Almé et al., 1977; Makino et al., 1975; Meng et al., 1997; Takikawa et al., 1984). However, these publications examined rather small study groups with less than 10 healthy subjects. Furthermore, most of these studies were carried out using GC(-MS), while nowadays LC-MS/MS is considered gold standard for measurement of conjugated steroids (Wudy et al., 2018). More recent publications working with larger study

groups and also using LC-MS/MS have shown results that are more similar to our own findings. For instance, there are reported sulfation percentages of 98.4-99.7% for derivatives of CDCA, DCA and LCA (Bathena et al., 2013). This study also reported sulfation rates of 96.9% for ursodeoxycholic acid derivatives and 55.1% for cholic acid derivatives. While we did not measure these particular bile acids in their sulfated forms, the fact that cholic acid has been reported to show a lower percentage of sulfation could still match our results, since CA and GCA were our most abundant unsulfated bile acids. With an overall sulfation rate of 88.5% (comprising all measured bile acids without distinction of individual bile acids), an additional LC-MS/MS study from the same institution using the same method showed similarly high results (Alamoudi et al., 2021).

In accordance with our results, another study also found that lithocholic acid derivatives GLCA and TLCA only appeared in their sulfated forms (GLCA-S and TLCA-S) (Humbert et al., 2012). The same study did not detect LCA-S, which was also markedly lower compared to GLCA-S and TLCA-S in our study. The small amount of LCA-S can probably be explained by hydrophobicity effects. Lithocholic acid derivatives are considered the most hydrophobic - and thus most toxic - bile acids (Monte et al., 2009; Perez and Britz, 2009). In addition to sulfation, amidation also lowers hydrophobicity (Perez and Britz, 2009). As a result - in spite of its sulfation - LCA-S is still more toxic than GLCA-S and TLCA-S and might therefore be less abundant in healthy human organisms.

In contrast to sulfation, the results for amidation state throughout different comparable publications were not always that clear-cut. Bathena et al. (Bathena et al., 2013) found that most urinary bile acids were predominantly present in their glycine-amidated form. The present study, on the other hand, did not see such a clear trend, since the unamidated and taurine-amidated forms prevailed for some analytes (see Table 13 and Figure 20).

The most distinct differences in amidation were visible for chenodeoxycholic acid: The present study found TCDCA the most, followed by CDCA and GCDCA (for GCDCA all results < LOQ). However, Bathena et al. published the opposite order of abundance (GCDCA > CDCA > TCDCA) (Bathena et al., 2013). The reasons for this discrepancy could be of methodical nature. While in the present study GCDCA had the highest limit of quantification (18.75 ng/mL; compared to 6.25 ng/mL for the 17 remaining analytes), Bathena et al. were able to achieve a limit of quantification of 1 ng/mL for each analyte (Bathena et al., 2013). The high limit of quantification could be the reason for a possible underrepresentation of GCDCA compared to CDCA and TCDCA in the present study. Apart from that, all forms of

unsulfated chenodeoxycholic acid generally appeared rarely - and if so, at very low levels. They were not even detected by Humbert et al. (Humbert et al., 2012).

	Present Results* Healthy	Present Results* Obese	Humbert et al., 2012*	Bathena et al., 2013**	Alamoudi et al., 2021**
CA	220.8 ± 245.2	367.0 ± 588.7	124.8 ± 193.2	194.2 ± 30.2	179.0 ± 30.0
GCA	138.0 ± 109.6	135.7 ± 201.7	47.7 ± 51.0	69.8 ± 5.5	67.0 ± 0.0
GCDCA-S	670.2 ± 444.1	483.1 ± 379.5	not included	2399.9 ± 126.2	2380.0 ± 130.0
GDCA-S	459.5 ± 498.2	484.8 ± 480.4	not included	2991.1 ± 151.5	2900.0 ± 140.0
GLCA-S	580.6 ± 637.6	429.8 ± 384.8	185.7 ± 151.6	808.2 ± 46.1	780.0 ± 40.0
TLCA-S	234.3 ± 232.5	127.7 ± 142.6	108.6 ± 95.4	222.9 ± 12.5	220.0 ± 10.0
	n=80, aged 3-18 years; 24-hour urine; HPLC-MS/MS	n=237, aged 8-17 years; 24-hour urine; HPLC-MS/MS	n=39, age not specified; 24-hour urine; HPLC-MS/MS	n=90, aged 19-65 years; spot urine***; UPLC-MS/MS	n=103, aged 19-65 years; spot urine; UPLC-MS/MS

Table 30. Comparison of our six most abundant bile acids with results from previous publications. Results are presented as mean concentration [nmol/L]. In general, the area of results is comparable. Distinct deviations are especially visible for GCDCA-S and GDCA-S.
**results presented as mean ± standard deviation*
***results presented as mean ± standard error (of mean)*
****fasting urines from 4 different visits were pooled*

When interpreting the results, we also looked into potential sex differences between urinary bile acids excretion in boys and girls. DCA stood out as the only analyte with a significant sex difference in all three units, having delivered higher results in female samples. Furthermore, GCDCA-S was significantly higher in males when looking at daily excretion [nmol/d]. Another study also investigated sex differences in urinary bile acids (Bathena et al., 2015b). When looking at our 18 analytes in their study, they found significant differences for CDCA and DCA. Concordant with our findings, Bathena et al. also reported significantly higher mean urinary concentrations for DCA in females. An explanation for the higher concentration of DCA amongst female probands remains uncertain.

While the results for healthy urinary bile acids concentration in nmol/L barely delivered any significant age correlation (only GCA significantly decreased with age), the units of creatinine-related bile acids concentration [mmol Crea/nmol BA] as well as daily excretion [nmol/d] in healthy children showed some more age trends. While creatinine-related bile acids concentration more likely decreased with age (5 analytes and the sum of the 18 analytes), daily bile acids excretion often appeared higher with age - even though only GCDCA-S and the sum of the 18 analytes reached significant results. However, these results could be misleading and it might be wrong to conclude that they are proof for ontogenetic changes in bile acids metabolism, since the numbers could be distorted due to physiological age-dependent increases of creatinine excretion (Remer et al., 2002) and daily urine production (Beckford et al., 2020).

