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Messung von 7α-Hydroxy-4-cholesten-3-on als Marker für Gallensäureverlust. Etablierung von Normalwerten bei Kindern und Anwendung bei Patienten mit Kurzdarmsyndrom und chronisch-entzündlichen Darmerkrankungen.

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1. Einleitung und Problemstellung

Gallensäuren werden von der Leber synthetisiert und ermöglichen im Darm die Emulgierung und Absorption von Nahrungsfetten sowie von fettlöslichen Vitaminen. Im terminalen Ileum werden ca. 95% der sezernierten Gallensäuren aktiv resorbiert und gelangen über die Pfortader erneut zur Leber. Dieser Mechanismus wird als enterohepatischer Kreislauf der Gallensäuren bezeichnet[1].
Ist die Resorption der Gallensäuren im terminalen Ileum eingeschränkt, kommt es zum vermehrten Übertritt von Gallesäuren in das Kolon. Dort führen sie über eine Sekretion von Wasser und Elektrolyten zu Diarrhoe[2]. Dieses Krankheitsbild wird als Gallensäuremalabsorption, Gallensäureverlust-Syndrom oder chologene Diarrhoe bezeichnet.
Eine Gallensäuremalabsorption wurde als entscheidender pathogenetischer Faktor von Durchfällen bei verschiedenen Erkrankungen identifiziert. Neben Erkrankungen wie dem Kurzdarmsyndrom, bei dem das terminale Ileum häufig reseziert ist, wurde ein Gallensäureverlust bei Patienten mit Reizdarmsyndrom oder Mikroskopischer Kolitis festgestellt[3, 4]. Auch bei erwachsenen Patienten mit M. Crohn (MC), einer Erkrankung, die auch als *Ileitis terminalis* bezeichnet wird, zeigten Studien einen Gallensäureverlust bei bis zu 50% der Patienten, während er bei Colitis ulcerosa (CU) selten war[3, 5].
Die Diagnose einer Gallensäuremalabsorption kann mittels verschiedener Verfahren gestellt werden, u.a. durch serologische Messung von 7α-Hydroxy-4-cholesten-3-on (C4), einem Intermediat der hepatischen Gallensäuresynthese. Der Vorteil der C4-Methode liegt in der fehlenden Strahlenbelastung sowie in einem Verzicht auf mehrtägige Stuhlsammlung. Dies macht den Test insbesondere in der pädiatrischen Diagnostik wertvoll[6]. Bei dem ^{75}*Selenium-Homotaurocholicacid*-Test (SeHCAT) wird dem Patienten eine radioaktiv-markierte Gallensäure oral zugeführt. Diese wird unverändert im Ileum resorbiert und in den enterohepatischen Kreislauf eingespeist. Nach sieben Tagen wird mittels Gamma-Kamera die verbliebene Restmenge im Körper bestimmt. Ferner kann radioaktives ^{14}C-Glykocholat eingenommen werden. Bei verminderter ilealer Gallensäureresorption kann nach bakterieller Dekonjugation im Kolon eine erhöhte Menge von ^{14}C-Glycin in einer 24 Stunden-Stuhlsammlung gemessen werden[6]. Die Gesamtausscheidung einzelner Gallensäuren kann auch direkt in einer Stuhlsammlung über 48 Stunden bestimmt werden. Allerdings ist hierfür eine aufwendige Analysetechnik notwendig[6].

Bislang fehlen in der Literatur alters- und geschlechtsspezifische Normalwerte für C4 bei gesunden Kindern und Jugendlichen. In der vorliegenden Dissertation soll die verwendete Methode als diagnostischer Test auf Gallensäureverlust durch Untersuchung eines großen Normalkollektivs bei pädiatrischen Patienten etabliert werden, als pathologische Kontrollen dienen hierfür Patienten mit Kurzdarmsyndrom.
Darüber hinaus soll überprüft werden, ob Gallensäuremalabsorption auch bei pädiatrischen Patienten mit chronisch-entzündlichen Darmerkrankungen (CED) auftritt. Zusätzlich zu den bereits publizierten Studien an erwachsenen CED-Patienten soll außerdem eine Korrelation zwischen laborchemischen Zeichen der Gallensäuremalabsorption und der klinischen Situation der Patienten vorgenommen werden.

Besondere Aufmerksamkeit liegt hierbei auf der Frage, inwieweit eine vermutete Gallensäuremalabsorption ursächlich am Symptom einer chronischen Diarrhoe beteiligt ist.

2. Literaturübersicht

A. Synthese der Gallesäuren und ihre Regulation

Die Synthese der primären Gallensäuren Cholsäure und Chenodesoxycholsäure erfolgt in den Hepatozyten ausgehend von Cholesterin auf einem klassischen/neutralen sowie auf einem alternativen/sauren Stoffwechselweg. Dabei werden ca. 90% der Gallensäuren über den klassischen Weg gebildet[7]. Der geschwindigkeitsbestimmende Schritt in diesem Syntheseweg wird durch das Enzym Cholesterin-7α-Hydroxylase (CYP7A1, Abb. 1) vermittelt. Es katalysiert die Reaktion von Cholesterin zu 7α-Hydroxycholesterin und unterliegt komplexen Regulationsmechanismen.
Zur Verbesserung der Löslichkeit werden die primären Gallensäuren vor der Sekretion ins Gallengangssystem mit den Aminosäuren Glycin oder Taurin konjugiert[7-9]. Im Darmlumen ermöglichen sie als polare Moleküle die spontane Bildung von Mizellen, indem sie hydrophobe Fette umschließen. So gelangen die unpolaren Moleküle in Kontakt zur Bürstensaum-Membran des Dünndarms und können resorbiert werden[10]. Durch bakterielle Dekonjugation und Dehydroxylierung entstehen im Darmlumen die sekundären Gallensäuren Desoxycholsäure und Lithocholsäure[6].

Die Neusynthese von Gallensäuren dient dem Ausgleich der geringen Verluste im enterohepatischen Kreislauf, den der gesamte Gallensäurebestand des Körpers ca. fünfmal täglich durchläuft[11]. Ziel ist die Aufrechterhaltung eines konstanten Gallensäurepools. Daher wird die *De-novo*-Synthese durch hepatisch wiederaufgenommene Gallesäuren gehemmt. Entscheidende Rolle dabei spielt der nukleäre Farnesoid-X-Rezeptor (FXR), der die Aktivität der Cholesterin-7α-Hydroxylase hemmt[12]. Auch in den Enterozyten des Ileums resorbierte Gallensäuren vermitteln über dortige FXR-Aktivierung und das Signalmolekül *fibroblast-growth-factor-19* eine Synthese-Hemmung[13]. Interessanterweise stellt die Gallensäuresynthese den effektivsten Mechanismus des Körpers zur Cholesterin-Exkretion dar[9, 12]. Dies wird therapeutisch durch den Einsatz von Gallensäure-Bindern wie Colestyramin oder Colesevelam bei Hypercholesterinämie, auch bereits im Kindesalter, genutzt[14, 15]. Bei entsprechend gestörter Resorption kann die Neusynthese von Gallensäuren um das Drei- bis Fünffache gesteigert werden[8, 16].

Weitere Faktoren beeinflussen die Gallensäuresynthese: Untersuchungen von Gälman et al. zeigten bei Probanden, die Standardmahlzeiten zu festgelegten Uhrzeiten aßen, zwei Gipfel der Synthese um 13 Uhr sowie um 21 Uhr. Die C4-Werte waren gegenüber dem Basiswert um 9 Uhr morgens zwei- bis vierfach erhöht[17]. Auch Kovár et al. ermittelten einen Peak der Aktivität der 7α-Hydroxylase um 13 Uhr, gemessen an den C4-Werten von zwölf gesunden Probanden, die ebenfalls Standardmahlzeiten zu sich nahmen[16]. In beiden Untersuchungen folgte der Synthesepeak um 13 Uhr dem Mittagessen. Doch auch bei Patienten, die auf jede Nahrungsaufnahme verzichteten, ließ sich ein Anstieg der Gallensäuresynthese in den Mittagsstunden feststellen, wenn auch in geringerem Ausmaß als nach Nahrungsaufnahme[17].
Aus diesen Ergebnissen wurde auf das Vorhandensein einer zirkadianen Rhythmik der Gallensäuresynthese geschlossen. Es muss davon ausgegangen werden, dass sowohl der Zeitpunkt der

Blutentnahme wie auch etwaige vorherige Nahrungsaufnahme bei der Bestimmung der C4-Werte von Bedeutung sind.

