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# Pediatric Autoimmune or Primary Sclerosing Cholangitis: Metronidazole Effectiveness on Biochemical Data, Bile Acid Profile, and Gut Microbiota: A Pilot Study

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**ABSTRACT**

**Objectives:** Autoimmune hepatitis and primary sclerosing cholangitis (PSC) can both be present, resulting in autoimmune sclerosing cholangitis (ASC). PSC physiopathology could be based on the cross-talk between gut microbiota and bile acids (BAs); antibiotics are an innovative therapy. This pilot study assesses metronidazole (MTZ)'s effectiveness in ASC or PSC patients according to the stage of the disease, and its effects on biochemical parameters, BA profiles, and gut microbiota.

**Methods:** ASC or PSC patients from Cliniques universitaires Saint-Luc's pediatric hepato-gastroenterology division were enrolled retrospectively and prospectively; both datasets were merged. MTZ was administered over at least 14 days on top of standard treatment (ursodeoxycholic acid, azathioprine, and steroids). Fecal and blood samples were collected before (T0) and at MTZ day 14 (T14). Sustained biochemical remission was defined by the reduction of transaminases (AST and ALT), gamma-glutamyl transferase (GGT), and CRP until 12 months post-MTZ.

**Results:** A total of 18 patients (mean age, 13.2±4.5 years) were enrolled (13 ASC and 5 PSC), and divided in remission or relapse patients. CRP, AST, ALT, and GGT levels decreased post-MTZ in both groups (excepting GGT in relapse patients), with decreases between T0 and T14 being significant for AST and ALT. Relapse patients were older ($P = 0.0351$) and in late-disease stage, with mainly large-duct PSC ($P = 0.0466$). In remission patients, the mean plasma relative abundance of hydrophilic BA increased by +6.3% ($P = 0.0391$) after MTZ. Neither at baseline nor T14, there were significant differences in gut microbiota recorded.

**Conclusion:** These data are likely indicative of long-term benefits following MTZ therapy at early-stage ASC or PSC, with increased hydrophilic BA abundance. Multicenter prospective studies are needed.

**What Is Known**

- Autoimmune sclerosing cholangitis is an overlap syndrome characterized by features of autoimmune hepatitis and sclerosing cholangitis.
- Therapeutic options are scarce.

**What Is New**

- Biochemical data (CRP, AST, ALT, and gamma-glutamyl transferase levels) decreased following metronidazole (MTZ) in remission patients; these decreases were higher in remission than in relapse patients.
- MTZ therapy could result in long-term benefit at early-stage autoimmune sclerosing cholangitis or primary sclerosing cholangitis.
- The mean plasma relative abundance of hydrophilic bile acids significantly increased to a greater extent in remission versus relapse patients post-MTZ therapy.
- The hypothesis of gut-liver axis involvement could not be proven, possibly due to the small sample size.

**Key Words:** primary sclerosing cholangitis, autoimmune sclerosing cholangitis, overlap syndrome, gut microbiota dysbiosis, bile acid profile, metronidazole

## INTRODUCTION

Autoimmune hepatitis (AIH) is a chronic liver disease caused by dysregulated, not yet fully elucidated, immune mechanisms (1). This inflammatory liver condition is associated with circulating autoantibodies, hypergammaglobulinemia, and distinctive features on liver biopsies (2). Primary sclerosing cholangitis (PSC) is a rare chronic cholestatic liver disease of unknown etiology, with variable progression (3). The term overlap syndrome is employed to describe variant forms that display characteristics of both AIH and PSC, which is also called autoimmune sclerosing cholangitis (ASC) mainly observed in children and young adults (4,5). This condition is often associated with inflammatory bowel disease, primarily ulcerative colitis (UC). Without treatment, overlap syndrome tends to progress toward end-stage biliary cirrhosis requiring liver transplantation (6). Its therapeutic management is still empiric, given the lack of controlled trials (7).

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The striking association between PSC and inflammatory bowel disease points toward the gut-liver axis. The lack of an ideal animal model explains the shortage of knowledge about the pathogenesis. Several studies have set out to elucidate the role of the gut microbiota, its metabolites, and its influence on host immune defenses in PSC (8). Gut microbes have been hypothetically implicated in PSC's pathogenesis through their role in the synthesis of various metabolites such as bile acids (BAs), which operate as signaling molecules with a crucial impact on the gut and liver (8). As illustrated in Figure 1, the basic hypothesis is that gut microbes or their metabolites pass through a leaky gut barrier. These agents then gain access to the liver via the portal circulation. In the liver parenchyma, they trigger inflammatory reactions and impaired immune responses, which both augment tissue injury and fibrosis.

A handful of small randomized trials have reported biochemical improvements with antibiotic therapy such as vancomycin and metronidazole (MTZ) in patients with PSC (9). Microbial metabolites, including hydrophobic BAs, possibly cause cellular hepatocyte injury.

