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Characterizing the biochemical response to *Schistosoma mansoni* infection and treatment with praziquantel in pre-school and school-aged children

Gordana Panic¹,², Jean T. Coulibaly¹,²,³, Nikita Harvey⁴, Jennifer Keiser¹,²*, Jonathan Swann⁴

¹ Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, CH–4002 Basel, Switzerland

² University of Basel, CH–4003 Basel, Switzerland

³ Unité de Formation et de Recherche Biosciences, Université Félix Houphouët-Boigny, 01 BP V34, Abidjan 01, Côte d’Ivoire

⁴ Division of Integrative Systems Medicine and Digestive Diseases, Department of Surgery and Cancer, Imperial College London, London SW7 2AZ, United Kingdom

*Corresponding author: Prof. Jennifer Keiser, Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, P.O. Box, CH-4002 Basel, Switzerland. Tel.: +41 78 284-8218, E-mail: jennifer.keiser@swisstph.ch
ABSTRACT

Schistosomiasis is a widespread chronic neglected tropical disease prevalent mostly in children in under-resourced rural areas. Its pathological effects have been clinically characterized, yet the molecular-level effects are understudied. In this study, the biochemical effects of *Schistosoma mansoni* infection and praziquantel treatment were studied in 159 pre-school aged and 130 school aged infected children and 11 non-infected children in Azaguié, Côte d’Ivoire. Urine samples were collected prior to receiving 20, 40 or 60 mg/kg of praziquantel or a placebo, as well as 24 hours post-treatment, and at the 3-week follow up. Urinary metabolic phenotypes were measured using $^1$H NMR spectroscopy and metabolic variation associated with *S. mansoni* infection and praziquantel administration was identified using multivariate statistical techniques. Discriminatory metabolic signatures were detected between heavily infected and non-infected children at baseline as well as according to the dose of praziquantel administered 24 hours post treatment. These signatures were primarily associated with the metabolic activity of the gut microbiota, gut health and growth biomarkers and energy and liver metabolism. These analyses provide insights into the metabolic phenotype of schistosomiasis and treatment with praziquantel in two important demographics.

Keywords: *Schistosoma mansoni*, metabolic profiling, metabolism, pre-school aged, school-aged, children, praziquantel

Introduction

With over 250 million people infected, resulting in 2.6 million Disability Adjusted Life Years (DALYs) lost, schistosomiasis is the third single most important parasitic disease next to malaria and intestinal nematode infections\(^1,2\). The disease is common throughout the tropics and sub-tropics and mostly affects people, especially children, living in poor rural areas of low and middle income countries\(^3\). The infective agent is a blood fluke of the *Schistosoma*
genus, where the intestinal form of the disease is caused predominantly by *Schistosoma mansoni*. Adult-stage worms reside in the veins of the mesenteries alongside the intestine, where they lay tens to thousands of eggs per day \(^1\). The bulk of the pathology is due to eggs getting lodged in proximal organs such as the liver or intestines where they provoke strong Th2-type responses that envelop the eggs in granulomas, which eventually fibrose \(^4\). This results in both chronic inflammation and degradation of organ tissue, which also leads to portal hypertension and blood shunting, anemia, intestinal polyps and abscesses, blood in stools, and esophageal varices. The severity and frequency of symptoms are thought to be associated with infection intensity \(^5\). In children, schistosomiasis is associated with malnutrition, and growth and cognitive stunting \(^6\).

As most of the disease burden is due to disability rather than lethality, the crux of the WHO control strategy is morbidity reduction by preventative chemotherapy in the form of regular mass drug administrations (MDA) to target populations \(^3\). The drug of choice is praziquantel, a safe, effective and easy to use oral tablet administered at a single dose. For a long-time, the main target population was school-aged children. However, cumulative evidence has shown that pre-school aged children (<6 years) are also significantly impacted \(^7,\,8\), leading to a WHO recommendation to treat them on a case by case basis \(^9\). Aside from a handful of efficacy and safety studies, little is known about the effects of praziquantel in this age group, which is pivotal in light of parallel efforts to develop a pediatric praziquantel formulation \(^10\).