B. Methoden zur Erfassung einer Gallensäuremalabsorption

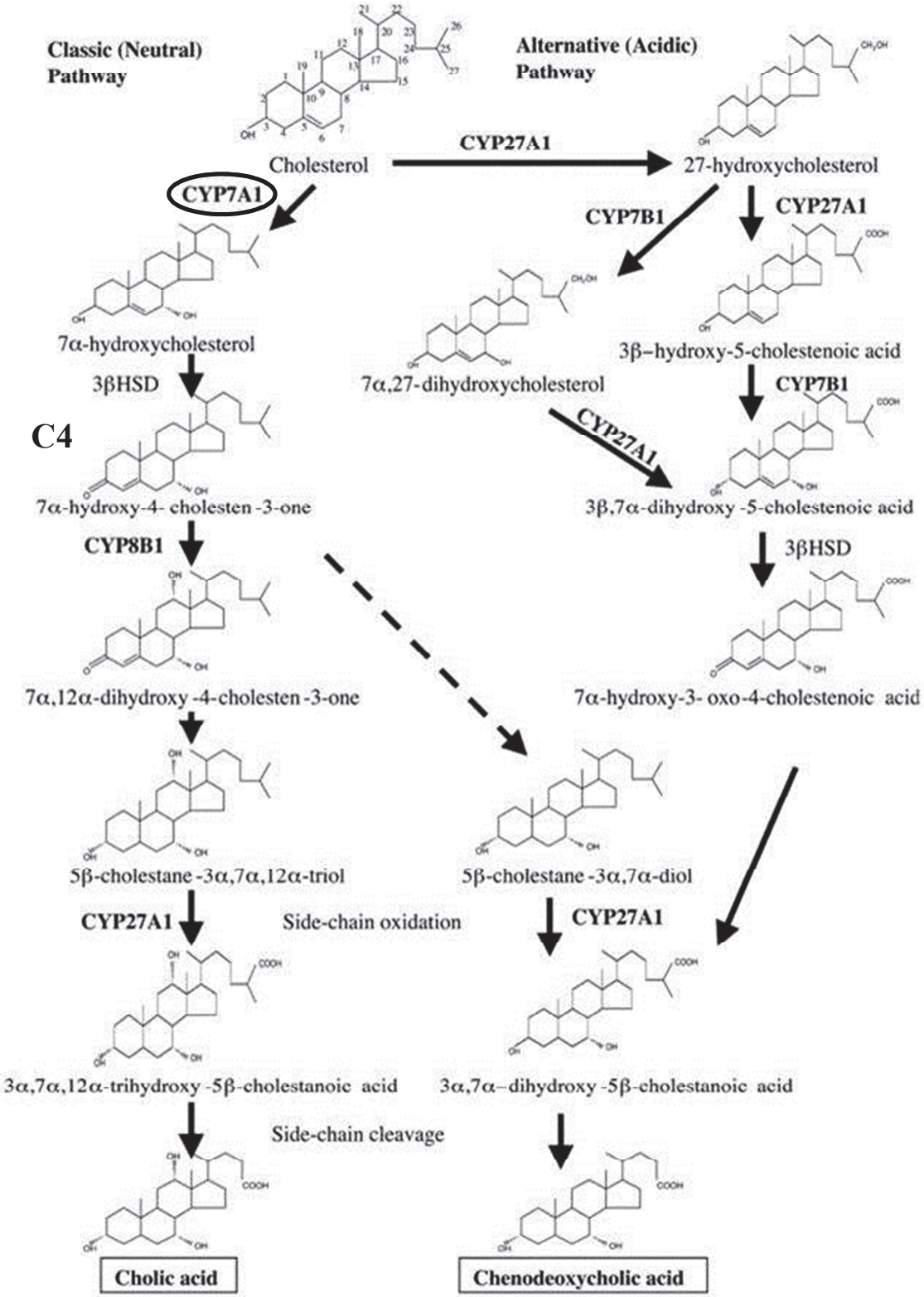
C4-Test:

Grundlegendes Prinzip dieses Tests ist die Tatsache, dass es bei einem Gallensäureverlust über den Darm kompensatorisch zu einer Steigerung der hepatischen Gallensäuresynthese kommt, um einen konstanten Gallensäurepool aufrechtzuerhalten. Als Maßstab für die Gallensäuresynthese wird die Aktivität des Schlüsselenzyms verwendet. C4 spiegelt die Aktivität der CYP7A1 mitsamt ihrer tageszeitlichen Schwankungen wider[18]. Der Metabolit entsteht im zweiten Schritt des klassischen Synthesewegs aus 7-Hydroxycholesterin unter Einfluss der 3β-Hydroxy-C_{27}-Steroid-Oxidoreduktase[7] und gelangt aus der Leber ins periphere Blut[17]. Der Vorteil der C4-Bestimmung liegt darin, dass C4 nicht wie 7α-Hydroxycholesterin auch bei anderen Stoffwechselvorgängen im Körper entsteht[19] (Abb. 1). Erstmals beschrieben wurde die C4-Bestimmung von Axelson *et al.* unter Verwendung einer Hochleistungsflüssigkeits-chromatographie (HPLC)[19]. Als methodische Fortentwicklung wurde 7β-Hydroxy-4-cholesten-3-one als interner Standard eingeführt[20]. Von Sauter wurde die Methode im Klinikum Großhadern etabliert[21].

In der vorliegenden Arbeit wurden Weiterentwicklungen des von Sauter *et al.* publizierten Versuchsaufbaus vorgenommen. Die Probenmenge konnte nach entsprechenden Vorversuchen und in Analogie zu aktuellen Methodenbeschreibungen von 1,5 ml auf 1,0 ml gesenkt werden[17, 22]. Die Trennsäule wurde wie in der Beschreibung von Gälman et al.[17] umgestellt und überdies durch eine entsprechende Vorsäule ergänzt. Zuletzt konnte durch vorherige Destillation des Elutionsmittels Chloroform die Genauigkeit der C4-Bestimmung verbessert werden, da seinen Inhaltsstoffen eine breitbasige Absorption im Detektionszeitraum nachzuweisen war.

Unter Berücksichtigung der zirkadianen Rhythmik der Gallensäuresynthese wurden die Blutentnahmen morgens zwischen acht und elf Uhr unter Nahrungskarenz durchgeführt.

Abb. 1: Gallensäuresynthesewege in der Leber. Modifiziert nach Chiang[8].



C. Kurzdarmsyndrom

Der Begriff Kurzdarmsyndrom beschreibt einen Malabsorptionszustand, bei dem die resorptive Kapazität des Darmes für eine angemessene Versorgung des Körpers mit Nährstoffen und Flüssigkeit nicht ausreicht. Er beschreibt also einen funktionellen Zustand, obgleich verschiedene Definitionen anatomische Kriterien zur Diagnosestellung anführen. Wales et al. definieren Kurzdarm als eine Dünndarmlänge von maximal 25% der für das Alter normalen Länge oder die Notwendigkeit parenteraler Ernährung über einen Zeitraum von mindestens sechs Wochen[23]. Meist resultiert ein Kurzdarmsyndrom nach ausgeprägten Resektionen des Darms. Ursächlich in Betracht kommen bei Kindern vor allem eine Darmresektion nach nekrotisierender Enterokolitis, Dünndarmatresie, Volvulus, Gastrochisis und bei langstreckiger Aganglionose des Darmes[24-27]. Bei der nekrotisierenden Enterokolitis sind in den meisten Fällen Ileum und proximales Kolon von einer Resektion betroffen[28]. Auch bei Dünndarmatresie, Volvulus und Gastroschisis kommt eine Ileumresektion häufig vor[29]. Zur Inzidenz des Kurzdarmsyndroms liegen nur wenige Daten vor, sie wird mit 2-5 Fällen/Million angegeben[24, 25]. Die klinische Symptomatik beinhaltet u.a. Durchfall, Flüssigkeits- und Elektrolytverluste, ferner eine Mangelversorgung mit Makro- und Mikronährstoffen und deren Konsequenzen bei noch wachsenden Kindern. Auch eine Hypersekretion von Magensäure ist häufig, außerdem besteht die Gefahr von Anastomosen-Ulzerationen und -insuffizienzen, jeweils abhängig von Länge und Beschaffenheit des verbliebenen Restdarms[30].

D. Chronischentzündliche Darmerkrankungen (CED) im Kindesalter

Die Gruppe der CED umfasst die Krankheitsbilder Morbus Crohn (MC), Colitis ulcerosa (CU) und die unklassifizierbare CED. Die genaue Ätiologie der CED konnte bisher nicht geklärt werden. Es wird ein Zusammenspiel aus genetischer Prädisposition, Umweltfaktoren und einer Barrierestörung des Darms mit gestörtem Mikrobiom angenommen[31]. Trotz großer regionaler Unterschiede konnte weltweit in den letzten Jahren eine steigende Inzidenz von CED, insbesondere von MC-Erkrankungen im Kinderalter festgestellt werden[32]. Insgesamt werden ca. 25% aller CED im Kindes- und Jugendalter diagnostiziert, die meisten davon wiederum im zweiten Lebensjahrzent[33, 34]. Beide Entitäten zeichnen sich durch einen schubhaften Verlauf mit zwischenzeitlichen Remissionen aus. Die typischen intestinalen Symptome eines MC sind Bauchschmerzen, Durchfall und Gewichtsverlust. Bei der CU sind häufiger blutige Durchfälle zu finden. Darüber hinaus zeigen viele der jungen Patienten Wachstumsstörungen und eine verzögerte Pubertätsentwicklung. Bei 30% der Kinder kommt es außerdem im Krankheitsverlauf zu extraintestinalen Manifestationen der CED[34]. Hier sind als häufigst Arthritiden, Hautveränderungen wie das *Erythema nodosum* oder das *Pyoderma gangraenosum*, entzündliche Augenerkrankungen sowie Lebererkrankungen zu nennen.
Die Diagnose einer CED wird durch obere und untere Endoskopie mit Biopsie-Entnahmen sowie Bildgebung des Dünndarms gestellt. Überdies werden serologische Marker und Stuhluntersuchungen herangezogen. Die Unterscheidung zwischen MC und CU erfolgt anhand der histologischen Beurteilung der entnommenen Biopsate sowie des Befallsmusters. Beim MC findet sich eine

diskontinuierliche Entzündung der ganzen Darmwand, die den gesamten Gastrointestinaltrakt erfassen kann. Im Kindesalter am häufigsten ist ein entzündlicher Befall von Ileum und Kolon[35, 36]. Bei mehr als 50% der pädiatrischen Patienten findet sich jedoch auch ein Befall des Dünndarms bzw. des oberen Gastrointestinaltrakts[36]. Histologisch lassen sich in der entzündeten Schleimhaut bei etwa 50% der MC Patienten bei Diagnosestellung nichtverkäsende Granulome nachweisen, während diese bei der CU fehlen[37]. Es besteht bei MC überdies das Risiko für die Bildung von Fisteln, Stenosen und Strikturen. So müssen bei 20-34% der Patienten innerhalb der ersten fünf Erkrankungsjahre Darmresektionen vorgenommen werden[36, 38].

Bei der CU zeigt sich ein kontinuierlicher Befall vom Rektum ausgehend, der auf Mukosa und Submukosa begrenzt ist. Bei Kindern findet sich häufig bereits zum Diagnosezeitpunkt eine Pankolitis, auch eine Mitbeteiligung des terminalen Ileums als *backwash ileitis* ist möglich[36, 39].