BA pool consists of multiple BA species, some of which are more hydrophobic than others, which accounts for their functional differences. ursodeoxycholic acid (UDCA) and CA—conjugated or free forms—were considered hydrophilic BA owing to their trihydroxylic structure, whereas chenodeoxycholic acid (CDCA), DCA, and lithocholic acid were hydrophobic (10).

Drug therapies that target BA pathways represent an active research area, with UDCA being widely employed. Besides, standard immunosuppression is currently proposed for managing ASC; this approach has been shown effective on parenchyma inflammation, yet not on cholangiopathy (11,12). UDCA can be added for managing bile duct lesions, and this agent is also used for PSC therapy (13). UDCA was shown to result in biochemical benefits, without improved long-term outcome, yet with serious undesirable effects at high doses (14). There has been scarce research on pediatric populations.

This pilot study sought to assess MTZ's effects in children with ASC or PSC based on the biochemical data, gut microbiota, and BA profile. The primary end point was to evaluate the MTZ's effectiveness according to the stage of the disease. The current study was prospective in nature, but data from a retrospective observation conducted in the same department were included to enhance data relevance.

## METHODS

### Participants

We prospectively included all patients with ASC or PSC attending the pediatric hepato-gastroenterology division of the Cliniques universitaires Saint-Luc in Brussels, Belgium from December 2017 to February 2020. The retrospectively collected data pertained to a first pilot study started in April 2016 with the same promotor and investigator Etienne Sokal, MD PhD, with the same medical staff. For BA analysis, both datasets were compared with data collected from healthy pediatric volunteers, recruited upon preoperative anesthesia consultation, who were not treated with MTZ and had no hepato-gastrointestinal disease (only stomatological disease, growth delay, or urological disease). For the BAs control group, only one blood sample was collected for BA analysis; collection of a stool sample was not feasible.

### Inclusion Criteria

Patients were included if AIH diagnosis was established based on an International Autoimmune Hepatitis Group score >15 (15); PSC diagnosis was defined by increased gamma-glutamyl transferase (GGT) levels above 1 to 2× upper limit of normal (ULN), with typical PSC findings on biopsies. The difference between small-duct and large-duct PSC was based on the presence (or absence) of intrahepatic lesions on magnetic resonance cholangiopancreatography (MRCP) if available or on liver ultrasound if MRCP was unavailable (3). UC diagnosis was confirmed by fecal calprotectin levels or biopsy findings upon colonoscopy. UC activity was established with pediatric UC activity index score (16,17).

### MTZ Treatment

Patients were treated using MTZ over 14 days at 500 mg three times a day (3×/day) or 7.5 mg/Kg 3×/day for children under 12 years old, on top of standard treatment. In the absence of biochemical improvement, repeated or extended treatments of MTZ could be considered by the clinician. Stool and blood samples were collected

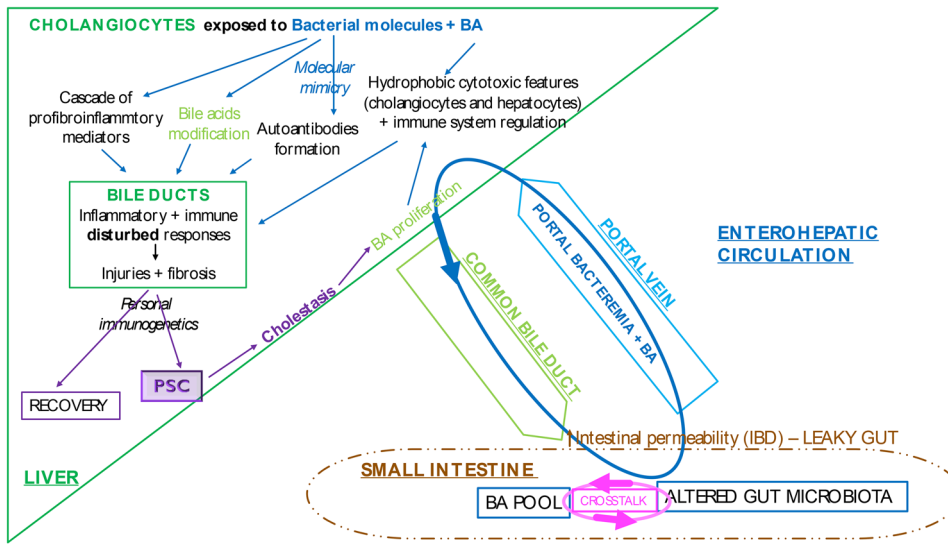


**FIGURE 1.** Primary sclerosing cholangitis pathogenesis based on the immune and nonimmune mechanisms, along with genetic susceptibility and environmental factors.

before MTZ treatment (T0) until after MTZ treatment, from 12 to 24 days postbaseline (T14).