In broad agreement with our findings of no distinct age trend in nmol/L for most analytes (except for GCA), Bathena et al. found no variation of absolute urinary bile acids levels with age in any of the analytes (Bathena et al., 2015b). But then again, that study only examined adults aged from 19 to 65 years instead of children, so this comparison might be flawed.

One of the studies that inspired us to use a creatinine-related unit also published absolute results for urinary bile acids in nmol BA/mmol Crea (Taylor et al., 2014). When comparing these results with our creatinine-related results, there appear clear deviations. While the median value of CA was higher in our findings and that of GCA was similar in both studies, the other bile acids that were included in both studies (TCA, CDCA, GCDCA, TCDCA, DCA, GDCA, TDCA, LCA, GLCA, TLCA, UDCA) were reported with higher median values by Taylor et al. This applies to both of our study groups compared to their results. However, there are several reasons why a plane comparison between these results is flawed. Firstly, while we analyzed samples from 80 healthy or 237 obese children, respectively, they looked into urine of 15 healthy adults with a median age of 50 years. In addition to that, there were

also relevant methodical differences. Although both studies used LC-MS/MS, Taylor et al. applied a method were they deconjugated the urinary bile acids prior to measurement. Consequently, physiologically present unconjugated, sulfated and glucuronidated derivatives of an individual bile acid were combined in their measurements, whereas we measured unconjucated and sulfated bile acids separately without prior deconjugation of the sulfate group. Bile acids glucuronides were not included in our method.

4.2.3 Urinary Bile Acids in obese Children and Comparison to healthy Children

For nanomolar concentration and creatinine-related concentration, the same 8 analytes showed significant group differences: While TCA, GCDCA-S and TLCA-S were more abundant in healthy children, CDCA, GCDCA, DCA, GDCA and UDCA achieved higher results in obese children. As for most of our rather particular significant findings, it is hard to find an explanation for these significant group differences. Bile acids sulfation mainly takes place in the liver (Alnouti, 2009). When looking at the fact that two of the more abundant bile acids in health (GCDCA-S and TLCA-S) are sulfates, one could speculate that obesity might lead to a lower extent of bile acids sulfation due to obesity-related limitations of the liver's sulfation capacity. A further argument supporting this hypothesis is the fact that there are also group differences in sulfation percentage. This does not apply for lithocholic acid derivatives, which were only quantifiable as sulfates. However, there were variations showing a slightly lower percentage sulfation of GDCA and GCDCA in the obese group (see Table 26). For GDCA, this group variation was statistically significant (see Table 27). On the other hand, limitations of the liver's functionality might only appear after a chronic period of obesity, eventually leading to conditions from the group of NAFLD. Since our probands were children, one might argue it may be doubted that there already are obesity-related complications restricting liver function after such a short lifetime. But by taking a look into literature, we see that NAFLD actually seems to play a relevant role in childhood obesity with reported prevalences up to almost 80% amongst obese children (Nobili et al., 2013). Altered serum bile acids in children with steatosis hepatis have already been published (Montagnana et al., 2020). It has been postulated that childhood obesity and concomitant NAFLD lead to an alignment of children's metabolism towards adult metabolism - resulting in altered serum bile acids in obese children (Giannini et al., 2022). To what extent this also transfers to urinary bile acids remains to be explored. When comparing the present results with elsewhere published urinary bile acids concentrations in adults, it does not seem like the obese group in the present study shows more similarities with the adult results than the healthy group (Table 30).

Contrary to our results in children's urine, another study that investigated sulfation percentages of urinary bile acids in adults found no differences when comparing normal-weight and obese probands (Bathena et al., 2015b), so this might actually be a phenomenon unique to children. Regarding absolute urinary bile acid amounts, their study also found no relevant changes with BMI.

Apart from sulfation, we also documented group differences in amidation percentage, which are illustrated by the pie charts in Figure 20. Here we generally see that the percentage of unamidated derivatives was higher in obesity compared to health. With bile acids amidation taking place in liver (Russell, 2003), this again suits the theory of a reduced liver synthesis capacity in obesity. Lithocholic acid forms an exception, since there hardly appeared any unamidated derivatives in both groups. A second important observation when looking from healthy to obese children is the fact that the amidation trend seems to shift towards glycine: While the relative amount of taurine-amidation decreases in obesity, the share of glycine-amidation rises. This may indicate that in case of impaired hepatic synthesis capacity, glycine-amidation still works more readily than taurine-amidation. Concordant with the present findings, the previously cited study from Nebraska also reported lower amidation percentages of urinary CA and DCA in obese adults (Bathena et al., 2015b). Thus, in contrast to the sulfation percentage, obesity-related changes in amidation seem to appear in childhood as well as in adulthood.
In conclusion, there is justified reason to believe that childhood obesity effects urinary bile acids and therefore this topic is definitively worth further investigation in the future.

Coming back to group differences of individual bile acids, the unit of nmol/d stands out. While it showed higher results for GCDCA-S in health (likewise to the two remaining units), it did not show significant results for TLCA-S, but instead for CA and GDCA-S – both of which were higher in obese children's urine. The higher results for GDCA-S in obesity speaks against the previously mentioned thought of reduced hepatic sulfation capacity in obesity. However, the group comparison in nmol/d should be made with caution, since the 24-hour urine volume itself significantly varied between the groups with higher volumes in the obese group (Table 25). Creatinine concentration on the other hand did not significantly change between the two study groups (Table 24).

In addition to the boxplots in Figures 14-16 and the linear regression analyses' p-values in Tables 23-25, the percentile curves in Figures 17-19 present the group differences for the six most abundant individual bile acids included in our method. They nicely showcase the lower amounts of GCDCA-S and TLCA-S in obese children regarding nanomolar concentration [nmol/L] and creatinine-related concentration [nmol BA/mmol Crea] as well as the respective group differences for CA, GCDCA-S and GDCA-S regarding daily excretion [nmol/d]. By implementing these percentile curves, we also aimed for portraying a potential practical

application of our bile acids data. Percentile curves are widely used in pediatrics for comparing an individual's state of development (e. g. regarding height or weight) to a reference population at a certain age. Of course, for claiming to be a reference collective, our healthy group should have included a larger number of probands. But still, it certainly sets a first mark, where the reference values for urinary bile acids in healthy children may lie and what a possible use in clinical routine in the form of percentile curves could look like.