Zusammenfassend sind pädiatrische CED gekennzeichnet durch einen stärker ausgeprägten intestinalen Befall und rasche Progression im Vergleich zu Patienten mit CED-Erstdiagnose im Erwachsenenalter[36].

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Serum 7-Alpha-Hydroxy-4-Cholesten-3-One as a Marker for Bile Acid Loss in Children

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Objective To establish age-related reference values for 7-alpha-hydroxy-4-cholesten-3-one (C4) in a pediatric population and to investigate bile acid malabsorption in children with short bowel syndrome (SBS).

Study design Serum was obtained between 8:00 a.m. and 11:00 a.m. from 100 healthy children (52% males, 9 months to 18 years of age) after 10 hours of fasting. Pediatric patients with SBS served as disease controls (n = 12). Following solid-phase extraction and purification, C4 was determined by high-performance liquid chromatography using a ultraviolet detector at a wavelength of 241 nm. The upper limit of normal for C4 concentrations was defined as the mean plus 2 SD of the log-normal distribution.

Results The mean concentration and SD of C4 in healthy children was 22.8 ± 15.8 ng/mL with no relation to age or sex and an upper limit of normal of 66.5 ng/mL. Normal C4 values were found in 97 of 100 healthy children, and all 12 patients with SBS had C4 concentrations above 100 ng/mL (mean 299.6 ± 167.8 ng/mL; range 105.7-562.1 ng/mL, $P < .0001$ compared with controls).

Conclusions The determined upper limit of normal for C4 concentration in healthy children corresponds to previously published levels in healthy adults and is independent of age and sex. The consistently elevated C4 concentrations in our patients with SBS confirm the reliability of this noninvasive, nonisotopic method to assess bile acid malabsorption in children. *(J Pediatr 2013;163:1367-71)*.

Bile acid loss syndrome, a consequence of disturbed reabsorption of bile acids in the terminal ileum, manifests as diarrhea, steatorrhea with malabsorption of fat soluble vitamins, and predisposes to formation of gallstones. At risk for bile acid loss are patients with chronic inflammation or surgical resection of the terminal ileum such as patients with Crohn's disease or short bowel syndrome (SBS).[1-3] Other conditions causing bile acid loss include a rapid small bowel transit time in diarrheal diseases of different etiologies such as bacterial overgrowth, blind loop syndrome, carbohydrate malabsorption, dysmotility, or after biliary surgery. Interruption of the enterohepatic circulation of bile acids results in loss of bile acids into the cecum and colon leading to watery osmotic diarrhea. The liver compensates partially by activating the de novo synthesis of bile acids. However, chronic bile acid loss often results in a decreased bile acid pool size and changed bile acid composition.

7-alpha-hydroxy-4-cholesten-3-one (C4) is a semiquantitative serum marker for bile acid synthesis in humans.[4] C4, a precursor of cholic acid in the classic (neutral) pathway of bile acid synthesis from cholesterol, correlates with the time-limiting enzymatic step catalyzed by the 7-alpha-hydroxylase.[4] In patients with bile acid loss, CYP7a1 is upregulated as reflected in a higher serum level of C4, which is relatively stable. In adults, normal values for C4 have been established and serum concentrations higher than 50-60 ng/mL indicate bile acid loss.[2,5-8] Two methods typically have been utilized to assess bile acid loss: the 75-SeHCAT scintigraphy and an assay to measure bile acids in the feces. The latter requires stool collection over 3 days, which is impractical in children.[9] For scintigraphy, the radioactive labeled bile acid 75-SeHCAT is orally administered and after 7 days the remaining 75-SeHCAT is measured. The 75-SeHCAT scan is currently the reference standard as a quantitative method. In adults, a good correlation between the 75-SeHCAT scan and the C4 measurement has been shown.[5,10-12] However, the 75-SeHCAT is cost-intensive, time consuming, and involves radiation exposure, and thus, rarely was used in children. The purpose of this prospective study was to evaluate the "C4 method" to assess bile acid loss in pediatric patients. We determined reference values for C4 in a large cohort of children of different age groups from infancy to 18 years of age. To test C4 as a marker for bile acid loss, we investigated serum samples from children with SBS.

BAM	Bile acid malabsorption
C4	7-alpha-hydroxy-4-cholesten-3-one
HPLC	High-performance liquid chromatography
ICV	Ileocecal valve
PN	Parenteral nutrition
SBS	Short bowel syndrome

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Methods

Of the 100 children and adolescents serving as healthy controls, 19 were volunteers and 81 were recruited from the Department of Pediatric Surgery at the Dr von Hauner Children's Hospital. They were scheduled for minor plastic surgeries or elective surgery of the urinary tract or extremities. A structured history was taken for any acute and chronic diseases. Drug intake, weight, length, and body mass index were assessed, and liver enzymes alanine aminotransferase and aspartate aminotransferase measured. Children were excluded if they had by history or laboratory values any evidence of a liver-, pancreatic-, bowel-, renal- or thyroid disease, or if they took any medication over the last 2 weeks.

Patients with SBS were recruited regardless of age from the gastrointestinal department. Exclusion criteria were elevated liver enzymes >2 times the upper limit of the normal or intake of any medication known to affect bile acid synthesis (eg, statins) or ursodeoxycholic acid. The diagnosis of SBS was defined according to the criteria of Wales et al as any child having a laparotomy with a residual small bowel length of less than 25% of the age adjusted small bowel length or the need of postoperative parenteral nutrition (PN) for at least 6 weeks.[13] The age-adjusted length of the small intestine was assessed according to Weaver et al.[14] Anatomic details of the remaining intestine were recorded from surgical records.

Blood samples were obtained in the morning (8:00 a.m.-11:00 a.m.) after overnight fast. In patients with SBS, fasting time was shorter. In 2 patients, it was only 2 hours after the last meal and after stopping PN, and in 4 other patients 3-4 hours. The remaining patients with SBS fasted for at least 8 hours. The blood samples were centrifuged immediately and serum stored at −20°C until analysis.

Written informed consent was obtained from the patient's parents or the patient itself. The study was approved by the local Ethics committee (093-11). The method described by Axelson et al[6] was adapted according to Sauter et al using 7β-hydroxy-4-cholesten-3-one as an internal standard.[5]

Solid-Phase Extraction/Purification

A measurement of 1 mL of serum was diluted with 2 mL of saline, 100 ng of the internal standard 7β-hydroxy-4-cholesten-3-one (Steraloids, Newport, Rhode Island) was added from a 1 mg/1 L stock solution prepared with methanol (LiChrosolv; Merck, Darmstadt, Germany). C4 was extracted in jacketed glass columns, connected to a water bath, using octadecylsilane-bonded silica (Preparative C18, 125Å, 55-105 μm; Waters, Michigan). Columns were prewashed with 2 × 5 mL methanol and 2 × 5 mL of water (LiChrosolv; Merck) and heated to a temperature of 64°C. Samples were sonicated in water for 20 minutes and incubated at a temperature of 64°C for 5 minutes. After loading the cartridges, they were allowed to pass through by gravity (1 mL/min). The extraction procedure was followed by washing the columns with 3 × 5 mL water at a temperature of 64°C and 2 × 5 mL 65% aqueous methanol at room temperature. C4 was then eluted with 2 × 4 mL hexane-chloroform (75:25, v/v, LiChrosolv; Merck and Rotisolv high-performance liquid chromatography [HPLC]; Carl Roth, Karlsruhe, Germany). The samples were dried under nitrogen at 60°C and the extract was reconstituted in 100 μL of methanol.

HPLC-Assay

The HPLC-system consisted of a pump (LC-6A; Shimadzu, Kyoto, Japan), a ultraviolet-detector at wavelength 241 nm, and an integrator (CR-6A; Shimadzu). A reversed phase silica column Nova-Pak C18 column, 3.9 × 300 mm, 4 μm particle size was used with a Nova-Pak C18 guard column, 3.9 × 20 mm, 4 μm particle size (Waters, Milford, Massachusetts). Acetonitril/water (97.5:2.5 v/v, LiChrosolv; Merck) served as mobile phase at constant flow rate of 1 mL/min. The injection volume was 20 μL. C4 was quantified in comparison with the respective peak of the known amount of internal standard.

Statistical Analyses

Results are given as mean ± SD. The D'Agostino and Pearson omnibus normality test was used to test for non-normality in healthy subjects.[15] The upper and the lower level of the reference limits of C4 were determined as mean ± 2 SD of the log-normal distribution. Correlations were tested using Spearman rank coefficient, and sex-dependence via Mann–Whitney U test. The comparison of C4 values in different age groups was performed using Kruskal–Wallis-test/one-way ANOVA. Data analysis was performed using GraphPad Prism 6. P values of <.05 were considered as statistically significant.

Results

C4 Concentrations in Healthy Subjects

The age of the 100 healthy (52 males) controls ranged from 9 months-18 years with a mean age of 9.7 ± 5.1 years and a median of 10.0 years. The mean concentration of C4 ± 1 SD in control children was 22.8 ± 15.8 ng/mL (range 4.7-80.3 ng/mL; median 19.0 ng/mL; 95% CI 19.7-25.9 ng/mL). The 5th to 95th percentile of C4 concentrations in healthy children ranged from 7.3-53.7 ng/mL. Because the serum concentrations of C4 did not follow a normal distribution ($P < .0001$), we used a logarithmic transformation of the C4 values to obtain a normal distribution ($P > .45$). The mean ± 2 SD of the log-normal distributions were 2.92 ± 1.27 ng/mL, resulting in a lower limit of 5.2 ng/mL and an upper limit of 66.5 ng/mL.