While the macro-level effects of schistosomiasis are well characterized, the disease is poorly understood at the molecular level. Metabolic phenotyping (metabonomics) is a field of “omics” defined as the study of the metabolic responses of multicellular organisms to pathophysiological stimuli \(^11\). With respect to schistosomiasis, metabonomic analyses in rodent models of infection have revealed disturbances in metabolites related to energy (glycolysis, TCA cycle) and amino acid metabolism, inflammation and collaborative gut microbial-mammalian co-metabolism, in infected versus non-infected rodents \(^12-14\). In contrast,
metabolic phenotyping of urine or stool samples from human schistosomiasis cases are scarce. A study by Ballog et al. investigated the biochemical response of *S. mansoni*-infected school-aged children and adults in the Mayuge district of Uganda pre and post-treatment with praziquantel. Differences were observed in metabolites associated with energy metabolism and gut microbial activity between heavily infected and non-infected adults, whereas differences in children were less certain.

In this present study, we have applied a metabolic phenotyping approach to determine the metabolic variation between *S. mansoni*-infected and non-infected pre-school and school aged children from the rural region of Azaguié in Côte d'Ivoire. We studied the metabolic responses of praziquantel treatment in a dose-response manner, with the aim of elucidating whether they differ between school-aged and pre-school aged children. Urinary metabolite profiles were measured using $^1$H nuclear magnetic resonance (NMR) spectroscopy and the metabolic variation associated with infection and treatment response was identified using multivariate statistical techniques.

**METHODS**

Study design and ethical considerations
This study was embedded within a pediatric praziquantel dose-response study in the health district of Azaguié in southern Côte d'Ivoire, which is detailed in a publication by Coulibaly et al. Ethical approval for the study was obtained by the National ethics committee of the Ministry of Health in Côte d'Ivoire (CNER, reference no. 037/MSLS/CNER-dkn) and the Ethical Committee of Northwestern and Central Switzerland (EKNZ; reference no. 162/2014). In brief, 161 pre-school aged children (aged 2-5) from the villages of Makouguié, Odoguié, M'Bromé and Elevi, and 180 school-aged children (aged 6-15 years) from Makouguié, infected with *S. mansoni* were eligible for the study. The children were stratified according to infection intensity (light (<100 eggs per gram feces) or moderate and heavy (100 to 400 eggs
per gram feces and >400 eggs per gram feces, respectively), and randomized to either 20, 40 or 60 mg/kg praziquantel or placebo. Complete urine samples were collected from 130 pre-school and 161 school aged children at 3 time-points: prior to treatment, 24 hours post-treatment and 3-weeks post-treatment. Urine samples were also collected from 11 non-infected children from Makouguié at parallel time-points, as a comparator group. Anthropometric and parasitological data from the sampling population are provided in Table S1. Samples were immediately stored on ice throughout the day and transported to a -80 °C freezer until cold-chain shipment, and then stored at -80 °C until use.

**¹H NMR spectroscopy**

Urine samples (630 µl) were thawed to room temperature and combined with phosphate buffer (70 µl; 1.5 M KH₂PO₄, 2 mM NaN₃ and 1% TSP in D₂O; Sigma-Aldrich, Switzerland). The samples were vortexed, centrifuged (13,000 x g for 10 minutes) and 600 µl of the supernatant was transferred to a 5 mm diameter NMR tube. A pooled urine sample was also created to monitor instrument stability. Spectral profiles were acquired using a 600 MHz Bruker Avance III spectrometer at the Clinical Phenotyping Centre (CPC), Imperial College London. ¹H NMR spectra were acquired using a standard one dimensional (1D) pulse sequence using the first increment of the NOE pulse sequence for water suppression as previously described ¹⁷. Raw spectra were phased, baseline corrected and calibrated to TSP using Topspin 3.2 (Bruker Biospin) before being digitized and imported into the MATLAB environment (Version R2014a; Mathworks Inc., USA). Redundant peaks derived from water, urea and TSP were removed from the spectra before manual alignment and normalization to the probabilistic quotient using in-house MATLAB scripts developed at Imperial College London.

