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# Gas chromatography-mass spectrometry (GC/MS) reveals urine metabolites associated to light and heavy infections by *Schistosoma mansoni* in mice

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ABSTRACT

High-throughput profiling of metabolites has been used to identify metabolic changes in murine models as a response to the infection by the parasitic trematode *Schistosoma*. These investigations have contributed to our understanding on the pathogenesis of this tropical neglected disease, with a potential of helping diagnosis. Here, our study aimed to investigate the application of gas chromatography–mass spectrometry (GC/MS) on the profiling of urine metabolites from mice carrying infections by *Schistosoma mansoni*. Two larval infection doses created distinctive infection intensities in mice, whereby the heavily infected animals were found to release 25 times more eggs in faeces than lightly infected animals. Over 200 urine metabolites were identified from these animals by GC/MS, following two complementary derivatisation methods. A list of 14 individual metabolites with altered relative abundances between groups were identified. Most of the altered metabolites showed a trend of increased abundances in response to infection intensity, indicating host-specific metabolic alterations as a result of the disease. Hippurate, a metabolite which concentration is intimately modulated by the gut microbiota, was found to be highly correlated to infection intensity. Our study showed that urine metabolic profiling by GC/MS can distinguish non-infected animals from those carrying light and heavy infections by *S. mansoni*, revealing metabolites associated to the infection and providing insights on the pathogenesis of schistosomiasis.

Schistosomiasis, the infection of a helminthic trematode of the genus *Schistosoma*, is a neglected tropical disease that affects over 200 million people worldwide. This infection is acquired by skin penetration of larvae (cercariae) which are released in water by the snail vector [1,2]. Metabolic profiling is a promising approach for better understanding pathogenesis of schistosomiasis, with a potential for helping diagnosis [3]. Functional correlations between metabolites and schistosomiasis in murine models of infection by *Schistosoma mansoni* have shown perturbations on glycolysis and the tricarboxylic acid (TCA) cycle [4–10]. Additionally, in agreement with recent observations that schistosomiasis is accompanied by gut dysbiosis [11], most of the correlational studies above have repeatedly reported common alterations in microbiota-related metabolites [4,5,12].

Among various technologies for metabolic profiling, each having different weaknesses and strengths [13], gas chromatography–mass spectrometry (GC/MS) has not been yet applied to understanding metabolic changes in murine infections by *S. mansoni*. Advantages of mass spectrometry are mostly low cost, high sensitivity and high-throughput capability in comparison to other technologies such as nuclear magnetic resonance (NMR). When coupled with high-resolution gas chromatography, a complex mixture of metabolites can be separated by GC/MS in a single run [14]. Combining different methods of chemical derivatization, GC/MS allows sensitive detection and relative quantification of various classes of components (e.g. organic and amino acids, sugars, sugar alcohols, fatty acids, and phosphorylated compounds) [15]. Importantly, previous studies on metabolic profiling of mice infected by *S. mansoni* have only considered high infection challenges of >80 cercariae per animal which are likely to produce heavy infections [4–10]. However, people living in low-endemic regions often carry light infections [16]. With the expectation of contributing to these gaps of knowledge, our study aimed to investigate the application of GC/MS on the profiling of urine metabolites from mice carrying light and heavy infections by *S. mansoni*.

In this study, 45-day old female BALB/cJ mice were grouped (8 animals per group) by their infection challenge: non-infected (NI) or infected with either a low (LI) or high (HI) larval dose; i.e. either 20 or