Figure 1 shows the C4 values of healthy children for males and females (20.0 ± 11.4 ng/mL for males and 25.9 ± 19.2 ng/mL for females). Age dependency was investigated by dividing into groups (0-<3 years; 3-<6 years; 6-<9 years; 9-<12 years; 12-<15 years; and 15-18 years). Mean in the 6 groups range from 18.2-30.9 ng/mL, medians 14.4-26.1 ng/mL, with no significant differences of C4 values in relation to age (Figure 2).

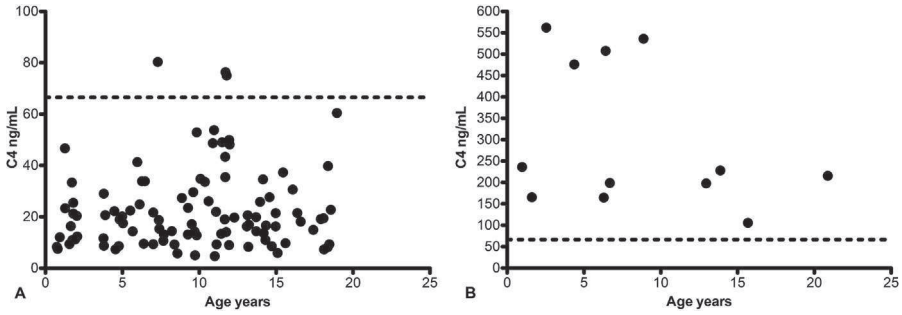

Figure 1. **A,** C4 concentrations of all healthy controls (n = 1 00). **B,** C4 concentrations in patients with SBS (n = 12). *Dashed line* indicates the upper limit of normal C4, defined as mean ± 2 SD of the log-normal distribution (66.5 ng/mL).

C4 Concentrations in SBS

Mean age of 12 patients with SBS was 8.4 ± 6.2 years, ranging from 1.0-20.9 years. Ten of the 12 patients had bowel resections during the first year of life (NEC, volvulus, extended Hirschsprung's disease, small bowel atresia); 2 patients underwent surgery after age 10. The ileocecal valve (ICV) was resected in 7 of 12 patients. The mean duration of the PN after the resection was 12.4 months (range 1-36 months), with 3 patients not weaned off the PN at time of analysis. All patients fulfilled the criteria of Wales et al[13] (**Table I**; available at www.jpeds.com). C4 values in all 12 patients with SBS were far above threshold value of 66.5 ng/mL (mean 299.5 ± 167.8 ng/mL; range 105.7-562.1 ng/mL; 95% CI 192.9-406.1 ng/mL; $P < .0001$). The length of remaining terminal ileum was negatively correlated to C4 concentration (r value: −0.73; $P < .0001$).

Discussion

This study established normal values for C4 concentrations in serum as a method for detection of bile acid loss in a pediatric population. Patients with SBS with a high clinical likelihood for bile acid loss served as disease controls to confirm the validity of elevated C4 values. In healthy controls, no significant differences were found in the different age groups or between girls and boys. The determined cutoff value for pathologic results corresponded to the upper limit of normal reported in healthy adult patients.[2,5] Control patients were equally distributed by age from 9 months-18 years with a mean age of 9.7 ± 5.1 years. The proportion of male to female patients (52:48) was balanced. Blood samples were drawn after fasting for at least 10 hours between 8:00 a.m. and 11:00 a.m. to avoid any interference with natural circadian rhythm of bile acid synthesis.[16,17] The C4 method has been used in several studies in adults (**Table II**) in comparison with 75-SeHCAT scan. Sauter et al investigated 106 patients with identical exclusion criteria to assess values 6.1 ng/mL to 48.4 ng/(mean ± 1.96 SD)[5]; Camilleri et al studied 111 patients with 5th to 95th percentile of 6-60.7 ng/mL.[2] None of the studies found a relation to age and sex, making the C4 method a very stable tool.

Due to ethical restriction, we were not able to validate the C4 methods against 75-SeHCAT. Several studies compared the 2 methods in adult patients with and without bile acid loss and found a good correlation. Eusufzai et al (r = 0.80; $P < .001$) and Brydon et al (r = 0.63; $P < .0001$) correlated C4 levels to the fractional catabolic rate of SeHCAT, and

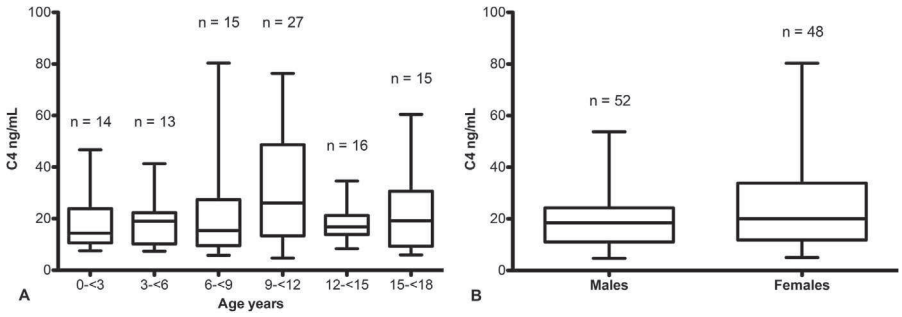

Figure 2. **A**, Comparison between C4 levels of different age groups and **B**, of males vs females, displayed as median and IQRs (*box*). *Whiskers* indicate minimum/maximum. No statistical differences could be seen using ANOVA/Kruskal–Wallis test ($P = .32$) for different age groups, or, for comparing males and females, using Mann–Whitney U test ($P = .27$), although male children had lower C4 levels (mean: 20.0 vs 25.9 ng/mL) and showed less variability (SD: 11.4 vs 19.2 ng/mL).

Table II. Available studies on C4 normal values

Author	No. of controls	Age range (y)	Males (%)	Sex difference	Method	Mean ± SD (ng/mL)	Median (ng/mL)	Range (ng/mL)
Axelson et al[6]	20	21-48	n.d.	No	HPLC	n.d.	12	3-40
Pettersson et al[7]	27	22-53	n.d.	n.d.	HPLC	n.d.	8.9	2-35
Sauter et al[5]	106	15-90	48%	No	HPLC	19.6 ± 10.4	17.9	4-55
Camilleri et al[2]	111	18-65	n.d.	n.d.	LC-MS	22.3 ± 19.7	14.3	2.9-128
Lenicek et al[3]	119	n.d.	53%	n.d.	HPLC	n.d.	12	n.d.
Present study	100	0-18	52%	No	HPLC	22.8 ± 15.8	19.0	4.7-80.3

n.d., not determined.

Bajor et al ($r = -0.71$; $P < .0001$) and Sauter et al ($r = -0.66$; $P < .001$) analyzed the retention or the half-life time of SeHCAT.[5,10-12] Compared with scintigraphy, the C4 method has several advantages: no radiation and only a single blood sample with a total of 1 mL serum required. For C4 measurement HPLC or LC-MS is required, but the inter-laboratory reliability seems high. We were not able to perform intraindividual comparisons because we could not take blood in healthy children at a second time point. In addition, it is not clear whether a shorter fasting period would influence the results.

The test results provide no information about composition of the bile acid pool, but they are indicators of upregulated bile acid synthesis. It is unresolved whether different diets or drugs affect the C4 levels in healthy persons, and how taurine or ursodesoxycholic acid administration may change the results in patients with SBS or cystic fibrosis. Genetic factors may also play a role. This could be an explanation for the slightly elevated levels (60 and 80 ng/mL) in 3 of the healthy children.

Children with SBS represent the extreme end of the spectrum of patients with bile acid malabsorption (BAM). Further studies are needed to prove sensitivity of this method in children with milder forms of BAM, such as in patients with ileocecal resection, inflammatory bowel disease, or irritable bowel syndrome. In a substantial proportion of adult patients with these conditions, BAM has been shown by C4 and 75SeHCAT methods, with a strong concordance of both tests.[18]

The consistently elevated C4 values in patients with SBS confirm the reliability of the method detecting bile acid loss in children. Although this is an inhomogeneous group of patients regarding extent and location of the removed bowel, the absence of the terminal ileum and/or the ICV and the persistent need for PN, they all had C4 concentrations above 100 ng/mL. Patients with removed ICV ($n = 7$) tended to have higher C4 values. Removal of the ICV is only a marker for a more extended resection of the terminal ileum.[19]

In conclusion, the standard normal values for C4, investigated in a pediatric population, allow for a new diagnostic tool for the relatively common pathologic condition of bile acid loss in children. We hope this method will be able to replace the more invasive alternative methods in the near future. This tool will also help to better understand the pathophysiology in cases of unexplained diarrhea, gallstones, or malabsorption syndromes. ■

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50 Years Ago in *The Journal of Pediatrics*

Cigarettes, School Children, and Lung Cancer

Medovy H. J Pediatr 1963;63:1060-2

As a child in the 1960s, I remember being particularly pleased with myself if I could score a pack or 2 of candy cigarettes, and maybe even a gum cigar, in my haul at Halloween. Smoking was admirable. Today such seems patently absurd. However, 50 years ago in *The Journal*, Medovy bemoaned the continuous-exposure of school-age children to television commercials and programs glorifying cigarette smoking with social and material success, romance, and even physical perfection. He blamed parents, the general public, government, and even the medical profession for allowing escalating numbers of children to embrace smoking. The Surgeon General's report was yet a year away, even though lung cancer death rates were skyrocketing and the United Kingdom's Medical Research Council had already acknowledged in 1957 the causal link between cigarettes and cancer.[1]

Medovy presciently wrote, "If the pediatrician is convinced, and we believe he is, that the long-term effects of cigarette smoking are likely to be harmful, even lethal, to the children under his care, then he has a real responsibility to make these facts known in the most persuasive manner at his disposal. The cumulative effect of anticigarette education in thousands of doctors' office, in the patient's home, and in the school is bound to bring about eventually a change in public attitude towards smoking." Indeed he was right. Pediatricians and parents successfully eradicated television shows glorifying smoking, advertising for cigarettes, and even Joe Camel. Now smoking rates in children are at their lowest in years, and deaths from cigarette-induced lung cancer are slowing.[2]

Like it or not, as pediatricians we are still a persuasive, critical mass for social justice and public health. We should do more than pass out advice and antibiotics. In the next 50 years, we should hope that *The Journal* chronicles declines in childhood obesity, gun violence toward children, and child trafficking. Are we convinced yet that these problems are harmful, even lethal, to the children under our care?