**Data analysis**

The clinical trial metadata and pre-processed spectra were imported into SIMCA (Version 14.1; Umetrics) for multivariate data analysis. Principal components analysis (PCA) was
initially performed on all the spectral profiles to identify outliers in the dataset, which were subsequently removed (13 samples were removed due to implausibly excessive acetate concentrations, which were suspected to be contaminated due to improper storage, and 2 due to the presence of large amounts of unidentified foreign metabolites). Supervised statistical analyses namely, orthogonal partial least squares (OPLS) and OPLS-discriminant analysis (OPLS-DA), were used to study the refined dataset and in the case of OPLS-DA to analyze inter-group variations, for categories of interest. The predictive ability ($Q^2_Y$) of the OPLS models was calculated using a 7-fold cross-validation approach. The significance of the $Q^2_Y$ values was assessed by permutation testing (1000 permutations, with a $p$ value threshold of $p < 0.05$). For all valid models, both an OPLS or OPLS-DA coefficients plot constructed in MATLAB were used to identify peaks that significantly influenced the model. Significant metabolic associations were identified using correlation coefficient (R) cut-off values ($p < 0.05$) of 0.115, 0.311, 0.116, 0.174, and 0.152 for the age, infection status, praziquantel all, praziquantel pre-school age and praziquantel school-age models, respectively. Metabolites were identified using in-house databases, referencing to previous literature and Statistical Correlation Spectroscopy (STOCSY).

RESULTS AND DISCUSSION

Metabolic differences associated with S. mansoni infection status

A PCA model was built on all pre-intervention profiles to identify metabolic variation of the study population, and thus also to identify potential confounders. From these PCA models, metabolites were found not to vary across village or sex but were observed to vary according to the age of the child (Fig S1). An OPLS model was constructed to identify metabolic variation associated with age (N= 289; 1 predictive and 1 orthogonal component; $Q^2_Y$= 0.698; $p$<0.01), from which an OPLS coefficients plot was constructed to identify significantly contributing metabolites (Fig S2; Fig 3). Creatinine and citrate excretion was positively associated with age, while the excretion of creatine, glycine and succinate was negatively
associated. This is largely consistent with findings from metabolic studies of aging in children by Gu and colleagues (2009). The excretion of several metabolites associated with the metabolic activity of the gut microbiota were also observed to change in an age-dependent manner, which differed from the Gu et al study. Hippurate excretion increased with age while 4-hydroxyphenylacetate (4-HPA), dimethylamine (DMA) and trimethylamine (TMA) (metabolites associated with gut microbial choline metabolism) decreased. Reduced excretion of 4-HPA, DMA and TMA was also incidentally observable in mice fed on a protein or zinc deficient diet, which potentially reflects progressive malnourishment with age. This is consistent with lower WAZ scores in the older children of this study (Table S1). Due to the above-described age-related variation, subsequent models were built using homogenously distributed age profiles, to prevent potential bias.

An OPLS-DA model built on pre-treatment profiles comparing 28 heavily infected (>400 eggs per gram feces) and 11 non-infected children identified discriminatory metabolites between the groups (1 predictive and 1 orthogonal component; \( Q^2 Y = 0.317, p < 0.05 \)) (Fig 1). Heavily infected children were observed to excrete lower amounts of 2-oxoisovalerate, TMA, creatinine, hippurate mannitol (Fig S3), compared to uninfected children (Fig 3), while 4-methyl-2-oxovalerate (ketoleucine), fumarate and 2 unknown metabolite peaks (\( \delta \) 2.85 singlet and \( \delta \) 3.02 singlet), which were found to correlate strongly by statistical correlation spectroscopy (STOCSY), were excreted in greater amounts. Some of these metabolites are associated with metabolic pathways of the liver, where schistosomiasis causes severe inflammation and fibrosis. For example, 2-oxoisovalerate is a branched chain organic acid and is a metabolite of valine produced in the liver. Aside from a positive association with muscle mass, decreased excretion of creatinine is associated with liver cirrhosis and renal function. Hippurate is a microbial-host co-metabolite produced in the liver: gut microbiota metabolize diet polyphenols to benzoic acid, which is subsequently conjugated with glycine in the liver of the host to produce hippurate. Lower excretion of hippurate in the presence of infection might indicate altered hepatic metabolism and/or a disruption to the metabolic
activity of the intestinal microbiota, which was also recently found to be perturbed by schistosomiasis \(^{22-24}\).