Analyzing urinary bile acids in obese children, we also investigated potential age- or sex-specific trends. Regarding age, the results in nmol/L and nmol/d were similar to those for healthy children: GCA concentration was significantly lower with increasing age (nmol/L) and daily excretion (nmol/d) of GCDCA-S and the sum of the 18 bile acids (Total) significantly rose with age. The study groups differed in creatinine-related concentration (nmol BA/mmol Crea), where obese children only delivered a significant negative age-correlation for CA, GCA and Total, while healthy children additionally showed this for TCA, GCDCA-S and TLCA-S.

Sexual patterns showed more inter-group variations: DCA, that was consistently higher amongst female samples throughout all three units in the healthy group, was not significantly altered by sex in the obese group. On the other hand, CA, GCDCA-S and Total were significantly higher in male probands of the obese group in two of the units (nmol/L, nmol/d), while in the third unit (nmol BA/mmol Crea) LCA-S was significantly more abundant in females.

Conclusively, we have no specific explanation for these distinct age- and sex-dependent alterations that only occurred for some specific bile acids and sometimes only in health or in obesity, respectively. What stands out is the fact that the sum of the 18 bile acids (Total) was significantly age-correlated for daily excretion (nmol/d) and creatinine-related concentration (nmol BA/mmol Crea) in both study groups (healthy and obese). However – as mentioned above – this could be due to ontogenetic alterations of 24-hour urine volume and creatinine excretion rather than age-dependent variations of bile acids metabolism itself. A comparison to other publications is currently not possible, as to our knowledge there is no other study that tested for urinary bile acids' age- and sex-correlations in obesity.

4.2.4 Nutrition and urinary Bile Acids

The six most abundant bile acids (CA, GCA, GCDCA-S, GDCA-S, GLCA-S, TLCA-S) within the reference group of 80 healthy children were tested for potential correlations with nutritional factors. For that purpose, regression analyses were done relating urinary bile acids to fat, protein or carbohydrate intake, respectively.

Since supporting the absorption of fatty food components is a key function of bile acids (Horn et al., 2019), it seems natural to assume that especially fat intake leads to alterations in circulating bile acids and consequently also in urinary bile acids. Furthermore, bile acids play a role in lipid, glucose and energy homeostasis via interactions with receptors FXR and TGR5 (Prawitt et al., 2011), indicating even stronger that changes in nutritional intake - also regarding proteins and carbohydrates - are followed by changes in urinary bile acids. However, significant correlations between nutritional intake and urinary bile acids were only found for one individual bile acid (GCDCA-S). Urinary concentration of GCDCA-S decreased with higher carbohydrate intake, while it increased with higher fat or protein intake, respectively. The reasons why these changes only occurred for GCDCA-S and why any significant alterations are absent in case of the other bile acids are yet to be determined.

Another study already looked into food effects on serum and urinary bile acids. However, the authors only compared fasting samples with samples obtained one hour after a meal instead of investigating the effects of major nutrients separately. They found increased levels of serum bile acids postprandial, whereas urinary bile acids were slightly decreased after food intake. While percentages of bile acids sulfation and amidation in the serum samples were altered by food effects, they remained virtually steady in the urine samples (Bathena et al., 2015b).

Taking these previous findings and our own results into account, it appears as if nutritional intake does not affect urinary bile acids as much as one might expect. It seems that under physiological conditions the organism is able to maintain bile acids homeostasis, resulting in only slight changes in urinary bile acids concentrations due to food effects. The main influences of nutritional intake on bile acids probably take place in serum and most certainly within the enterohepatic circulation. This may change in disease. As urinary bile acids have already been reported to be elevated in hepatobiliary diseases (Alamoudi et al., 2021; Bathena et al., 2015a; Ferslew et al., 2015; Makino et al., 1975) or diabetes (Taylor et al., 2014), these pathologies could also make the organism more susceptible to nutritionally induced alterations in urinary bile acids.

5 Conclusions

- A new LC-MS/MS method for quantification of urinary bile acids has been developed, successfully validated and applied to urine samples of 80 healthy and 237 obese children.
- Reference values (percentiles) have been created for children and adolescents.
- Sulfated bile acids made up for the vast majority of urinary bile acids in both study groups.
- Sulfation and amidation seemed to be impaired by obesity. Glycine-amidation appeared to be less prone to obesity-related impairments than taurine-amidation. Sulfation rates seem to decrease with obesity.
- Urinary concentration of GCDCA-S decreased with higher carbohydrate intake, while it increased with higher fat or protein intake, respectively.

6 Summary

Bile acids (BA) are C24 steroids synthesized from cholesterol in the liver. Apart from emulsification of fatty food components, they function as endocrine signaling molecules. As such, bile acids bear great potential as future biomarkers in diagnosis and monitoring of metabolic diseases. However, hardly any data exist on BA in urine. Therefore, the present study aimed at developing and implementing a new method for the quantification of urinary bile acids using targeted liquid chromatography-tandem mass spectrometry (LC-MS/MS).
The targeted approach included 18 BA: the primary BA cholic acid (CA) and chenodeoxycholic acid (CDCA), and the secondary BA deoxycholic acid (DCA) and lithocholic acid (LCA) as well as glycine and taurine conjugates of these four BA. Furthermore, ursodeoxycholic acid (UDCA) and five BA in their sulfated forms (LCA-S, GLCA-S, TLCA-S, GCDCA-S, GDCA-S) were analyzed.
Another goal consisted in presenting first reference values of urinary bile acids during childhood and to investigate their excretion patterns in obese children and adolescents. Finally, potential correlations between urinary bile acids and nutrition were examined.
The method required 2 mL of urine and sample preparation consisting of protein precipitation and solid phase extraction. Stable isotopes of BA were included as internal standards (IS). The method was successfully validated and then applied to samples of 80 healthy children as well as 237 obese children of various age groups. The results were presented in three different units ([nmol/L], [nmol BA/mmol Crea] and [nmol/d]).
Regardless of the unit, sulfated bile acids (GCDCA-S, GLCA-S, GDCA-S, TLCA-S) dominated in both study groups, CA and GCA were the two dominant non-sulfated BA. Lower bile acid sulfation and amidation in obese children may point to obesity-related limitations in hepatic metabolic capacity. Glycine-amidation appeared to be less prone to obesity-related impairments. Urinary concentration of GCDCA-S decreased with higher carbohydrate intake, while it increased with higher fat or protein intake, respectively.