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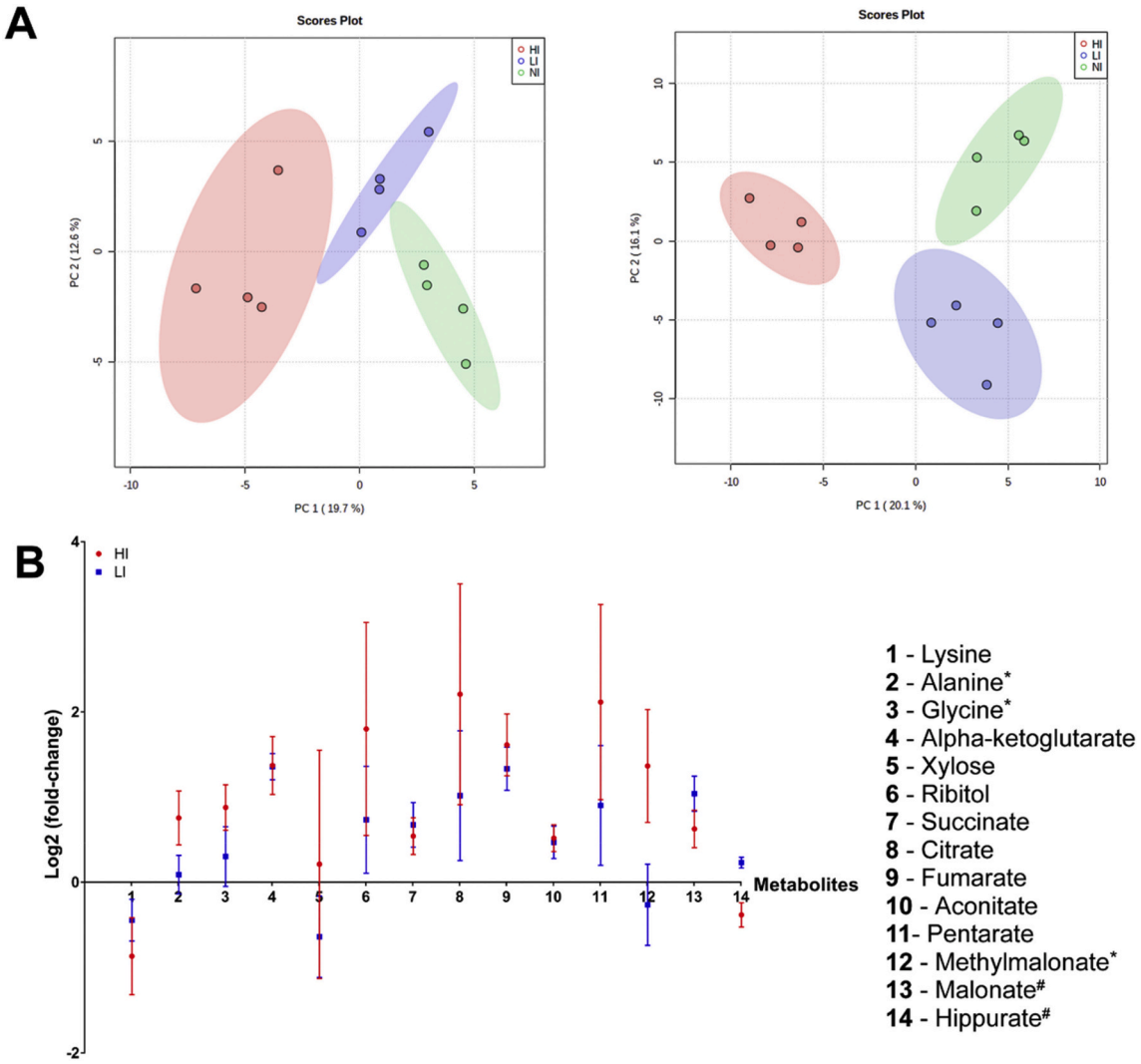


**Fig. 1.** Metabolic profiling of urine by GC/MS can indicate infection by *S. mansoni* in mice. Red, blue and green colours were applied to animals carrying heavy, light or no infection (HI, LI and NI respectively). (A) Principal component analysis of urine metabolites, derivatised by either MCF (left) or TMS (right). Animal groups formed separate clusters within 95% confidence regions regardless of the derivatization method. MCF provided the best discrimination between infection intensity groups analyzing the PC 1 eigenvalue. (B) Fold-changes on the relative concentrations of metabolites that were altered in LI (blue) and HI (red) in comparison to the NI group. Only altered metabolites that have passed statistical tests (Supplementary Table 3) are shown here. After normalization, the relative abundances of metabolites detected in HI and LI were compared to NI and transformed using the eq. Y = Log2(Y). Pearson correlation tests ($p < 0.05$; Supplementary Table 3) revealed three metabolites directly correlated with infection intensity (*) and two metabolites inversely correlated with infection intensity (#). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Metabolic profiling of urine by GC/MS can indicate infection by *S. mansoni* in mice. Red, blue and green colours were applied to animals carrying heavy, light or no infection (HI, LI and NI respectively). (A) Principal component analysis of urine metabolites, derivatised by either MCF (left) or TMS (right). Animal groups formed separate clusters within 95% confidence regions regardless of the derivatization method. MCF provided the best discrimination between infection intensity groups analyzing the PC 1 eigenvalue. (B) Fold-changes on the relative concentrations of metabolites that were altered in LI (blue) and HI (red) in comparison to the NI group. Only altered metabolites that have passed statistical tests (Supplementary Table 3) are shown here. After normalization, the relative abundances of metabolites detected in HI and LI were compared to NI and transformed using the eq. Y = Log2(Y). Pearson correlation tests ($p < 0.05$; Supplementary Table 3) revealed three metabolites directly correlated with infection intensity (*) and two metabolites inversely correlated with infection intensity (#). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

80 cercariae/animal respectively. Transcutaneous infections were achieved by containing each mouse in an individual beaker with 60 ml of the cercaria dose in water under light for 1 h (IAM/Fiocruz; Animal Ethics approval 94/2016). Samples were collected at day 47 post-infection. Faeces were collected within 60 min after individual caging and Kato-Katz diagnostics was performed to determine the infection intensity, as number of eggs per gram of faeces. As per previous studies [4–10], urine was chosen as the biological sample for being easily obtainable if wished to be applied to human population surveys. Urination was induced by gently rubbing the mouse abdomen individually [5]. Urine was collected directly into sterile plastic Petri dishes, transferred into cryogenic vials, immediately snap-frozen and kept at −80 °C. To guarantee sufficient urine volume (250 μl) for GC/MS analyses, samples from two animals within a group were pooled together. Therefore, each animal group was represented by four urine samples. After removing urea by treating samples with 100 U of urease (Sigma-Aldrich) at 37 °C for 60 min, metabolite chemical derivatisation of urine metabolites was achieved by methyl chloroformate (MCF) and trimethylsilyl (TMS) protocols [15,17,18]. The Zebron ZB-1701 column (Phenomenex, Torrance, CA, USA) was used for separation of metabolites. The GC/MS analysis was performed on the Agilent GC 7890A coupled to the MS 5975C (Agilent Technologies, Santa Clara, CA, USA)