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Table I. Features of patients with SBS

Patient	Actual age (y)	Diagnosis	Gestational age at birth (wk + d)	Age at resection (y)	Duration of PN (mo)	Remaining small bowel length (%)	ICV	Remaining colon (%)	Stoma	C4 (ng/mL)	Defecation (per d)
1	11/12	Volvulus	28 + 4	0/12	10 until now	12%	No	100%	No	236.3	2 times (formed)
2	1	Small bowel atresia	36	0/12	20 until now	10%	No	100%	No	165.4	2-6 times (liquid-formed)
3	2	Long segment Hirschsprung disease	40	1/12	10	29%	No	0%	Jejuno	562.1	While feeding (liquid-mushy)
4	4	Volvulus	27 + 3	0/12	3	62%	Yes	100%	No	476.1	1-3 times (liquid)
5	5	Volvulus	24 + 0	0/12	32	16%	Yes	100%	No	164.8	3-4 times (liquid-mushy)
6	6	Volvulus	40	1	18	5%	Yes	100%	No	198.8	1-2 times (liquid-mushy)
7	6	NEC	25 + 3	1/12	36	11%	No	100%	No	507.7	1-4 times (liquid-mushy)
8	8	Volvulus	28	0/12	3	23%	No	100%	No	535.9	2 times (mushy)
9	12	NEC	25	0/12	3	10%	No	50%	No	198	3 times (mushy-formed)
10	13	Volvulus, Hirschsprung disease	40	4	1.5	40%	Yes	25%	No	228.1	3 times (liquid-formed)
11	15	Septic bowel necrosis	n.k.	15	7	22%	Yes	100%	Ileo	105.7	Total PN
12	20	NEC	31	0/12, 8 re-resections last in 3/2011	6	30%	No	60%	No	215.7	4 times (mushy)

n.k., not known.

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Bile acid malabsorption assessed by 7 alpha-hydroxy-4-cholesten-3-one in pediatric inflammatory bowel disease: Correlation to clinical and laboratory findings☆

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KEYWORDS
BAM;
C4-Test;
7α-Hydroxy-4-cholesten-3-one;
Pediatric IBD

Abstract

Background and aims: Measurement of 7 alpha-hydroxy-4-cholesten-3-one (C4) in serum is a semiquantitative test for bile acid malabsorption (BAM). We have previously established pediatric normal values for C4 with an upper limit of normal of 66.5 ng/mL, independent of age and sex. Here we performed the C4 test in 58 pediatric patients with Crohn's disease (CD) and ulcerative colitis (UC).
Methods: C4 was measured using high performance liquid chromatography (HPLC) in fasting serum samples of 44 patients with CD (range 7–19 years) and 14 with UC (4–18 years). Disease activity was assessed by the pediatric CD and UC activity indices (PCDAI and PUCAI, respectively) plus serum (CRP, ESR) and fecal inflammatory markers (calprotectin).
Results: C4 concentrations were increased in 10 CD (23%) (range: 70.8–269.3 ng/mL) but only one UC patient (72.9 ng/mL). CD patients with diarrhea (n = 12) had higher C4-values compared to those without (76.9 vs. 30.4 ng/mL; p = 0.0043). Ileal resection in CD patients (n = 10) was associated with increased C4 concentrations (81.2 vs. 24.3 ng/mL, p = 0.0004). No correlation

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was found between C4 values and inflammatory markers. Six of 7 CD patients with persistent diarrhea but quiescent disease (PCDAI $\leq$12.5) had C4 values indicating BAM.
Conclusion: Elevated C4 concentrations indicating BAM are common in children with CD. They are associated with ileal resection and non-bloody diarrhea in the absence of active disease or elevated inflammatory markers. The C4-test identifies a subgroup of CD patients with persistent diarrhea in spite of clinical remission which may benefit from bile acid binding therapy.

1. Introduction

Bile acid malabsorption (BAM) has been reported in up to 50% of adult patients[1,2] with Crohn's disease (CD), predisposing to diarrhea, steatorrhea with malabsorption of fat soluble vitamins and formation of gallstones and kidney stones.[3–5]

The gold standard in diagnosing BAM is the TauroH-23-(^{75}Se) selena-25-homocholic acid 23-seleno-25-homo-tauro-cholic-acid-test (SeHCAT).[6] The radio-labeled bile acid ^{75}SeHCAT is administered orally, and after seven days the remaining radioactivity is measured by a gamma camera. A retention of less than 10–15% of the administered tracer indicates BAM.[7]

The measurement of the serum marker 7 alpha-hydroxy-4-cholesten-3-one (C4) to assess bile acid loss was first described by Axelson et al.[8] C4 is an intermediate in the classical pathway of bile acid synthesis reflecting the activity of the rate-limiting step catalyzed by the 7 alpha-hydroxylase (CYP7A1).[9] In patients with BAM, CYP7A1 is upregulated in order to compensate, resulting in an increased synthesis of bile acids and their precursors. C4 is a relatively stable precursor and can be measured by HPLC (high-performance liquid chromatography). Although it is a semi-quantitative test, it has some obvious advantages compared to the SeHCAT method[10]: it is easier to perform, less invasive, less time and cost intensive. The C4 method has been modified by Pettersson[11] and Sauter[12] and showed in several studies a good inverse correlation with remaining tracer in the SeHCAT-test.[12–15] Recently, we have established normal values for the C4 test in a large pediatric population of healthy children (n = 100) of different age groups (0–18 years).[16] C4 concentrations above 66.5 ng/mL were defined pathological indicating BAM. This upper limit of normal was independent of age and gender and corresponds to previous published cut off values in adults.[2,12]

In pediatric IBD (PIBD), only few data are available regarding BAM. Childhood onset IBD occurs in up to 25% of all IBD cases and is characterized by extensive intestinal involvement and rapid early progression.[17,18] While no studies have been performed in PIBD applying the C4-tests, two series including a small number of patients looked at BAM by measuring fecal bile acid excretion. In one study investigating BAM in 31 pediatric IBD patients by measuring the fecal excretion of the intravenous administered radio-labeled bile acid carboxyl-^{14}C-cholic acid, there was no difference between pediatric CD and UC patients (n = 15 and 16, respectively). BAM was detected in patients with radiographically abnormal terminal ileum and a high inflammatory activity in the ascending colon assessed by colonoscopy. No influence of clinical disease activity and stool consistency could be detected.[19] The other study revealed significantly increased total fecal excretion of bile acids in 18 pediatric IBD patients (16 UC, 2 CD, age 10–17 years), all of them were in clinical remission and had normal stools.[20]

In this study, we wanted to clarify the following questions: is bile acid malabsorption a problem in pediatric IBD patients, and if so, is it related to the type of disease? Is it influenced by previous ileocecal-resection, the presence of diarrhea or high disease activity? We speculate that the measurement of C4 concentrations allows identifying children with CD or UC with non-bloody diarrhea that is due to BAM and not a sign of mucosal inflammation. This would have major therapeutic implication.

2. Patients and methods

2.1. Subjects

A total of 58 patients were recruited from the IBD clinic of the Division of Pediatric Gastroenterology and Hepatology at the Dr. von Hauner Children's Hospital, Munich. Forty-four patients with CD (median age 15.5 years, range 7–19 years) and 14 with UC (median 15.8 years, range 4–18 years) were recruited consecutively. There were no special inclusion criteria like suspected BAM. Exclusion criteria were intake of bile acids or bile acid sequestrants, bloody diarrhea and elevated liver enzymes (alanine aminotransferase and aspartate aminotransferase) of more than two times the upper limit of normal. The healthy control group consisted of 100 children (median age 10.0, range 9 months to 18 years, 52% males) recently described in detail.[16]

Disease location was assessed in all patients by upper and lower endoscopy and MRI-enterography. Symptoms and disease activity were assessed at the time of blood sampling by calculation of the pediatric Crohn's disease activity index (PCDAI)[21] in CD patients and the pediatric ulcerative colitis activity index (PUCAI)[22] in UC patients. The PCDAI defines inactive disease by a maximum of 10 out of 100 possible points, *mild activity* between 11 and 30 points and *moderate to severe disease* above 30 points. The PUCAI requires less than 10 points for *inactive*, 10 to 34 points are considered as *mild activity* and *moderate to severe activity* as >35 points. In both indices the presence of non-bloody diarrhea increases the score by 5 to 10 points. For example, the presence of three liquid, non-bloody stools per day causes 5 additional points in PCDAI.

Ten of the 44 CD patients had a previous resection of the terminal ileum. The resected bowel length ranged from 10 to 30 cm with inclusion of the ileocaecal valve (ICV) in 9/10 patients.

CD patients were assessed for the presence of persistent diarrhea, which was defined as two or more liquid non-bloody stools per day over the last two weeks.

Written informed consent was obtained from the patient's parents and the patient itself above the age of 14 years. The study was approved by the local Ethics committee (project no. 093-11).

2.2. Measurement of C4

Blood samples were obtained in the morning (8.00–11.00) after an overnight fast. The samples were centrifuged immediately and serum was stored at −20 °C until analysis. C4-concentrations were measured as recently described.[12,16] Briefly, 100 ng 7β-hydroxy-4-cholesten-3-one (Steraloids, Newport, RI, USA), serving as an internal standard, was added to 1 mL of serum. Extractions were undertaken in jacketed glass columns at a temperature of 64 °C using octadecylsilane-bonded silica (Preparative C18, 125 Å, 55–105 μm, Waters, MI, USA). After washing processes C4 was eluted with hexane–chloroform (75:25, v/v, LiChrosolv®, Merck, Darmstadt, Germany/ Rotisolv® HPLC, Carl Roth, Karlsruhe, Germany). Analysis was performed using high performance liquid chromatography on a reversed phase silica column Nova-Pak® C18 column, 3.9 × 300 mm, 4 μm particle size (Waters, Milford, MA, USA) connected to a UV detector at the wavelength of 241 (SPD-10, Shimadzu, Kyoto, Japan). Acetonitrile/water (97.5:2.5 v/v, LiChrosolv®, Merck, Darmstadt, Germany) served as the mobile phase at a constant flow rate of 1 mL/min. C4 was quantified according to the internal standard 7β-hydroxy-4-cholesten-3-one.