7 Zusammenfassung

Gallensäuren sind Steroide mit 24 Kohlenstoffatomen, die ausgehend von Cholesterin in der Leber synthetisiert werden. Neben ihrer Funktion als Emulgatoren für fetthaltige Nahrungsbestandteile erfüllen sie Aufgaben als endokrine Signalmoleküle. Als solche bieten Gallensäuren ein großes Potenzial als zukünftige Biomarker in Diagnostik und Verlaufskontrolle von metabolischen Erkrankungen. Daten zu Gallensäuren in Urin existieren jedoch kaum. Daher zielte die vorliegende Studie darauf ab, eine neue Methode zur Quantifizierung von Gallensäuren im Urin mittels Flüssigkeitschromatographie mit Tandem-Massenspektroskopie zu entwickeln. Die Methode misst gezielt 18 Gallensäuren: Die primären Gallensäuren Cholsäure (CA) und Chenodesoxycholsäure (CDCA), die sekundären Gallensäuren Desoxycholsäure (DCA) und Lithocholsäure (LCA) sowie mit Glycin und Taurin amidierte Konjugate dieser vier Gallensäuren. Zudem wurden Ursodesoxycholsäure (UDCA) und fünf Gallensäuren in ihrer sulfatierten Form (LCA-S, GLCA-S, TLCA-S, GCDCA-S, GDCA-S) analysiert. Ein weiteres Ziel bestand darin, erste Referenzwerte für Gallensäuren im Urin während der Kindheit zu erheben und die renale Gallensäurenausscheidung von adipösen Kindern und Jugendlichen zu untersuchen. Schließlich wurden auch potentielle Korrelationen zwischen Gallensäuren im Urin und der Ernährung analysiert. Für die Methode wurden 2-mL-Urinproben verwendet, die anschließend mittels Proteinausfällung und Festphasenextraktion für die Analyse vorbereitet wurden. Stabile Isotope der Gallensäuren wurden als interne Standards benutzt. Die Methode durchlief eine erfolgreiche Validierung. Anschließend wurden Urinproben von 80 gesunden sowie 237 adipösen Kindern verschiedener Altersgruppen untersucht. Die Ergebnisse wurden in drei verschiedenen Einheiten dargestellt ([nmol/L], [nmol BA/mmol Crea] und [nmol/d]). Unabhängig von der verwendeten Einheit traten sulfatierte Gallensäuren (GCDCA-S, GLCA-S, GDCA-S, TLCA-S) in beiden Gruppen am häufigsten auf. CA und GCA waren die am häufigsten vorkommenden unsulfatierten Gallensäuren. Niedrigere Gallensäuren-Sulfatierung und -Amidierung in der adipösen Gruppe könnten auf Adipositas-assoziierte Einschränkungen der hepatischen Stoffwechselaktivität hinweisen. Die Amidierung mit Glycin schien hiervon weniger beeinträchtigt zu sein als die Taurin-Amidierung. Die renale Ausscheidung von GCDCA-S sank mit höherer Kohlenhydratzufuhr und stieg mit höherer Fett- bzw. Protein-Zufuhr in der Ernährung.

8 List of Abbreviations

APCI	atmospheric-pressure chemical ionization
BMI	body mass index
CA	cholic acid
CDCA	chenodeoxycholic acid
DC	direct current
DCA	deoxycholic acid
DONALD study	Dortmund Nutritional and Anthropometric Longitudinally Designed study
ESI	electrospray ionization
FDA	Food and Drug Administration
GC	gas chromatography
GCA	glycocholic acid
GCDCA	glycochenodeoxycholic acid
GCDCA-S	glycochenodeoxycholic acid sulfate
GC-MS	gas chromatography – mass spectrometry
GDCA	glycodeoxycholic acid
GDCA-S	glycodeoxycholic acid sulfate
GLCA	glycolithocholic acid
GLCA-S	glycolithocholic acid sulfate
HPLC	high-performance liquid chromatography
IS	internal standard
LC	liquid chromatography
LCA	lithocholic acid
LCA-S	lithocholic acid sulfate
LC-MS/MS	liquid chromatography – tandem mass spectrometry
MRM	multiple reaction monitoring
MS	mass spectrometry
MULTIOMICS	Personalized Approach to Non-Syndromic Childhood Obesity using Multi-Omics Disease Signature
NPLC	normal-phase liquid chromatography

RF	radio-frequency
RPLC	reversed-phase liquid chromatography
SD	standard deviation
SRM	selected reaction monitoring
TCA	taurocholic acid
TCDCA	taurochenodeoxycholic acid
TDCA	taurodeoxycholic acid
TLCA	taurolithocholic acid
TLCA-S	taurolithocholic acid sulfate
UDCA	ursodeoxycholic acid

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12 Publikationsverzeichnis

- Publikation 2025 (Schauermann M, Wang R, Pons-Kuehnemann J, Hartmann MF, Remer T, Hua Y, Bereket A, Wasniewska M, Shmoish M, Hochberg Z, Gawlik, Wudy SA, 2025. **Targeted quantitative analysis of urinary bile acids by liquid chromatography-tandem mass spectrometry: Method development and application to healthy and obese children**. J Steroid Biochem Mol Biol 2025 Feb 21;249:106712. doi: 10.1016/j.jsbmb.2025.106712.)
- Publikation 2022 (Schauermann M, Wachter UA, Hua Y, Hartmann MF, Remer T, Wudy SA, 2022. **Excretion of oxidated cortisol metabolites is markedly lower than previously assumed: An analysis of urinary cortoic acids in healthy children by GC-MS**. J Steroid Biochem Mol Biol. 2022 Nov;224:106163. doi: 10.1016/j.jsbmb.2022.106163. Epub 2022 Aug 20. PMID: 35995415.)
- Poster, Symposium "Steroids, Mass Spectrometry and Endocrinology – Past, Present and Future", Birmingham, 2022
- Abstract, JLU Science Day, 2021
- Zwei Poster, Kongress European Society For Paediatric Endocrinology (ESPE), Liverpool (online), 2021
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Targeted quantitative analysis of urinary bile acids by liquid chromatography-tandem mass spectrometry: Method development and application to healthy and obese children

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ABSTRACT

Bile acids (BA) are C24 steroids synthesized from cholesterol in liver. Hardly any data exist on BA in the most accessible human biofluid urine. As bile acids bear great potential as future biomarkers in diagnosis and monitoring of metabolic diseases, we aimed at developing and implementing a new method for the quantification of urinary bile acids using targeted liquid chromatography-tandem mass spectrometry (LC-MS/MS). A second goal consisted in creating first reference values of urinary bile acids during childhood and to investigate their excretion patterns in obese children and adolescents.