Lower mannitol excretion with infection is of particular interest. Mannitol is present in large concentrations in cassava, a local staple and is not readily metabolized. The mannitol-lactulose test is frequently employed as a measure of gut permeability where low urinary excretion of mannitol following the intake of a set dose is reflective of malabsorption \(^{25}\). Lower mannitol excretion has also been associated with gut inflammation \(^{26}\). Conversely, in a recent study of malnourished children in Brazil, where the mannitol-lactulose test was also employed, higher excretion of mannitol was positively associated with healthy growth rate \(^{27}\).

Low urinary excretion of mannitol in the infected children may therefore be a product of infection-induced gut pathology and its resultant impairment of gut function, which can lead to growth stunting. Consistently, infected children displayed lower TMA excretion, which was also associated with decreased villus height (a metric of intestinal health) in a group of malnourished Zambian children \(^{28}\), and higher urinary excretion of ketoleucine, which is directly associated with a lower BMI \(^{29}\).
Fig 1 Urinary metabolic profiles of heavily infected (>400 eggs per gram feces) and non-infected children at baseline. Coefficients plot from an OPLS-DA model comparing heavily-infected and non-infected children (N= 39, 1 predictive and 1 orthogonal component, Q^2_Y = 0.317, p < 0.05). Significant metabolites (correlation coefficient (R) > 0.311) are shown in red and listed in Fig 3. U1, unidentified metabolite 1; U2, unidentified metabolite 2.

Metabolic responses to praziquantel treatment

In the clinical trial on which this study was based, praziquantel had a slightly lower efficacy in pre-school aged children compared to school aged children. While the 40 mg/kg dose met the WHO drug efficacy benchmark for 90% egg reduction rate in the school aged children, none of the doses administered achieved this threshold in the pre-school aged children (Fig 2A; Coulibaly et al. 2017). We were thus interested in the immediate metabolic responses to praziquantel treatment and how they differed between the two age groups at 24 hour post-treatment. An OPLS model was built to identify significant urinary metabolic alterations associated with increasing praziquantel doses in all study children 24 hours post-treatment (Fig 2B; 1 predictive and 1 orthogonal; Q^2_Y = 0.371 p < 0.01). The excretion of citrate,
creatinine, pyruvate, trimethylamine-oxide (TMAO) and scyllo-inositol was negatively correlated with praziquantel dose 24 hours after treatment (Fig 3). A decrease in citrate and pyruvate indicate perturbations to the TCA cycle. The depletion of creatinine could reflect an increase in the glomular filtration rate in response to treatment, which may also explain the increased excretion of scyllo-inositol, a sugar alcohol from the coconut palm, which is an abundant part of the children’s diet. No traces of praziquantel or praziquantel metabolites were found at this time point, which is consistent with its clearance rate ($t_{1/2}$ of 2-4 hrs).^31

Fig 2 Dose-response to praziquantel. (A) Egg reduction rate based on arithmetic mean for pre-school aged children (blue) and school aged children (red). The red dotted line is the WHO drug efficacy benchmark of a 90% egg reduction rate. (B) OPLS model ($n = 285; 1$ predictive and $1$ orthogonal component; $Q^2Y = 0.371, p = 0.01$) comparing the dose-response of urinary metabolic profiles 24 hours post praziquantel administration for both pre-school and school aged children (yellow= 0 mg/kg (placebo), green = 20 mg/kg, blue= 40 mg/kg and red= 60 mg/kg).