2.3. Markers of inflammation

C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), albumin in serum and hematocrit were determined on the day of C4 testing. A stool sample was provided for measurement of fecal calprotectin by enzyme-linked immunosorbent assay (ELISA) (PhiCal® Calprotectin ELISA Kit, Immundiagnostik AG, Bensheim, Germany).

2.4. Statistics

Results are either presented (as mean ± standard deviation (SD)) or as medians plus range (in data not following a normal distribution). Kruskal–Wallis-test/one-way-ANOVA was applied comparing C4 levels in healthy controls and IBD patients as well as the status of the terminal ileum in CD patients. Correlations were tested using Spearman's rank coefficient. The effect of determinants like ileal resection or the presence of diarrhea was assessed by Mann–Whitney-*U* test. Data were analyzed by Graph Pad Prism 6. p-Values < 0.05 were considered as statistically significant.

3. Results

The studied 58 patients included 44 patients with CD and 14 with UC. The disease characteristics of the 44 patients with CD and 14 with UC are given in Table 1.

10 of 44 CD patients had resections of the terminal ileum. 12 CD patients suffered from persistent non-bloody diarrhea. None of the CD patients had bloody diarrhea at the time of investigation. PCDAI-Scores varied between 0 and 52.5 (median 15). 4 of 14 UC patients had diarrhea at the time studied. The mean duration of inflammatory bowel disease in the investigated group of patients was 3.7 ± 2.6 years (CD: 3.4 ± 2.6 years; UC: 4.4 ± 2.9 years).

3.1. C4-concentration in patients with CD

Compared to the previously reported values of healthy control children (n = 100, median 19.0 ng/mL, range 4.7–80.3 ng/mL),[16] we found higher C4 concentrations in CD patients (median 32.8 ng/mL, range 5.8–269.3 ng/mL, $p < 0.001$). 23% of the CD patients (10/44) had elevated C4 concentrations with values above the limit of 66.5 ng/mL (Fig. 1).[16]

CD patients with former ileal resections (n = 10) had significantly higher C4-concentrations than patients with ileal inflammation only (n = 21) and patients without ileal involvement (n = 10) (p = 0.0023) (Fig. 2).

3.2. Relation of C4 concentrations with diarrhea and disease activity

CD patients with persistent non-bloody diarrhea (n = 12) had higher C4-concentrations than those with formed stools (n = 31) (Fig. 3, median: 76.9 vs. 30.4 ng/mL, respectively, p = 0.0043). Characteristics of all 12 CD patients with diarrhea are summarized in Table 2. Elevated C4 concentrations were found in 6 of 7 CD patients with persistent diarrhea in spite of being in remission indicated by a PCDAI ≤12.5 (Fig. 4). The relations between C4 concentrations and stool patterns in all CD patients are shown in Table 3.

Table 1 Clinical features of PIBD patients.

	CD n = 44	UC n = 14
Median age, range	15.5 (7–19)	15.8 (4–18)
Male (%)	27/44 (61%)	8/14 (57%)
Median duration of disease (range)	3.3 (0–10.3)	3.8 (0.1–9.1)
Ileal resection n (%)	10 (23%)	n/a
Ileal involvement n (%)	21 (48%)	n/a
Persistent non-bloody diarrhea n (%)	12 (28%)	4 (29%)
Median disease activity score (range)	15 (0–52.5)	10 (0–55)
Remission [a]	21/44 (48%)	9/14 (64%)

This table displays clinical features, intestinal involvement and disease activity of the 58 pediatric IBD patients studied.
[a] Remission in CD: PCDAI ≤10, in UC PUCAI ≤10.

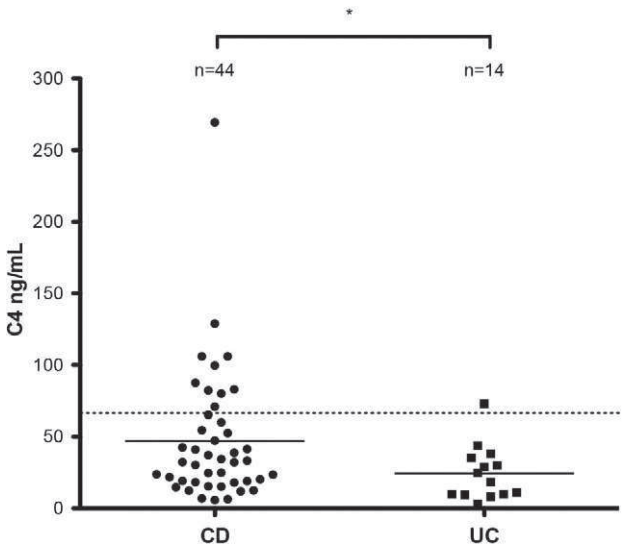

Figure 1 C4 levels in PIBD patients. C4 levels of patients with Crohn's disease (CD) and ulcerative colitis (UC). The dashed line indicates the upper limit of normal (66.5 ng/mL). Data are expressed as median plus range. Mann–Whitney-Test: p = 0.04.

C4 concentrations in CD patients showed no significant correlations to investigated markers of inflammation CRP, ESR and fecal calprotectin (CRP: r = −0.27, n = 44, p = 0.0775; ESR: r = −0.055, n = 41, p = 0.7314; Fecal calprotectin: r = −0.24, n = 27, p = 0.2381).

3.3. C4-concentration in patients with UC

C4 concentrations in UC patients were not significantly different from healthy controls (median: 21.7 ng/mL, range: 3.2–72.9 ng/mL vs 19.0 ng/mL, range: 4.7–80.3 ng/mL, respectively, p = 0.8731). Four of 14 UC patients had persistent non-bloody diarrhea (29%), including the only patient with a slightly increased C4 concentration of 72.9 ng/mL.

No correlations could be found in UC patients between C4 concentrations and inflammatory markers CRP (r = 0.27, n = 14, p = 0.3566), ESR (r = 0.01, n = 13, p = 0.9657) and fecal calprotectin (r = 0.30, n = 7, p = 0.5238). There was no correlation between C4 levels and PUCAI (r = 0.33, n = 14, p = 0.2436).

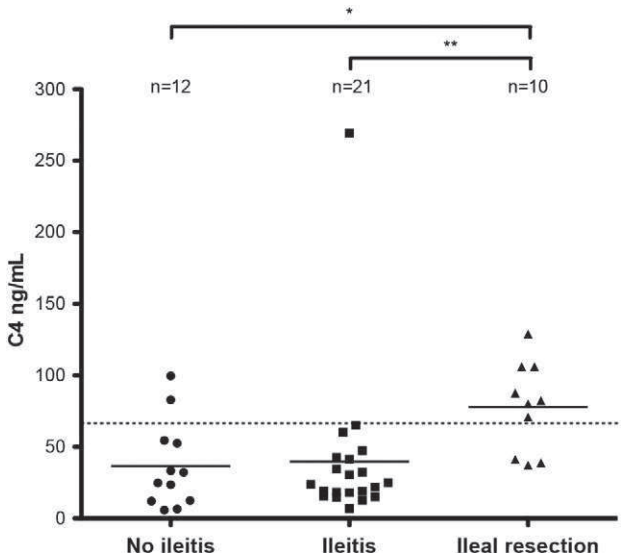

Figure 2 C4 values in CD. Fig. 2 shows C4 levels in CD patients divided up to ileal involvement. C4 levels are not altered by the presence of ileitis, but after ileal resection. Data are shown as dot-plots plus median, the dashed line marks the threshold value. Kruskal–Wallis-Test/1 way ANOVA: p = 0.0023. One patient with ileostoma was excluded.

4. Discussion

In this study, BAM in pediatric IBD patients was investigated applying the C4 test.[12–14] Pediatric CD patients had elevated C4 levels in 23% including those with previous ileal resection, while UC patients did not have an increased risk to have BAM. A risk for BAM in pediatric CD was associated with previous ileal resection and with presence of diarrhea, but not with markers of inflammation.

As the presence of diarrhea as one important factor associated with highly elevated C4 concentrations (Fig. 3), it raises the question if diarrhea is the cause or the consequence of BAM in those patients. To clarify this question, we studied the subgroups with diarrhea in detail.

When looking at all pediatric CD patients suffering from persistent, non-bloody diarrhea (n = 12), 58% (7/12) showed C4 concentrations suspicious for BAM (Table 2). In the group of ileal resected children, only four of ten had loose stools.