Our method required 2 mL of urine and sample preparation consisting of protein precipitation and solid phase extraction. Stable isotopes of BA were included as internal standards (IS).

Our method is capable of simultaneously determining 18 BA: the primary BA cholic acid (CA) and chenodeoxycholic acid (CDCA), and the secondary BA deoxycholic acid (DCA) and lithocholic acid (LCA) as well as glycine and taurine conjugates of these four BA. Furthermore, ursodeoxycholic acid (UDCA) and five BA in their sulfated forms (LCA-S, GLCA-S, TLCA-S, GCDCA-S, GDCA-S) were analyzed.

After successful validation (intra-day precision 1.02 % - 11.07 %; inter-day precision 0.42–11.47 %.; intra-day accuracy 85.75 % - 108.90 %; inter-day accuracy 86.76 % - 110.99 %; no significant matrix effect; recovery 90.49 % - 113.99 %)., the method was applied to samples of 80 healthy children as well as 237 obese children of various age groups.

Sulfated BA showed the highest concentrations, with GCDCA-S (nmol/L, medians, controls vs. obese 588.4 vs. 360.2) being the most abundant among all BA, followed by GLCA-S (353.9 vs. 344.8) and GDCA-S (319,3 vs. 323.9). CA (135.1 vs. 174.6) and GCA (100.2 vs. 92.4) were the two dominant non-sulfated BA.

In conclusion, we developed a LC-MS/MS method for the simultaneous determination of 18 urinary bile acids in children and adolescents. We created reference values and investigated obese children. Sulfated bile acids dominated in both study groups. Lower bile acid sulfation and amidation in obese children point to limitations in their hepatic metabolic capacity.

Schauermann M, Wang R, Pons-Kuehnemann J, Hartmann MF, Remer T, Hua Y, Bereket A, Wasniewska M, Shmoish M, Hochberg Z, Gawlik, Wudy SA, 2025. **Targeted quantitative analysis of urinary bile acids by liquid chromatography-tandem mass spectrometry: Method development and application to healthy and obese children**. J Steroid Biochem Mol Biol 2025 Feb 21;249:106712. doi: 10.1016/j.jsbmb.2025.106712.)

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Excretion of oxidated cortisol metabolites is markedly lower than previously assumed: An analysis of urinary cortoic acids in healthy children by GC–MS

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ABSTRACT

Discovered about 50 years ago, the four C_{21} steroidal acids (α-)cortolic acid, β-cortolic acid, (α-)cortolonic acid and β-cortolonic acid present the oxidative end products of cortisol metabolism. Undergoing renal elimination, these cortoic acids have been assumed to constitute up to 25 % of total urinary cortisol metabolites. However, their analysis has been difficult, only few data has been published in adults, and this class of steroids has become practically forgotten. Since data in children are lacking and nothing is known about their metabolism during human development, we aimed at establishing a more practical analytical method and determined their urinary concentrations in a high number of healthy subjects. In our method, 5-mL-aliquots of 24-hour urine samples were subjected to solid phase extraction (C18 cartridges), followed by strong anion exchange chromatography, and formation of 2-propylester-trimethylsilylether derivatives (2-PR/TMS). The cortoic acids were quantified by targeted gas chromatography–mass spectrometry (GC–MS) using a nonpolar GC column and selected ion monitoring (SIM). Baseline separation of all cortoic acids was achieved. Calibration graphs were linear ($R^2 >$ 0.98). Variations in precision and accuracy were less than 15 %, respectively. The detection limit was 100 pg (injected) with a signal-to-noise ratio of 3. 240 specimens from 24-hour urine collections from healthy children (120 boys, 120 girls, aged 3–18 years; DONALD study) were analyzed for cortoic acids and neutral cortisol metabolites to create first reference ranges. The profile of cortoic acids was dominated by α-cortolonic acid with excretion rates up to 70 μg/d. Absolute excretion rates of cortoic acids increased with age, their total excretion rates ranged between 11.0 and 127.3 μg/d (median 45.7 μg/d), but did not show any sexual dimorphism. Since cortoic acids make up only about 1 % of total urinary cortisol metabolites, determination of neutral urinary steroids reliably allows assessment of cortisol production. However, cortoic acids might present potential biomarkers of the body's redox state.

Schauermann M, Wachter UA, Hua Y, Hartmann MF, Remer T, Wudy SA, 2022. **Excretion of oxidated cortisol metabolites is markedly lower than previously assumed: An analysis of urinary cortoic acids in healthy children by GC-MS**. J Steroid Biochem Mol Biol. 2022 Nov;224:106163. doi: 10.1016/j.jsbmb.2022.106163. Epub 2022 Aug 20. PMID: 35995415

Excretion of Oxidated Cortisol Metabolites is Markedly Lower than Previously Assumed: An Analysis of Urinary Cortoic Acids in Healthy Children by GC-MS

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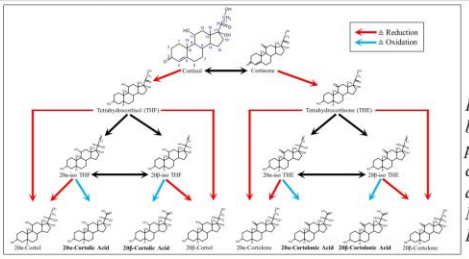
JUSTUS-LIEBIG-UNIVERSITÄT GIESSEN

INTRODUCTION

Discovered in the 1970s, the C21 steroidal acids (α-)cortolic acid, β-cortolic acid, (α-)cortolonic acid and β-cortolonic acid present the oxidative end products of cortisol metabolism[1,2]. These **cortoic acids** have been assumed to constitute up to 25% of total urinary cortisol metabolites[1-5]. However, their **analysis has been difficult**[3,6], only few data has been published, and these steroids have become practically forgotten. **Data in children are lacking completely** and nothing is known about their metabolism during human development.