## Biochemical Tests

Stool and blood samples were collected before MTZ treatment (T0), with serum BA samples obtained following blood centrifugation, and fecal BA samples extracted from lyophilized feces. BA concentrations were analyzed via HPLC-mass spectrometry using an LTQ-Orbitrap mass spectrometer coupled to an Accela HPLC system (ThermoFisher Scientific). For liver function tests, bilirubin, CRP, serum proteins, and hemograms, blood samples were taken at T0 and T14; 30 ± 14 days; 3, 6, and 12 months (T12 months). Sustained biochemical remission was defined as AST, ALT, and GGT levels below 1.5× ULN, with CRP concentrations below laboratory standards.

## Gut Microbiota

The gut microbiota was obtained from fecal samples, collected at T0 and T14, which were kept frozen until analysis. Bacterial DNA was extracted using QIAamp DNA Stool Mini Kit (QIAGEN). The 16S rRNA gene V1–V3 regions were amplified with primers 27Fmod519Rmodbio and sequenced using an Illumina MiSeq sequencing platform and reagents at MRDNA (Shallowater, TX). The data were processed using QIIME2 (18), including the calculation and evaluation of alpha- and beta-diversity metrics.

## Statistical Analysis

All data were expressed as mean ± SD. Comparison of categorical variables was conducted using Pearson's $\chi^2$ test; continuous variables were compared using the Wilcoxon signed-rank test. The latter and also the Kruskal-Wallis test were applied for comparisons of gut microbiota alpha-diversity, and PERMANOVA and the Kruskal-Wallis test for those of beta-diversity metrics.

Statistics and curve fitting were conducted using JMP software (v15.0; SAS Institute, Cary, NC). For fecal analysis, only descriptive statistics were conducted. A $P$ value <0.05 was considered statistically significant.

## Ethical and Regulatory Features

The study was performed in accordance with the Declaration of Helsinki principles. The protocol and patient consent forms were approved by the institutional (UCLouvain) and hospital (CUSL) ethics committees, with approval obtained on August 1, 2016 for the retrospective study and on June 17, 2019 for the prospective study (Belgian registration number: B403201628564). The signed informed consent was obtained from the children or parents/guardians. Patient data were anonymized under an established code, in line with the 1992 Belgian Law on privacy protection, as amended by the General Data Protection Regulation Belgian Law in 2018. The registration of the trial on clinicaltrial.gov was on March 3, 2017 (identifier: NCT03069976).

The statistical analyses, the BA profile, and the gut microbiota analyses were self-funded by the laboratories themselves. MTZ and regular control blood tests were at the patient's expense.

# RESULTS

## Study Populations

Overall, 18 patients were enrolled with a mean age of 13.2 ± 4.5 years, 7 in the retrospective study part, and 12 in the prospective study part, with their main diagnostic findings detailed in Supplemental Digital Content Table 1, http://links.lww.com/PG9/A122 and Supplemental Digital Content Table 2, http://links.lww.com/PG9/A123. One patient had previously participated in the retrospective study part (BE1 006), but he received additional MTZ 1 year later and underwent blood and fecal sampling for BA and microbiota analysis in the prospective study part (BE1 014), as illustrated in Supplemental Digital Content Figure 1, http://links.lww.com/PG9/A127. Another patient (BE1 013) received 5 MTZ treatment cycles: only the first one and the last one (18 months apart) were included in the prospective study part with blood and feces collected for both study inclusions.

Considering 18 recruited patients, their mean age at diagnosis was 11.6 ± 4.7 years. Thirteen exhibited ASC and 5 PSC, with UC recorded in thirteen. Of these, 10 patients fully met the 4 inclusion criteria; for the remaining 8, MRCP criteria were not fulfilled. In December 2020, the mean follow-up duration was 895 ± 482 days. For diagnosis, patients underwent biochemical analysis, abdominal ultrasound, colonoscopy with biopsy (except 3 patients), and liver biopsy. Two patients were excluded from analysis, one owing to absent PSC and the other due to a too short follow-up (16 days).

The 6-patient BA control cohort's mean age was 7.7 ± 5.7 years; 2 of them withdrew their consent, and their medical data were not considered.

## Drug Administrations

In the retrospective study, all 7 patients underwent MTZ therapy for at least 14 days, excepting one (BE1 006) being only treated for 12 days. MTZ was the initial treatment for one patient (BE1 001). In the prospective study, all 12 patients underwent MTZ therapy for at least 14 days. MTZ was the initial treatment for 6 patients. For most, several MTZ cycles were required to achieve sustained biochemical remission, as illustrated in Supplemental Digital Content Figure 2, http://links.lww.com/PG9/A128. MTZ was sometimes administered long-term on several days each month; hence, the patient who participated to both studies (BE1 006–BE1 014) reached remission status at T6 months of second study inclusion. At data analysis, attaining biochemical remission was associated with 2 MTZ cycles, on average.