Of the 12 CD patients involved in this study that are suffering from diarrhea, 5 had PCDAI scores >15, suggesting an active mucosal inflammation. Interestingly, the other 7 patients with diarrhea had PCDAI scores ≤12.5 (Fig. 4). None

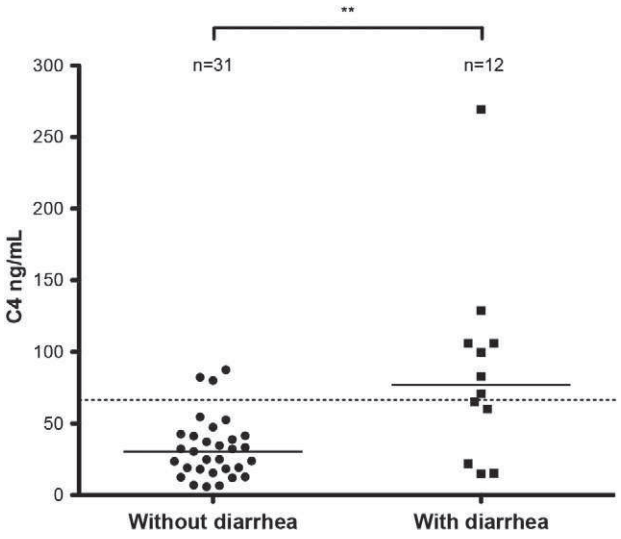

Figure 3 Influence of diarrhea in CD. Fig. 3 shows the influence of non-bloody diarrhea on C4 in CD patients. CD patients with diarrhea (n = 12) had higher serum C4-concentrations than patients with formed stools (n = 31) (p = 0.0043 Mann–Whitney-Test). Data are presented as dot-plots and median, the cut off value is indicated by the dashed line. Mann–Whitney-*U*-test p = 0.0043. One patient with ileostoma was excluded.

Table 2 CD patients with diarrhea.

Patient number	Sex	Actual age (years)	Ileal involvement	Severity of diarrhea (number of stools per day)	C4 (ng/mL)	PCDAI
1	Female	9	Ileitis	2–3 mushy stools	65.2	17.5
2	Male	11	Ileitis	6 liquid stools	14.8	52.5
3	Male	13	Ileitis	4 mushy stools	21.9	27.5
4	Female	13	Ileitis	3–4 liquid stools	15.2	52.5
5	Female	14	No affection	2 liquid stools	99.6	10
6	Female	15	Ileitis	4–5 liquids stools	269.3	12.5
7	Male	15	Resection of 30 cm including ICV	4–5 mushy stools	70.8	10
8	Female	16	Resection of 25 cm including ICV	2 liquid stools	106.0	10
9	Female	16	Resection of 10 cm including ICV	3 mushy-liquid stools	106.0	10
10	Male	16	Ileitis	2–3 liquid stools	60.1	10
11	Female	17	Resection of 15 cm excluding ICV	2 mushy-liquid stools	128.9	7.5
12	Male	17	No affection	2 liquid stools	83.0	17.5

Characteristics of the CD patients with chronic, non-bloody diarrhea (n = 12). 6 of 7 CD patients (86%) with PCDAI scores ≤12.5 showed elevated serum C4 concentrations.

of these 7 patients had elevated CRP levels, and fecal calprotectin was within the normal range in 5 of 7 patients. In this group of patients with persistent diarrhea despite low disease activity clinical or even remission, increased C4 concentrations were observed in 6 of 7 cases (86%), with a borderline result in the remaining patient (60.1 ng/mL). Thus it is very likely that BAM is the cause of their symptoms and not the consequence.

This is supported by our data on pediatric UC patients. Here, BAM was observed only in one of 14 patients, while 4 of 14 patients suffered from diarrhea at the time tested. This patient suffered from persistent diarrhea while exhibiting elevated inflammatory markers. In adult UC patients, BAM also seems to be a very rare condition (1%, n = 71).[1] We conclude from these results that diarrhea alone does not lead to BAM but certain conditions like backwash-ileitis in UC patients may probably increase the risk for BAM in UC patients.

The percentage of BAM in pediatric CD of 23% is relatively low compared to other studies, mostly performed on adults. Camilleri et al. found increased C4 levels in 46% of CD patients with ileal disease and in 55% of those with ileal resections.[2] Lenicek et al. observed laboratory signs of BAM by C4 test in 42% of 276 CD patients examined.[1] Significantly elevated C4-levels were not only found in CD patients with inflammation or resection of the terminal ileum, but also in 14% of CD patients with only colonic involvement.[1]

One possible explanation for the lower prevalence of BAM in our pediatric patients compared to adult studies is the fact that we have a selection of patients, excluding for example those with bloody diarrhea. Other possible causes are the relatively short duration of the disease (3.4 ± 2.6 years) in our cohort, and the lower percentage of patients with previous ileal resections (23% vs. 61% in the adult study).[1] As expected, we found a significantly higher risk for BAM in ileal resected patients (Fig. 2). Nonetheless, 30% (3/10) of our ileal resected patients showed normal concentrations of C4, according to the findings in adults, where 38% of the CD patients did not manifest BAM after ileal surgery.[1] Conversely, BAM was also found in non-resected CD patients (3/34 in children vs. 13/109 in adult CD patients).[1]

There are different hypotheses to explain the pathophysiology of BAM in CD. Impaired fibroblast growth factor (FGF19) feedback inhibition of bile acid synthesis is one explanation on the molecular level.[23] Normally, FGF 19 inhibits the bile acid de-novo-synthesis when bile acids are reentering the portal circulation. Accordingly, impaired FGF 19 feedback inhibition leads to up-regulated bile acid synthesis as proven in adult CD patients.[1] Furthermore,

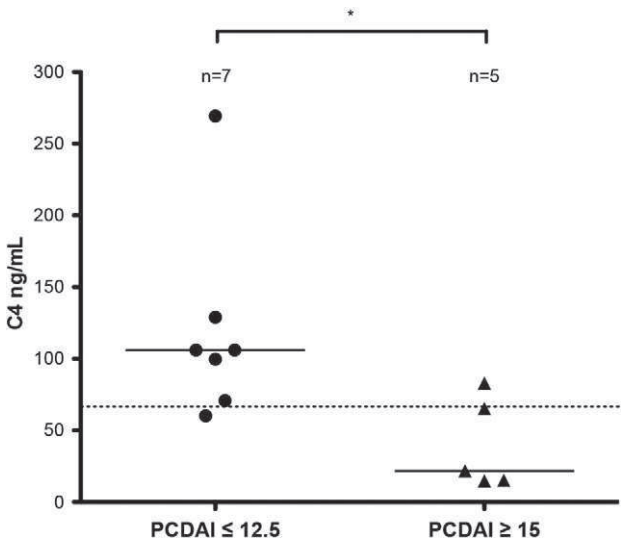

Figure 4 Disease activity in CD with diarrhea. CD patients with persistent diarrhea divided by disease activity. All but one patient with quiescent disease indicated by a PCDAI ≤ 12.5 showed C4 levels above the threshold value (dashed line) Mann–Whitney-*U*-test p = 0.023.

Table 3 C4 and stool patterns in CD.

	Diarrhea	No diarrhea	
C4 levels elevated	7	3	10 (23%)
C4 levels normal	5	28	33 (77%)
	12 (28%)	31 (72%)	43 (100%)

This table shows the numbers of patients examined for C4 and stool patterns, proportions in parentheses. The limit of C4 concentrations considered normal is 66.5 ng/mL. One patient with ileostoma was excluded.

there is evidence that BAM is induced by accelerated small bowel and colonic transit time, which can be assumed in the situation of persistent diarrhea.[24] As another hypothesis, a diminished expression of the ileal bile acid transporter "ASBT" (apical sodium dependent bile acid transporter) in patients with active CD has been observed, which was independent of ileal inflammation.[25] On the other hand, suspected mutations in the apical sodium-dependent bile acid transporter (ASBT) gene (*SLC10A2*) in patients with BAM have not been found.[1,26]

The study was limited to the fact that the C4 test is an indirect method, reflecting the up-regulation of bile acid synthesis instead of the amount of fecal bile acid loss. So we cannot exclude that there is an increased bile acid synthesis despite a normal bile acid reuptake in the terminal ileum. Although a negative correlation of C4 and FGF-19 has been found in adult patients,[1] FGF-19 assessment is needed to clarify this question.[17]

Neither the serum inflammatory markers ESR, CRP nor fecal calprotectin correlated with increased C4 values in pediatric CD patients. While both CRP and ESR are unspecific markers, fecal calprotectin, a neutrophil cytosolic protein, has shown to be more accurate in reflecting disease activity[27,28] than CRP and ESR. There was also no positive correlation with disease activity score PCDAI.[21] In conclusion of these results, we found that active CD alone does not necessarily lead to BAM.

In this study, we confirmed the clinical importance to test for BAM in pediatric IBD patients, especially in children with CD suffering from persistent diarrhea despite clinical remission or very limited disease activity. Our data support the hypothesis that malabsorbed bile acids in the colon cause diarrhea in these patients. In the case of proven BAM, a therapeutic intervention with bile acid binders should be considered, although these potent drugs may exert adverse side effects. Especially the decrease of absorption of fat and fat-soluble vitamins[29] and interactions with other drugs like oral contraceptives should be taken into consideration when discussing long term treatment. Prospective studies are needed to proof the benefit of this treatment in pediatric IBD patients with BAM associated diarrhea. In conclusion, the C4 test is a valuable tool to easily differentiate between diarrhea related to disease activity or to BAM in IBD and contributes to find the appropriate treatment.

Conflict of interest

This is to state that there is no conflict of interest or ethical adherence regarding the submitted manuscript "Bile acid malabsorption assessed by 7 alpha-hydroxy-4-cholesten-3-one in pediatric inflammatory bowel disease: Correlation to clinical and laboratory findings" for me or any of the co-authors.

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6. Zusammenfassung

Hintergrund:
Eine verminderte Resorption von Gallensäuren im terminalen Ileum führt zu einem vermehrten Anfall von Wasser und Elektrolyten im Dickdarmlumen mit der Folge einer chronischen Diarrhoe. Eine Gallensäuremalabsorption ist bei Patienten nach Resektion des terminalen Ileums beschrieben, außerdem bei ca. der Hälfte der erwachsenen Patienten mit Morbus Crohn (MC).
Bei Kindern sind Daten zu Gallensäuremalabsorption sehr limitiert, da die meisten Untersuchungsmethoden eine Strahlenexposition oder Stuhlsammlung erfordern. Die Bestimmung von 7α-Hydroxy-4-cholesten-3-on (C4) im Serum als Marker der Gallensäuresynthese wurde bei Kindern bisher nicht durchgeführt. Es fehlen alters- und geschlechtsspezifische Normalwerte ebenso wie Daten zur Gallensäuremalabsorption bei pädiatrischen Patienten mi chronisch-entzündlichen Darmerkrankungen (CED).