Preferred biosynthetic pathway of cortoic acids according to Monder and Bradlow (1980)[2]

GOALS

- Developing a **practical analytical method** for quantification of urinary cortoic acids
- Establishing **reference values** for urinary cortoic acids excretion in healthy children, adolescents and young adults

METHOD

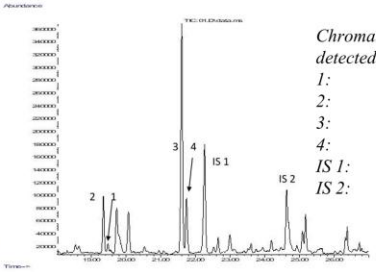
5 ml aliquots of 24-hour urine samples were used for sample work up consisting of **solid phase extraction** (C18 cartridges), strong **anion exchange** and **derivatization** to 2-propylester-trimethylsilylether derivatives. Subsequently, the cortoic acids were quantified by targeted GC-MS using a nonpolar GC column and selected ion monitoring.

CONCLUSIONS

- Introduction and successful validation of a new and less complicated method for quantification of urinary cortoic acids by targeted GC-MS.
- Establishment of first reference values in healthy children.
- Excretion of cortoic acids increased with age.
- **Cortoic acids only constituted about 1% of total urinary cortisol metabolites, a percentage much lower than previously assumed.**

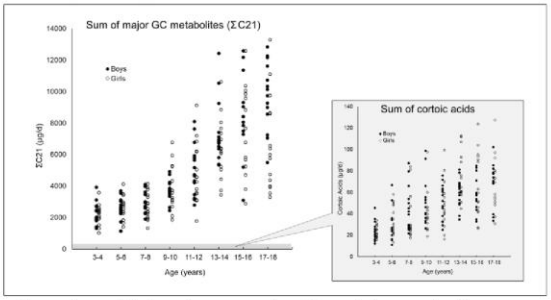
RESULTS

Baseline separation of all four cortoic acids was achieved. Calibration graphs were linear ($R^2 > 0.98$). Variations in precision and accuracy were less than 15%, respectively. The detection limit was 100 pg (injected) with a signal-to-noise ratio of 3.



Chromatogram: Cortoic acids, detected in a typical urine sample
1: α-cortolic acid
2: β-cortolic acid
3: α-cortolonic acid
4: β-cortolonic acid
IS 1: 5α,3β-cortolic acid
IS 2: 5α,3β-cortolonic acid

The method was used to analyze 24-hour urine collections from 240 healthy children (120 boys, 120 girls, aged 3-18 years; DONALD study). The cortoic acids' profile was dominated by α-cortolonic acid with excretion rates up to 70 µg/d. When excretion rates of the four cortoic acids were summed, all subjects excreted less than 130 µg/d. **Cortoic acids represented about 1%** of total urinary cortisol metabolites. While **higher age led to increased excretion rates**, sex did not significantly affect the amount of daily excreted cortoic acids.



Comparison of daily urinary excretion: Neutral glucocorticoid metabolites (ΣC21) vs. cortoic acids

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Endocri-nology 2022 Steroids Mass Spec

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Poster, Symposium "Steroids, Mass Spectrometry and Endocrinology – Past, Present and Future", Birmingham, 2022

PG_25: Urinary Bile Acids as Potential Biomarkers of Human Metabolism: Reference Values in Children by Targeted LC-MS/MS

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Abstract:

Bile acids (BA) are steroids synthesized from cholesterol in liver. Having so far been mainly considered as emulsifiers, BA's additional hormonal functions in regulation of metabolism have only recently come into focus. Since there are hardly any data on BA in urine, the most easily accessible body fluid, a targeted LC-MS/MS method was developed to analyze 18 urinary BA simultaneously. The method contains the measurement of two primary BA (CA, CDCA) and two secondary BA (DCA, LCA) as well as glycine and taurine conjugates of these four BA (GCA, TCA, GCDCA, TCDCA, GDCA, TDCA, GLCA, TLCA). Furthermore, UDCA and five BA in their sulfated forms (LCA-S, GLCA-S, TLCA-S, GCDCA-S, GDCA-S) were analyzed. The method required 2 mL of urine which underwent protein precipitation and solid phase extraction. Our method meets the requirements for Bioanalytical Method Validation by the FDA. The method was applied to samples of 80 healthy children aged 3-18 years. CA (median: 55.2 ng/mL) was the dominant non-sulfated BA. However, sulfated BA showed much higher concentrations, with GCDCA-S (337.5 ng/mL) being the most abundant among all BA. Concentrations of glycine amidated BA were higher than taurine amidated and non-amidated ones. No obvious trends between urinary BA concentrations and age or sex, respectively were observed. Our data show that urinary BA are mostly present in their sulfated form in children's urine, indicating sulfation to be a major pathway for urinary BA excretion. Our reference data for healthy children lay the foundation for further investigations into BA's potential role as metabolic biomarkers.

Abstract, JLU Science Day, 2021

ESPE 2021
The 59th Annual Meeting Online
22-26 September 2021

EXPLORING URINARY BILE ACIDS AS POTENTIAL MARKERS OF METABOLISM: REFERENCE VALUES IN CHILDREN BY TARGETED LC-MS/MS

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JUSTUS-LIEBIG-UNIVERSITÄT GIESSEN

INTRODUCTION

Bile acids (BA) are C_{24} steroids synthesized in liver from cholesterol[1]. They can be conjugated by amidation[1] and sulfation[2].

While BA's role as emulsifiers has been known for long, their additional endocrine functions have lately aroused interest[3].

In comparison to BA in blood, it is surprising that hardly any data exist on BA in the most accessible human biofluid urine – especially when it comes to children.

AIM

- Development and validation of a **targeted LC-MS/MS method** for measurement of 18 urinary BA
- Establishment of **reference values** for urinary BA in healthy children aged 3-18 years

METHOD

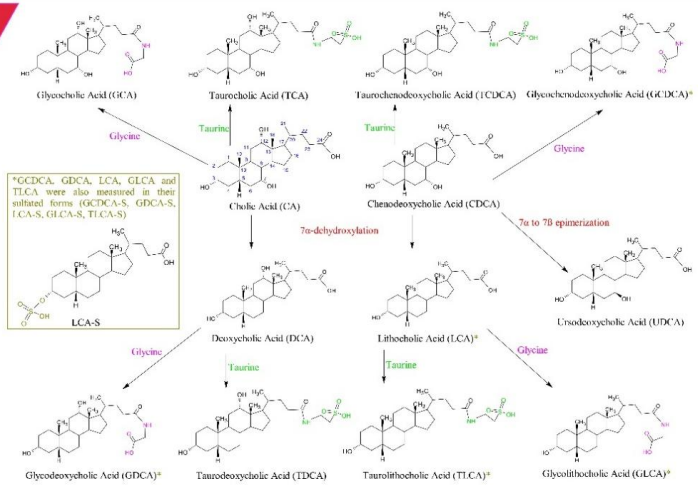
2 mL of 24-hour urine were used for sample preparation comprising protein precipitation (acetonitrile-$ZnSO_4$) and solid phase extraction (C18 cartridges).