All patients received concomitant medications before, during, and after study drug administration, including methylprednisolone or azathioprine for AIH, UDCA for PSC, and mesalazine, budesonide, or tumor necrosis factor alpha blockers for UC. These additional treatments were different for each patient, as illustrated in Supplemental Digital Content Table 3, http://links.lww.com/PG9/A124.

## Clinical Evolution

During follow-up, patients were assigned to remission or relapse groups, dependent on sustained biochemical response. Clinical remission, as listed in Supplemental Digital Content Table 4, http://links.lww.com/PG9/A125, was not a discriminatory criterion. Of the 18 treated patients, 12 were classified into remission, remission being maintained over 850-day period on average, versus 6 into relapse, as illustrated in Supplemental Digital Content Figure 1, http://links.lww.com/PG9/A127. During follow-up, all treated patients displayed stable disease or favorable evolution, although 7 still exhibited clinical signs/symptoms at T12 months. The relapse and remission patients were compared with each other regarding several criteria at inclusion (Table 1). For these analyses, only the first study inclusion was considered for patients BE1 006 and BE1 013; their second inclusions were considered as additional data.

The differentiating findings at T0 were as follows. Relapse patients were 16.2 ± 3.0 years old at inclusion T0, while remission patients were 11.7 ± 4.5 years old ($P$ = 0.0351). Total bilirubin levels were higher ($P$ = 0.056) in relapse versus remission patients, as were between-group differences in large-duct or small-duct PSC ($P$ = 0.0466) (19). The major between-group differences pertained to bile duct proliferation/damage. Such proliferation was severe in 50% of relapse patients, affecting mainly the large bile ducts, versus 0% of remission patients ($P$ = 0.0544), bile duct damage was observed in

**TABLE 1.** Comparison of patient characteristics and disease features between remission and relapse group patients

| | Total (n = 18) | Remission group (n = 12) | Relapse group (n = 6) | *P* value |
|---|---|---|---|---|
| Girls, n | 6/18 (33%) | 4/12 (33%) | 2/6 (33%) | 1.00 |
| Boys, n | 12/18 (67%) | 8/12 (67%) | 4/6 (67%) | |
| Age at inclusion T0, y; mean (SD) | 13.20 (±4.49) | 11.71 (±4.46) | 16.18 (±2.97) | 0.0351 |
| Age at diagnosis, y; mean (SD) | 11.63 (±4.17) | 10.63 (±4.75) | 13.62 (±1.56) | 0.2417 |
| Small-duct PSC, n (MRCP) | 7/18 (38.89%) | 5/12 (41.67%) | 2/6 (33.33%) | |
| Large-duct PSC, n (MRCP) | 5/18 (27.28%) | 1/12 (8.33%) | 4/6 (66.67%) | 0.0466 |
| Small-duct PSC, n (echography) | 5/18 (27.28%) | 5/12 (41.67%) | 0/6 (0%) | |
| Large-duct PSC, n (echography) | 1/18 (5.56%) | 1/12 (8.33%) | 0/6 (0%) | |
| OS only, n | 4/18 (22.22%) | 1/12 (8.33%) | 3/6 (50%) | |
| OS + UC, n | 9/18 (50%) | 7/12 (58.33%) | 2/6 (33.33%) | 0.2664 |
| PSC only, n | 1/18 (5.56%) | 1/12 (8.33%) | 0/6 (0%) | |
| PSC + UC, n | 4/18 (22.22%) | 3/12 (25%) | 1/6 (16.67%) | |
| UC, n | 13/18 (72.22%) | 10/12 (83.33%) | 3/6 (50%) | 0.1657 |
| T0: total bilirubin, mg/dL; mean (SD) (ULN: 1.2 mg/dL) | 1.40 (±1.81) | 0.78 (±0.61) | 2.88 (±2.86) | 0.0562 (n = 17) |
| T0: direct bilirubin, mg/dL; mean (SD) (ULN: 0.3 mg/dL) | 2.00 (±2.54) | 0.63 (±0.45) | 3.37 (±3.22) | 0.2683 (n = 6) |
| Liver biopsy: bile duct damage, n | 11/18 (61.11%) | 5/12 (41.67%) | 6/6 (100%) | 0.0167 (invalid Pearson test) |
| Liver biopsy: bile duct proliferation | | | | |
| Absence of bile duct proliferation | 4/18 (22.22%) | 3/12 (25%) | 1/6 (16.67%) | |
| Mild bile duct proliferation limited to portal tract | 9/18 (50%) | 7/12 (58.33%) | 2/6 (33.33%) | 0.0544 |
| Significant portal tract bile duct proliferation | 2/18 (11.11%) | 2/12 (16.67%) | 0/6 (0%) | |
| Portal tract and periportal bile duct proliferation | 3/18 (1.67%) | 0/12 (0%) | 3/6 (50%) | |

MRCP = magnetic resonance cholangiopancreatography; OS = overlap syndrome; PSC = primary sclerosing cholangitis; SD = standard deviation; UC = ulcerative colitis; ULN = upper limit of normal.

