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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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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Bile acid malabsorption (BAM) has been reported in up to 50% of adult patients with Crohn's disease (CD), predisposing to diarrhea, steatorrhea with malabsorption of fat soluble vitamins and formation of gallstones and kidney stones. The gold standard in diagnosing BAM is the TauroH-23-(75Se) bile acids or 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 7 alpha-hydroxy-4-cholesten-3-one method; it is easier to perform, less invasive, less time consuming and cost intensive. Although it is a semi-quantitative test, it has some obvious advantages compared to the C4 test in a large pediatric population of healthy children (n = 100) as well as different age groups (0–18 years). C4 concentrations above 66.5 ng/mL were defined as pathological. This upper limit of normal was independent of age and gender and corresponds to previous published cut-off values in adults. 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. 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). In patients with BAM, CYP7A1 is upregulated in the pathway of bile acid synthesis reflecting the activity of the acid-test (SeHCAT). The radio-labeled bile acid 75SeHCAT 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. 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. 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). 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 7 alpha-hydroxy-4-cholesten-3-one method; it is easier to perform, less invasive, less time consuming and cost intensive. The C4 method has been modified by Pettersson and Sauter and showed in several studies a good inverse correlation with remaining tracer in the SeHCAT-test. 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). C4 concentrations above 66.5 ng/mL were defined as pathological indicating BAM. This upper limit of normal was independent of age and gender and corresponds to previous published cut-off values in adults.

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. 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-14C-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. 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.

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 a 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.

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) in CD patients and the pediatric ulcerative colitis activity index (PUCAI) 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 ileocecal 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. 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, Milford, MA, 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/Rotisolv®) 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) (PhCal@ 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), 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).

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>
<thead>
<tr>
<th>Table 1</th>
<th>Clinical features of PIBD patients.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD n = 44</td>
</tr>
<tr>
<td>Median age, range</td>
<td>15.5 (7–19)</td>
</tr>
<tr>
<td>Male (%)</td>
<td>27/44 (61%)</td>
</tr>
<tr>
<td>Median duration of disease (range)</td>
<td>3.3 (0–10.3)</td>
</tr>
<tr>
<td>Ileal resection n (%)</td>
<td>10 (23%)</td>
</tr>
<tr>
<td>Ileal involvement n (%)</td>
<td>21 (48%)</td>
</tr>
<tr>
<td>Persistent non-bloody diarrhea n (%)</td>
<td>12 (28%)</td>
</tr>
<tr>
<td>Median disease activity score (range)</td>
<td>15 (0–52.5)</td>
</tr>
<tr>
<td>Remission a</td>
<td>21/44 (48%)</td>
</tr>
</tbody>
</table>

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.
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 ($r = 0.33$, $n = 14$, $p = 0.2436$).

4. Discussion

In this study, BAM in pediatric IBD patients was investigated applying the C4 test. 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 $\leq 12.5$ (Fig. 4). None

![Figure 1](http://ecco-jcc.oxfordjournals.org/) 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: $p = 0.04$.

![Figure 2](http://ecco-jcc.oxfordjournals.org/) 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.

![Figure 3](http://ecco-jcc.oxfordjournals.org/) 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.
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,

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

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.
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. 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. On the other hand, patients with active CD have been observed, which was suspected mutations in the apical sodium-dependent bile acid transporter (ASBT) gene (SLC10A2) in patients with BAM have not been found.

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, FGF-19 assessment is needed to clarify this question.

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 cytotoxic protein, has shown to be more accurate in reflecting disease activity than CRP and ESR. There was also no positive correlation with disease activity score PCDAI. 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 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 IBD\" for me or any of the co-authors.

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