Methoden:
Nach geringen methodischen Modifikationen wurde C4 bei 100 Darm-gesunden Kindern zwischen 0-18 Jahren bestimmt. Hierbei erfolgte die C4-Messung nach Festphase-Extraktion und Aufreinigung mittels Hochleistungsflüssigchromatographie unter Verwendung eines UV-Detektors der Wellenlänge 241 nm. An zwölf Kurzdarmsyndrom-Patienten mit vermutetem Gallensäureverlust wurde die Zuverlässigkeit der Methode geprüft. In einem zweiten Schritt wurden 58 pädiatrische CED-Patienten mittels C4-Test untersucht, zugleich wurden klinische und laborchemische Daten wie Entzündungsmarker, Krankheitsaktivität, Stuhlgewohnheiten oder Befallsmuster erhoben. Es sollte geklärt werden, ob ein Gallensäureverlust auch bei Kindern mit CED serologisch nachzuweisen ist, mit welchen klinischen Charakteristika er assoziiert ist und inwieweit ein solcher ursächlich am häufigen Symptom einer chronischen Diarrhoe beteiligt ist.

Ergebnisse:
Der C4-Grenzwert bei den 100 Darm-gesunden Kindern wurde als Mittelwert plus zwei Standardabweichungen der log-Normalverteilung festgelegt. Dieser lag mit 66,5 ng/ml im Bereich bisher publizierter Normalwerte für Erwachsene und war unabhängig von Alter und Geschlecht der Probanden. In der heterogenen Gruppe der zwölf untersuchten Kinder mit Kurzdarmsyndrom fielen durchweg Werte oberhalb des ermittelten oberen Grenzwertes auf. Somit scheint die verwendete Methode geeignet, auch bei Kindern die Diagnose eines Gallensäureverlusts zu stellen.
In der Gruppe der CED-Patienten zeigten sich bei MC-Patienten im Vergleich zur Kontrollgruppe erhöhte C4-Werte, während sich die der CU-Patienten nicht von denen gesunder Kinder unterschieden. Pathologisch erhöhte Werte entsprechend einem Gallensäureverlust fanden sich bei 23% der Kinder mit MC. Bei der Korrelation der C4-Werte mit klinischen und serologischen Charakteristika der MC-Patienten konnte nachgewiesen werden, dass erhöhte Messwerte mit vorangegangener ilealer Resektionen und dem Vorhandensein persistierender Durchfälle einhergingen. Von zwölf MC-Patienten mit chronischer Diarrhoe waren sieben Kinder in Remission und fünf litten unter einer aktiven

Entzündung. Trotz geringer Stichprobengröße waren die C4-Werte der Kinder in Remission signifikant höher als die der Patienten im Krankheitsschub. Dies deutet darauf hin, dass die Durchfälle bei den Kindern in Remission durch einen Gallensäureverlust verursacht wurden.

Schlussfolgerungen:
In der vorliegenden Dissertation wurden Normwerte der C4-Methode für Kinder erstellt und der Wert der Methode bei Kindern mit verschiedenen Erkrankungen des terminalen Ileums nachgewiesen. So konnte eine Subgruppe von MC-Patienten mit persistierenden Durchfällen identifiziert werden, bei denen die Diarrhoe nicht durch eine aktive Entzündung, sondern durch einen Gallensäureverlust verursacht zu sein schien. Diese Differenzierung hat therapeutische Konsequenzen für die betroffenen Patienten.

7. Abstract

Background:
An impaired resorption of bile acids in the terminal ileum leads to a higher amount of water and electrolytes in the colonic lumen and consequently to chronic diarrhea. Bile acid loss is a well described phenomenon in patients after surgical resection of the terminal ileum and also common in adult patients suffering from Crohn`s disease (CD).
In children there is only very limited data on bile acid malabsorption (BAM) due to the need of radiation exposure or stool collection in most published detection methods. The measurement of 7α-hydroxy-4-cholesten-3-one (C4) in serum as a marker of bile acid synthesis has not been performed in pediatric patients so far, therefore age- and sex-related normal values are lacking as well as data on BAM in pediatric inflammatory bowel disease (IBD) patients.

Methods:
After minor methodical changes 100 healthy children between 0 and 18 years of age were examined. Following solid-phase extraction and purification, C4 was determined by high-performance liquid chromatography using a UV detector at a wavelength of 241 nm**.** The reliability of the method was tested using twelve patients suffering from short bowel syndrome (SBS) with an assumed BAM. Afterwards 58 pediatric IBD patients were investigated. Additionally inflammatory markers, disease activity, bowel habits and disease localization were assessed. The aim was to clarify if BAM can also be serologically diagnosed in pediatric patients. And if so, is it related to certain clinical features and does it contribute to the symptom of chronic diarrhea?

Results:
An upper limit of normal for C4 in 100 healthy children was determined as mean plus two standard deviations of the log-normal distribution. The cutoff value of 66.5 ng/mL was independent of sex and age and corresponded to previous published normal values in healthy adults. The consistently elevated C4 values in the twelve heterogeneous patients with SBS confirmed the reliability of the method detecting bile acid loss in children.
In the group of the pediatric IBD patients elevated levels compared to healthy children could be detected in patients with CD, while no differences could be observed in Ulcerative colitis (UC) patients. Pathologic C4-levels suggesting BAM were found in 23% of the children suffering from MC. Comparing these results to clinical and laboratory findings the risk for BAM in pediatric CD was associated with previous ileal resection and the presence of diarrhea. Among the twelve patients with chronic diarrhea seven were in clinical remission while five had an active mucosal inflammation. Despite the low sample size patients in clinical remission showed statistically significant higher C4-levels than those in acute disease relapse. These data suggest that chronic diarrhea in patients with quiescent disease is caused by BAM.

Conclusions:
In this dissertation normal values of the C4-method in pediatric patients were established and the clinical relevance of this method was confirmed in children with different pathologies of the terminal ileum. Furthermore a subgroup of CD patients could be identified, in which not inflammation but BAM is very likely to cause the persistent, non-bloody diarrhea seen in these patients. This distinction has a therapeutic implication in the affected patients.

8. Verzeichnis der Abkürzungen und Akronyme

BAM	*Bile acid malabsorption*
C4	7α-Hydroxy-4-cholesten-3-on
CD	*Crohn`s disease*
CED	Chronisch-entzündliche Darmerkrankungen
CU	Colitis ulcerosa
CYP7A1	Cholesterin-7α-Hydroxylase
FXR	Farnesoid-X-Rezeptor
HPLC	Hochleistungsflüssigchromatographie
IBD	*Inflammatory bowel disease*
MC	Morbus Crohn
SBS	*Short bowel syndrome*
SeHCAT	^{75}*Selenium-Homotaurocholicacid*-Test
UC	*Ulcerative colitis*

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10. Veröffentlichungen

Originalartikel:

Freudenberg F, Gothe F, Beigel F, Rust C, Koletzko S. 7 alpha-hydroxy-4-cholesten-3-one as a serum marker for bile acid loss in children. J Pediatr. 2013 Nov;163(5):1367-71.

Anteil Gothe F: Patientenrekrutierung, Probensammlung, C4-Messungen, statistische Auswertung, Interpretation der Ergebnisse, Mitwirkung an Manuskript

Gothe F, Koletzko S, Beigel F, Rust C, Freudenberg F. Bile acid malabsorption assessed by 7 alpha-hydroxy-4-cholesten-3-one in pediatric inflammatory bowel disease: Correlation to clinical and laboratory findings. J Crohns Colitis. 2014 Sep 1;8(9):1072-8.

Anteil Gothe F: Patientenrekrutierung, C4-Messungen, statistische Auswertung, Interpretation der Ergebnisse, Schreiben des Manuskriptes

Vorträge und Posterpräsentationen:

Freudenberg F, Gothe F, Beigel F, Rust C, Koletzko S. 7 alpha-hydroxy-4-cholesten-3-on als Serummarker für Gallensäureverlust bei Kindern.
Vortrag auf der 27. Jahrestagung der *Gesellschaft für pädiatrische Gastroenterologie und Ernährun*g, Wien, 27.04.2012

Gothe F, Beigel F, Rust C, Hajji M, Koletzko S, Freudenberg F. Gallensäureverlust bei Morbus Crohn.
Poster auf der 28. Jahrestagung der *Gesellschaft für pädiatrische Gastroenterologie und Ernährun*g, Heidelberg, 22.03.2013

Freudenberg F, Gothe F, Beigel F, Rust C, Koletzko S. Determination of 7 alpha-hydroxy-4-cholesten-3-one in serum as marker for bile acid loss in children.
Poster auf dem 46th Annual Meeting of *The European Society for Pediatric Gastroenterology, Hepatology and Nutrition*, London, 10.05.2013

Gothe F, Koletzko S, Rust C, Beigel F, Freudenberg F. Bile acid malabsorption assessed by the C4-test in pediatric IBD patients.
Vortrag und Poster of Distinction auf dem 46th Annual Meeting of *The European Society for Pediatric Gastroenterology, Hepatology and Nutrition*, London, 11.05.2013

Eidesstattliche Versicherung

Gothe, Florian Eberhard Karl

Name, Vorname

Ich erkläre hiermit an Eides statt,

dass ich die vorliegende Dissertation mit dem Thema

Messung von 7α-Hydroxy-4-cholesten-3-on als Marker für Gallensäureverlust. Etablierung von Normalwerten bei Kindern und Anwendung bei Patienten mit Kurzdarmsyndrom und chronisch-entzündlichen Darmerkrankungen.

selbständig verfasst, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

Ich erkläre des Weiteren, dass die hier vorgelegte Dissertation nicht in gleicher oder in ähnlicher Form bei einer anderen Stelle zur Erlangung eines akademischen Grades eingereicht wurde.

München, den 10.12.2015

Ort, Datum

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