100% and 42% of patient group biopsies, respectively ($P = 0.0167$). There was no difference between-group about concomitant UC.

## Biochemical Evolution

Following MTZ therapy, liver enzyme levels decreased in both groups, except for GGT in relapse patients, as illustrated in Figure 2. For patients BE1 006 and BE1 013, only their first inclusion in the study was considered.

In remission patients (n = 12), AST decreased by –44.4 U/L as mean difference ($P = 0.0497$), ALT by −64.7 U/L ($P = 0.0182$), and GGT by −139.1 U/L ($P = 0.1264$). In relapse patients (n = 5, results missing at T14 for one patient BE1 018), the AST decrease was −35.8 U/L ($P = 0.0338$) and ALT decrease −55.6 U/L ($P = 0.0088$), whereas GGT increased by +14.8 U/L ($P = 0.6961$).

## Bile Acids

The BA detected in blood samples comprised primary BA such as CA and CDCA, with their taurine (T) conjugates TCA and TCDCA, and glycine (G) conjugates GCA and GCDCA; secondary BA such as DCA with its taurine-conjugate TDCA and glycine-conjugate GDCA; tertiary BA such as UDCA with its taurine-conjugate TUDCA and glycine-conjugate GUDCA. An additional secondary BA, namely lithocholic acid, was detected in feces samples. The plasma BA pool from treated (remission + relapse) patients (n = 14) was compared with that of the controls at T0 (n = 6). Consequently, the mean relative abundance of glycine conjugates was significantly higher in treated versus control patients (38% versus 16%; $P = 0.0119$), contrary to taurine conjugates. The BA pool size was significantly smaller in control (1637 versus 34 833 pmol/mL; $P = 0.0006$) versus treated patients. In remission patients (n = 8), the mean plasma relative abundance of hydrophilic BA increased significantly by +6.3% ($P = 0.0391$) post-MTZ, whereas any significant difference has been observed in relapsing patients (+2.8%; $P = 0.2500$; n = 3). More detailed data about plasma BA profile were illustrated in Tables 2–3 and Supplemental Digital Content Table 5, http://links.lww.com/PG9/A126.

Concerning fecal BA, the analyses on feces samples were performed only for the prospective study part, without any control group. Few samples were available, particularly in relapse group. The fecal BA pool at baseline was mainly hydrophobic with a mean relative abundance of hydrophobic BA of 69% and 87% in remission and relapse groups (n = 4 and n = 1), respectively. The pool was mostly composed of secondary BA (68% and 87%) and unconjugated BA (94% and 97%) in both groups, respectively, whereas conjugated BA in the form of taurine conjugates were only detected in remission patients. Following MTZ, the BA profile markedly changed in the remission group, reflected by mainly hydrophilic BA detected (+42% from T0 to T14; $P = 0.1751$), with a major rise in primary BA (+42%; $P = 0.1814$), while tertiary BA stayed stable (+0.05%; $P = 0.994$), and secondary BA decreased (−42%; $P = 0.1685$). For the single patient in the relapse group, the mean relative abundance of hydrophilic BA decreased slightly (−4%). More detailed data about