Reversed-phase liquid chromatography was done on a phenyl-hexyl column, followed by tandem mass spectrometry with a triple quadrupole mass spectrometer using electrospray ionization (ESI) in the negative mode.

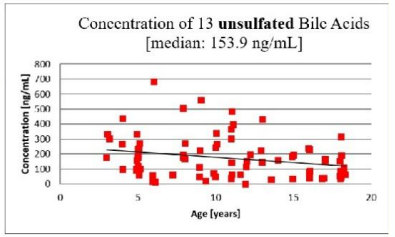
RESULTS

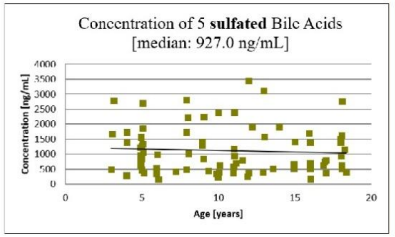
The method achieved good linearity (R^2 > 0.99) and recovery (90.49% - 113.99%). Intra-day/inter-day precision and accuracy ranged from 0.42% to 11.47% and 85.75% to 110.99%, respectively. No significant matrix effect was observed.

CA (median: 55.2 ng/mL) and GCA (48.9 ng/mL) were the two dominant non-sulfated BA. However, sulfated BA showed much higher concentrations, with GCDCA-S (337.5 ng/mL) showing the highest levels among all BA, followed by GLCA-S (197.4 ng/mL) and GDCA-S (183.2 ng/mL). In total, 86.5% of quantified BA were sulfated. The total concentrations of glycine amidated BA measured were higher than taurine amidated and non-amidated ones. No obvious trends between urinary BA and age or sex, respectively were observed.



Chemical structures of the 18 analyzed BA: The presented 13 BA plus 5 BA sulfates (see small box) can be measured with our targeted LC-MS/MS method.





The median concentration of the group of sulfated BA was markedly higher compared to the median of the group of unsulfated BA.

CONCLUSIONS

- A new method for measuring 18 BA by targeted LC-MS/MS was successfully developed, validated and applied to 24-hour urine samples of 80 healthy children.
- Urinary BA concentrations neither changed with age nor showed a sex difference.
- BA were mostly present in their sulfated form in children's urine, indicating hepatic sulfation to be a major metabolic pathway for urinary BA excretion in humans.

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Poster, Kongress European Society For Paediatric Endocrinology (ESPE), Liverpool (online), 2021

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CORTOIC ACIDS: RENAISSANCE OF A FORGOTTEN CLASS OF STEROIDS

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INTRODUCTION

The C21 steroidal acids (**cortoic acids**) (α-)cortolic acid, β-cortolic acid, (α-)cortolonic acid and β-cortolonic acid present the oxidative end products of cortisol metabolism[1,2]. They have been assumed to constitute up to 25% of total urinary cortisol metabolites[1-5].
However, their analysis has been difficult[3,6], few data has been published in adults, and this class of steroids has become practically forgotten. Data in children are lacking completely.

AIM

- Developing a **practical analytical method** for quantification of urinary cortoic acids
- Establishing **reference values** for urinary cortoic acids excretion in healthy children

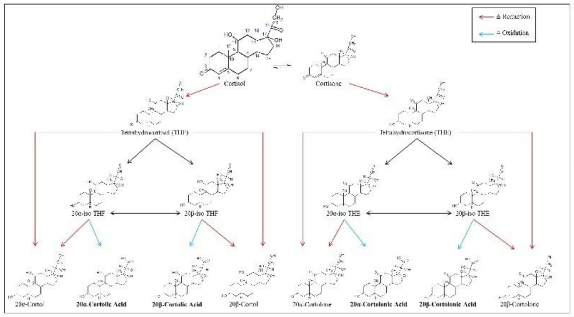
METHOD

5 ml aliquots of 24-hour urine samples were used. Sample work up consisted of solid phase extraction (C18 cartridges), strong anion exchange and derivatization.
Cortoic acids were measured as 2-propylester-trimethylsilylether derivatives.
The quantification was done by targeted GC-MS using a nonpolar GC column.

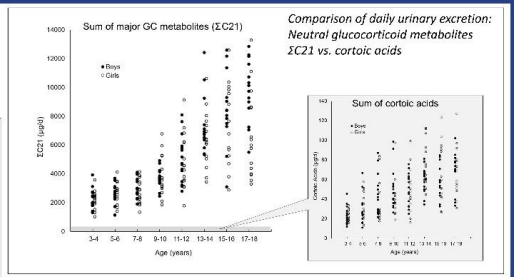
RESULTS

Baseline separation of all four cortoic acids was achieved on a nonpolar GC column. This enables a simultaneous sample work up and gas chromatographic determination of neutral and acidic cortisol metabolites – not in the same GC run, but with the same instrumental setup. Calibration graphs were linear ($R^2 > 0.98$). Variations in precision and accuracy were less than 15%, respectively. The detection limit was 100 pg (injected).

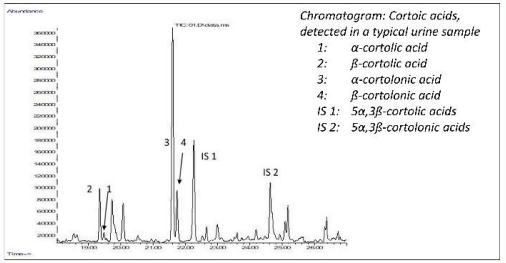
When excretion rates of the four cortoic acids were summed, all subjects excreted less than 130 µg/d (range: 11.0 µg/d - 127,3 µg/d). **Cortoic acids only represented about 1%** (range: 0,69% - 1,51) of total urinary cortisol metabolites. While **higher age led to increased excretion rates**, the children's sex did not significantly affect the amount of daily excreted cortoic acids.