To identify age-dependent differences in the metabolic response to praziquantel, separate OPLS models were constructed on the urine profiles collected at 24 hours for pre-school and school aged children, using praziquantel dose as the response variable (Fig 3). Praziquantel intake reduced the excretion of pyruvate, citrate, creatinine, TMAO, scyllo-inositol in both age groups. In the pre-school aged children, praziquantel also reduced the excretion of 2-
oxoisovalerate, and hippurate but increased the excretion of TMA (N = 127; 1 predictive component; $Q^2 Y = 0.358; p < 0.05$). This was not observed with school-aged children, who excreted less phenylacetylglutamine (PAG) following praziquantel intake (N = 168; 1 predictive and 1 orthogonal component; $Q^2 Y = 0.507; p < 0.05$). As TMA, hippurate and PAG are gut microbial co-metabolites, these differences might indicate that gut microbes are differently affected by praziquantel intake between the two age groups. Indeed, a microbial analysis of 24-hour post-treatment stool samples from the same children found that praziquantel intake did affect the abundance of the *Bacilli* and *Erysipelotrichi* classes, the ML615J_28 order and *Actinobacillus* genus $^{24}$. However, the abundances did not vary between age groups. This suggests that although praziquantel did not differentially affect the microbial community structure between the two age groups, it did have a different impact on its functional status.
Fig 3 Metabolites associated with age, infection status and praziquantel intake.

Dose refers to metabolites associated with increasing praziquantel dose 24 hours post-treatment and are shown also separately for preschool (Dose preschool) and school aged children (Dose school). R (P(cor)) values were extracted from the OPLS models constructed for each comparison.

No praziquantel-associated changes were observed at the 3-week follow up time-point. This indicates that the biochemical perturbations induced by a single dose of praziquantel do not persist. It also suggests that the functional modifications to the gut microbiota induced by praziquantel are transient, consistent with findings from Schneeberger et al. \(^{24}\).
A limitation of this study is that it is difficult to differentiate whether these biochemical alterations are due to the metabolic impact of praziquantel or due to clearance of the infection or both. The linear dose-response metabolic effect observed in pre-school aged children, which stands in contrast to the rather flat dose-response observed in praziquantel efficacy in this age group, suggest that most of these metabolic alterations are due to the metabolic impact of praziquantel. Nonetheless, the differential metabolic responses to praziquantel treatment between pre-school and school aged children, especially with regard to microbial co-metabolites, may provide first insights with regard to the differing praziquantel efficacy in these two demographics.

CONCLUSION

For the first time, we have conducted a metabonomics study embedded in a clinical trial for dose determination of praziquantel in pre-school and school-aged children infected with *S. mansoni*, in order to extend our understanding of both disease and treatment dynamics. A number of metabolites were shown to differ between non-infected and heavily infected children in pre-treatment urine samples. The metabolic alterations observed were indicative of disruptions to metabolism in the liver, functional changes in the gut microbiota and the gut itself and impoverished nutrition status. These observations may help to understand the biochemical mechanisms through which schistosomiasis can contribute to growth and developmental stunting. Moreover, several metabolic perturbations were identified 24 hours after praziquantel intake, some of which differed between pre-school and school-aged children, which then resolved 3 weeks post-treatment. These were primarily related to gut microbial-host metabolic interactions. A combined analysis of these metabolic profiles with gut microbial composition from matching stool samples and matching pharmacokinetic profiles from the study is envisioned to shed light on these interactions.
SUPPORTING INFORMATION:

The following files are available free of charge at ACS website:

http://scanmail.trustwave.com/?c=6967&d=hKKK2sie33eTaswt7FYdNhmdxgKnBX9XoADeQZc89g&u=http%3a%2f%2fpubs%2eacs%2eorg%3a

Table S1. Baseline characteristics of sampled population.

Figure S1. Metabolite distribution of sampled population at baseline.

Figure S2. OPLS model of metabolite distribution according to age.

Figure S3. STOCSY for the identification of mannitol.

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