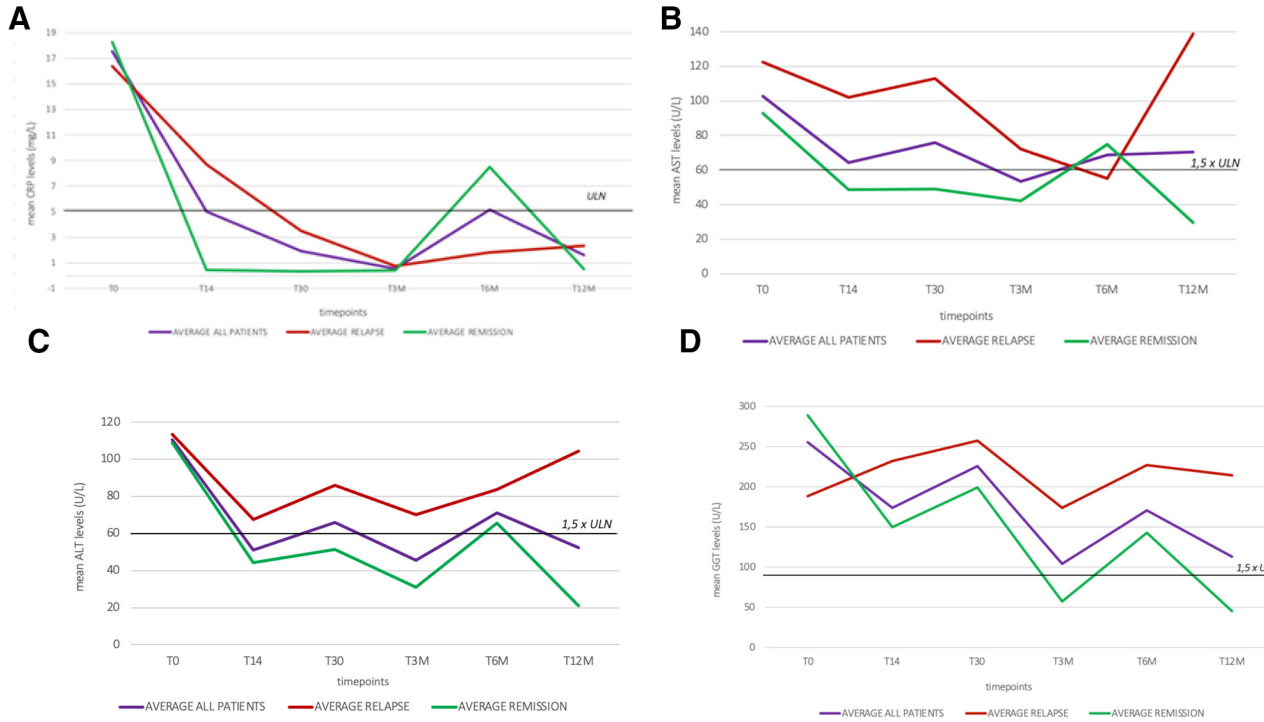


**FIGURE 2.** CRP, AST, ALT, and GGT level evolution during the 12-month follow-up. GGT = gamma-glutamyl transferase.

**TABLE 2.** Plasma bile acids: treated group vs control group: mean relative abundance, %

| | No. of patients | Average at T0, % | SD | *P* value (between groups) |
|---|---|---|---|---|
| **Primary BA** | | | | 0.3025 |
| Control group | 6 | 91.98% | 6.56% | |
| Treated group | 14 | 81.36% | 18.45% | |
| Secondary BA | | | | 0.0617 |
| Control group | 4 | 8.68% | 6.35% | |
| Treated group | 13 | 2.72% | 2.09% | |
| Tertiary BA | | | | 0.1795 |
| Control group | 5 | 2.68% | 1.88% | |
| Treated group | 14 | 16.12% | 18.88% | |
| Glycine conjugates | | | | 0.0119 |
| Control group | 6 | 15.87% | 9.09% | |
| Treated group | 14 | 38.22% | 19.62% | |
| Taurine conjugates | | | | 0.0526 |
| Control group | 6 | 79.89% | 10.21% | |
| Treated group | 14 | 59.74% | 21.15% | |
| Conjugated BA | | | | 0.3018 |
| Control group | 6 | 95.76% | 6.01% | |
| Treated group | 14 | 97.96% | 3.14% | |
| Unconjugated BA | | | | 0.0866 |
| Control group | 5 | 5.09% | 6.30% | |
| Treated group | 14 | 2.04% | 3.14% | |
| Hydrophilic BA | | | | 0.4833 |
| Control group | 6 | 61.04% | 30.91% | |
| Treated group | 14 | 73.96% | 10.58% | |
| Hydrophobic BA | | | | 0.4833 |
| Control group | 6 | 38.96% | 30.91% | |
| Treated group | 14 | 26.04% | 10.58% | |

BA = bile acid.

**TABLE 3.** Plasma bile acids: treated group–remission vs relapse patients mean relative abundance, %

| | No. of patients | T0, % | T14, % | *P* value (between T0 and T14 for each group) | Mean difference T14–T0, % | SD | p-value (mean differences between groups) |
|---|---|---|---|---|---|---|---|
| Primary BA | | | | | | | 0,7595 |
| Relapse | 3 | 70.19% | 54.07% | 0.5000 | −16.12% | 16.48% | |
| Remission | 8 | 83.56% | 69.51% | 0.3125 | −14.05% | 21.54% | |
| Secondary BA | | | | | | | 0,0814 |
| Relapse | 2 | 2.14% | 2.92% | 0.5000 | 0.78% | 0.10% | |
| Remission | 5 | 2.06% | 1.00% | 0.1875 | −1.06% | 1.80% | |
| Tertiary BA | | | | | | | 0,9187 |
| Relapse | 3 | 28.39% | 43.99% | 0.5000 | 15.60% | 16.06% | |
| Remission | 8 | 13.91% | 29.89% | 0.1953 | 15.98% | 23.39% | |
| Glycine conjugates | | | | | | | 0,6098 |
| Relapse | 3 | 50.41% | 54.40% | 1.0000 | 3.99% | 10.26% | |
| Remission | 8 | 37.80% | 44.73% | 0.9453 | 6.92% | 22.46% | |
| Taurine conjugates | | | | | | | 0,9187 |
| Relapse | 3 | 45.94% | 38.51% | 1.0000 | −7.43% | 16.42% | |
| Remission | 8 | 60.07% | 49.36% | 0.3828 | −10.70% | 19.40% | |
| Conjugated BA | | | | | | | 0,6098 |
| Relapse | 3 | 96.35% | 92.91% | 0.7500 | −3.44% | 6.72% | |
| Remission | 8 | 97.87% | 94.09% | 0.7422 | −3.78% | 11.45% | |
| Unconjugated BA | | | | | | | 0,6098 |
| Relapse | 3 | 3.65% | 7.09% | 0.7500 | 3.44% | 6.72% | |
| Remission | 8 | 2.13% | 5.91% | 0.7422 | 3.78% | 11.45% | |
| Hydrophilic BA | | | | | | | 0,6098 |
| Relapse | 3 | 82.90% | 85.74% | 0.2500 | 2.84% | 3.47% | |
| Remission | 8 | 70.30% | 76.57% | 0.0391 | 6.27% | 7.30% | |
| Hydrophobic BA | | | | | | | 0,6098 |
| Relapse | 3 | 17.10% | 14.26% | 0.2500 | −2.84% | 3.47% | |
| Remission | 8 | 29.70% | 23.43% | 0.0391 | −6.27% | 7.30% | |