Preferred biosynthetic pathway of cortoic acids according to Monder and Bradlow (1980)[2]



Comparison of daily urinary excretion: Neutral glucocorticoid metabolites ΣC21 vs. cortoic acids



Chromatogram: Cortoic acids, detected in a typical urine sample
1: α-cortolic acid
2: β-cortolic acid
3: α-cortolonic acid
4: β-cortolonic acid
IS 1: 5α,3β-cortolic acids
IS 2: 5α,3β-cortolonic acids

CONCLUSIONS

- Successful development, evaluation and application of a new and less complicated method for quantification of urinary cortoic acids using GC-MS
- Establishment of reference values by using data from 240 healthy children, adolescents and young adults
- Excretion of cortoic acids increased with age.
- **Cortoic acids' share in total urinary cortisol metabolites only added up to about 1%, a percentage much lower as hitherto estimated.**

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Poster, Kongress European Society For Paediatric Endocrinology (ESPE), Liverpool (online), 2021

Abstract-ID: 21. STEROID METABOLISM REVISITED: ONTOGENESIS OF URINARY CORTOIC ACIDS' EXCRETION

Discovered about 50 years ago, the four C21 steroidal acids (cortoic acids) (α-)cortolic acid, β-cortolic acid, (α-)cortolonic acid and β-cortolonic acid present the oxidative end products of cortisol metabolism. They have been assumed to constitute up to 25% of total urinary cortisol metabolites. However, their analysis has been difficult, few data has been published in adults, and this class of steroids has become practically forgotten. Data in children are lacking completely. In our new analytical method 5 ml aliquots of 24-hour urine samples were used for sample work up. Cortoic acids were quantified by targeted GC-MS using a nonpolar GC column and selected ion monitoring. Baseline separation of all cortoic acids was achieved. Calibration graphs were linear (R2 > 0.98). Variations in precision and accuracy were less than 15%, respectively. The detection limit was 100 pg (injected). The method was used to analyze 24-hour urine collections from 240 healthy children (120 boys, 120 girls, aged 3-18 years; DONALD study). When excretion rates of the four cortoic acids were summed, all subjects excreted less than 130 µg/d (range: 11.0 µg/d - 127,3 µg/d). Cortoic acids only represented about 1% (range: 0,69% -1,51) of total urinary cortisol metabolites. While higher age led to increased excretion rates, the children's sex did not significantly affect the amount of daily excreted cortoic acids. Conclusion: A new and less complicated method for quantification of urinary cortoic acids using GC-MS was developed, evaluated and successfully applied to 240 specimens from healthy children, adolescents and young adults. Baseline separation was achieved on a nonpolar GC column, reference values were established. Excretion of cortoic acids increased with age. However, cortoic acids' share in total urinary cortisol metabolites only added up to about 1%, a percentage much lower as hitherto estimated.

Abstract, Deutscher Kongress für Endokrinologien (DGE), Berlin (online), 2021

Abstract-ID: 40. SIMULTANEOUS QUANTIFICATION OF 18 SULFATED AND NON-SULFATED BILE ACIDS BY TARGETED LC-MS/MS IN HUMAN URINE

Bile acids (BA) are C_{24} steroids synthesized in liver. While their role as emulsifiers has been known for long, their potential endocrine functions have lately aroused interest. To simultaneously determine levels of 18 BA in human urine, a targeted LC-MS/MS method was developed, validated and applied to establish reference values in children, adolescents and young adults. The method comprises analysis of the primary BA cholic acid (CA) and chenodeoxycholic acid (CDCA), and the secondary BA deoxycholic acid (DCA) and lithocholic acid (LCA), as well as their respective glycine (G) and taurine (T) amides. Ursodeoxycholic acid (UDCA) and five sulfoconjugated BA were analyzed, too.

2 mL of urine were used for sample preparation comprising protein precipitation (acetonitrile-$ZnSO_4$) and solid phase extraction (C18 cartridges). The method showed excellent linearity ($R_2 > 0.99$) and recovery (90.5% - 114.0%). Limit of quantification was 6.3 ng/mL for each compound, except for GCDCA (18.8 ng/mL). Precision and accuracy of the method ranged from 0.4% to 11.5% and 85.8% to 111.0%, respectively. No significant matrix effect was observed. The method was applied to 24-hour urine samples of 80 healthy children of various age groups (median (range); 10.6 years (3.0-18.4)). The group of non-sulfated BA (n=13; median (range); 153.9 ng/mL (0.0-680.0)) was dominated by CA (55.2 (0.0-501.1)). However, sulfated BA showed much higher concentrations (n=5; 927.0 (153.3-3441.1)) with GCDCA-S (337.5 (20.3-1312.6)) as prevailing BA.

We established a new method for profiling urinary BA in humans. Urinary BA concentrations neither changed with age nor showed a sex difference, whereas 24-hour excretion rates increased with age. Our data showed that sulfated BA were the prevailing BA in urine, indicating sulfation a major pathway for urinary BA excretion. Amidation with glycine was preferred over taurine. Our work lays the foundation to further explore BA as biomarkers of endocrine and metabolic diseases.

Abstract, Deutscher Kongress für Endokrinologien (DGE), Berlin (online), 2021

13 Ehrenwörtliche Erklärung

„Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig und ohne unzulässige Hilfe oder Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten oder nichtveröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten sowie ethische, datenschutzrechtliche und tierschutzrechtliche Grundsätze befolgt. Ich versichere, dass Dritte von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen, und dass die vorgelegte Arbeit weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde zum Zweck einer Promotion oder eines anderen Prüfungsverfahrens vorgelegt wurde. Alles aus anderen Quellen und von anderen Personen übernommene Material, das in der Arbeit verwendet wurde oder auf das direkt Bezug genommen wird, wurde als solches kenntlich gemacht. Insbesondere wurden alle Personen genannt, die direkt und indirekt an der Entstehung der vorliegenden Arbeit beteiligt waren. Mit der Überprüfung meiner Arbeit durch eine Plagiatserkennungssoftware bzw. ein internetbasiertes Softwareprogramm erkläre ich mich einverstanden.“

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Ort/Datum Unterschrift

14 Danksagung

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