BA = bile acid.

fecal BA profile were illustrated in Table 4 and Supplemental Digital Content Table 5, http://links.lww.com/PG9/A126.

## Gut Microbiota

Sample sequencing from retrospective and prospective studies were analyzed at T0 and T14. Within each group (remission or relapse), no statistically significant difference was observed between alpha-diversity metrics before (12 remission patients and 5 relapse patients) and after MTZ (7 remission patients and 3 relapse patients). Moreover, the delta of alpha-diversity T14–T0 did not differ between relapse and remission patients. No statistically significant differences were highlighted regarding beta-diversity metrics between the remission or relapse patients. It must, however, be stressed that the microbiota analyses were hampered by several missing T14 samples and insufficient sequencing depth for a few samples, resulting in decreased statistical power. Consequently, our results cannot rule out gut dysbiosis involvement in the PSC/ASC pathogenesis.

# DISCUSSION

We have investigated the MTZ effects on biochemical parameters, BA profiles, and gut microbiota in overall 18 patients with PSC/ASC. The underlying hypothesis was that MTZ therapy had to be initiated at an early-disease state and it would be associated with beneficial effects on these study parameters. Several but not all aspects of this hypothesis were confirmed.

Of the 18 MTZ-treated patients, 12 were classified into the remission and 6 into relapse groups, according to pre-established biochemical criteria. Of note is that UC was unlikely to have impacted the outcome. A first conclusive outcome was that all treated patients displayed stable disease or favorable evolution, although 7 of them still exhibited clinical signs at 12 months post-MTZ even if some of them had reached biochemical remission.

We observed that patients in late-disease stage were significantly older, while presenting with evidence of sclerosing cholangitis on liver biopsies and large-duct PSC upon imaging. Moreover, these patients poorly responded to MTZ. Their relapse could be due to antibiotic therapy being administrated too late in time, with already more severe hepatic and biliary lesions. Hence, MTZ therapy could result in long-term benefit in early-stage PSC or ASC (20). Moreover, small-duct PSC could be considered a primary stage of large-duct PSC, with better prognosis (8), as seen in MTZ-treated remission patients.

In all 18 treated patients, AST, ALT, GGT, and CRP levels decreased after a mean 18-day MTZ (excepting GGT in relapse

**TABLE 4.** Fecal bile acids: treated group–remission vs relapse patients–mean relative abundance, %

| | No. of patients | T0, % | T14, % (or T30, % for relapse group) | Mean difference T14–T0, % | SD | *P* value (between T0 and T14 for remission group) |
|---|---|---|---|---|---|---|
| Primary BA | | | | | | |
| Relapse | 1 | 0.65% | 0.74% | 0.08% | | |
| Remission | 4 | 6.20% | 48.28% | 42.09% | 48.55% | 0.1814 |
| Secondary BA | | | | | | |
| Relapse | 1 | 86.70% | 90.56% | 3.86% | | |
| Remission | 4 | 67.93% | 25.61% | −42.33% | 46.85% | 0.1685 |
| Tertiary BA | | | | | | |
| Relapse | 1 | 9.47% | 5.47% | −3.99% | | |
| Remission | 4 | 24.90% | 24.95% | 0.05% | 11.01% | 0.994 |
| Taurine conjugates | | | | | | |
| Relapse | 0 | | | | | |
| Remission | 2 | 7.55% | 4.58% | −2.97% | 5.99% | 0.6103 |
| Conjugated BA | | | | | | |
| Relapse | 0 | | | | | |
| Remission | 2 | 7.55% | 4.58% | −2.97% | 5.99% | 0.6103 |
| Unconjugated BA | | | | | | |
| Relapse | 1 | 96.82% | 96.49% | −0.33% | | |
| Remission | 4 | 94.50% | 96.55% | 2.05% | 3.57% | 0.3348 |
| Hydrophilic BA | | | | | | |
| Relapse | 1 | 10.12% | 5.92% | −4.20% | | |
| Remission | 4 | 30.47% | 72.94% | 42.47% | 48.03% | 0.1751 |
| Hydrophobic BA | | | | | | |
| Relapse | 1 | 86.70% | 90.85% | 4.15% | | |
| Remission | 4 | 68.57% | 25.90% | −42.67% | 47.18% | 0.1682 |

BA = bile acid.

patients), illustrating the decrease in hepatic cytolysis, cholestasis, and intra and extrahepatic inflammation, respectively. The decrease between T0 and T14 was significant for AST and ALT. This was associated with an improvement in clinical signs and symptoms. Full response to treatment reflected by enzyme levels below 1.5× ULN during the 3 months posttreatment end were likely predictive of long-term biochemical remission. Partial response was likely associated with subsequent relapse.

Sustained biochemical remission was associated with a mean of 2 MTZ cycles. The intrapatient variation (BE1 006–BE1 014) suggested the potential benefit of repetition of MTZ cycles to increase the possibility of remission. However, some patients relapsed despite several MTZ cycles while other patients reached sustained biochemical remission with only one MTZ cycle.

BA are likely to play a role in the disease, being the intermediate between gut microbiota and liver damage. At baseline, the BA pool of ASC/PSC patients differed from that of controls. The former displayed a greater BA pool with elevated plasma BA levels and higher abundance of glycine conjugates. These latter are less hydrophilic than taurine conjugates, yet more hydrophilic than free BA (21). BA hydrophobicity is a key determinant of toxicity. Retention of hydrophobic BA inside hepatocytes during cholestasis have long been thought to be major hepatic disease drivers, as they were shown to induce injury to isolated hepatocytes (22), cultured hepatocytes (23), and whole liver (24). Assumedly, the BA pool could even constitute a biomarker for diagnosing cholestasis; and ASC/PSC should be suspected in the case of other typical features.

Following MTZ therapy, the BA pool's hydrophilicity significantly increased in the remission group (+6.3%; $P = 0.0391$) but any significant difference was detected in the relapse group (+2.8%; $P = 0.2500$), without significant between-group differences. A possible explanation of the impact of antibiotics such as MTZ is related to their effects on intestinal anaerobic bacteria; antibiotics such as MTZ would prevent the deconjugation and dehydroxylation of primary BA, thereby increasing the hydrophilicity of the BA pool. Another option is that they could stimulate the synthesis of UDCA by gut bacteria, such as *Bacteroides distasonis*. Bacteria belonging to *Bacteroides* were indeed detected in the fecal microbiota analysis. Oral UDCA administration in mice reduced the overall BA pool, with high-abundance of conjugated UDCA species (25).

According to the increased BA hydrophilicity found in remission patients versus no major changes in relapse patients, the hydrophilicity/hydrophobicity ratio may serve as a remission/relapse biomarker in clinical practice. The increment of hydrophilicity could be biased by starting UDCA treatment concomitantly to MTZ for 3 remission patients; UDCA treatment being able to rise the BA pool hydrophilicity (26). However, 3 relapse patients had also UDCA treatment initiated before study inclusion, with no significant change in the hydrophilicity of their BA pool.

Concerning fecal BA, the pretreatment BA pool was mainly hydrophobic, mostly composed of secondary BA and unconjugated

BA in remission and relapsing groups. After MTZ, the fecal BA pool changed in the remission group by becoming mostly hydrophilic with a major rise in primary BA, along with a decrease in secondary BA, in line with the collected plasma data. The change following MTZ therapy was more visible in the fecal BA pool, corresponding to intestinal BA where antibiotics directly acted against intestinal anaerobic bacteria, compared with the plasma BA pool, corresponding to reabsorbed and circulating BA (27). For relapsing patients, a fecal sample post-MTZ was available for only one single patient, rendering data interpretation difficult.

Considering gut microbiota, no difference in either alpha- or beta-diversity was observed between remission and relapsing patients or pre and posttreatment samples. We failed to demonstrate that the significant change in BA profile after MTZ was associated to a significant change in the gut microbiota. The hypothesis of gut-liver axis involvement could not be proven.

This study had several limitations, which include the sample size, the monocentric study, the lack of an ASC/PSC control group, and the partial retrospective inclusion to reach sufficient statistical power, but nevertheless bring the proof of concept that MTZ is able to induce partial or complete remission in early stage of overlap syndrome. According to the low incidence of ASC/PSC in children, the cohort of patients could be enlarged by making this trial multicentric in order to improve these study limitations.

## CONCLUSIONS

The discriminating biochemical data collected herein are likely indicative of long-term clinical benefits following MTZ therapy for patients at early-stage PSC/ASC. This was reflected by a significant increase in hydrophilic BA abundance in plasma and fecal samples of remission patients. These preliminary positive results warrant the implementation of large-scale multicenter prospective studies, especially involving pediatric ASC/PSC patients.

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