[15,18]; which was operated in scan mode starting after 5 min (mass range of 40 to 650 amu at 1.47 scans/s). The internal standard 2,3,3,3-$d_4$-DL-alanine was used for normalization and to determine the relative abundance of metabolites between samples [17]. Data were analyzed using the AMDIS report (http://www.amdis.net/), ChemStation (http://www.agilent.com), in-house reference ion library and the NIST (https://www.nist.gov/) reference library and the R software (https://www.r-project.org/) with the MassOmics package (version 2), as described [17]. Principal component analysis was done with MetaboAnalyst v3 (https://www.metaboanalyst.ca) and statistical analysis was obtained with GraphPad Prism v6.

We found that animals from the HI group produced in average 25 times more eggs than animals from the LI group, as quantified by Kato-Katz ($p < 0.0001$; 1-way ANOVA), indicating that the infection challenge created two distinctive groups with animals carrying either light or heavy infections. A total of 81 and 158 metabolites were identified via MCF and TMS chemical derivatization, after passing our verification criteria (Supplementary Tables 1 and 2). Principal component analysis indicated that animals within a group share a similar metabolic profile and that these groups can be distinguished by either the MCF or TMS profile (Fig. 1A left and right). Therefore, urine metabolic profiling using GC/MS is useful to indicate *S. mansoni* infection and can distinguish animals carrying light versus heavy infections.

A list of 14 individual metabolites with altered relative abundances between groups were identified, after deconvolution of the data [17] and statistical analyses (Supplementary Table 3). These metabolites were found to differ in their relative abundances between one or both of the infected groups (LI or HI) and the non-infected group (NI) significantly ($p < 0.01$; 2-way ANOVA with Bonferroni correction). Most of the altered metabolites showed a trend of increased abundances in response to infection (Fig. 1B). On the other hand, hippurate and malonate were inversely correlated with the intensity of infection (Fig. 1B and Supplementary Table 3).

These findings revealed host metabolic alterations due to the infection that are indicative of altered glycolysis, TCA activity and general disturbances on amino acid metabolism [3]. This is supported by alterations on the level of metabolites such as succinate and citrate and the amino acids lysine, glycine and alanine. Except for lysine, the relative concentrations of these metabolites were higher in infected than non-infected animals (Fig. 1B and Supplementary Table 3). The observed changes of alanine and glycine, both directly correlated with infection intensity (Supplementary Table 3), corroborate with the majority of the previous studies [5,7,10,19] in contrast to succinate, citrate and lysine [6,7,9,12]. Garcia-Perez et al. [9] highlighted the low sensitivity of capillary electrophoresis when compared to NMR, also pointing out to the variation among animals, as a reproducibility issue of metabolomics [6]. Increased levels of alanine and glycine were also reported in mice infected by the malaria parasite *Plasmodium berghei* due to liver damage [20]; also a typical pathological feature of schistosomiasis by *S. mansoni*.

Our findings also revealed alteration of microbial-related metabolites, particularly hippurate (Fig. 1B and Supplementary Table 3). The concentration of this metabolite, absent in the urine of germ-free mice [19], is strikingly modulated by the gut microbiota [21]. Similarly to gut-associated pathologies, *S. mansoni* infections are accompanied by an overall reduction in gut microbial alpha diversity [11]. Hippurate was previously found to be depleted in animals infected by *Schistosoma* [5,9] and other trematodes [22], a feature also shared among patients with schistosomiasis by *S. mansoni* [12]. In our study, hippurate was the altered metabolite with the highest confidence level of correlation to infection intensity ($R^2 = 0.95$ and $p < 0{,}0001$; Supplementary Table 3). The depletion of hippurate in high intensity infections is a consensus in the literature, despite biological variations and analytical methodologies [4–9,12]. As this is the first study to compare metabolic profiles between animals carrying light and heavy infections, our findings revealed that hippurate depletion is indeed a feature of heavy infections but conversely accumulates in the urine of lightly-infected animals. The gut microbiota of infected animals change with the time course of the infection qualitatively, likely due to the development of inflammatory responses caused by the transiting of schistosome eggs through the intestines [11]. Therefore, we envisage that the fluctuation of hippurate between lightly- and heavily-infected animals could represent a differential response to these gut microbial alterations.

Malonate is another metabolite associated with specific gut microbial taxa, either positively or negatively [23]. We found that levels of malonate and methyl malonate are altered during infection, varying between lightly and heavily infected animals (Fig. 1B and Supplementary Table 3). These metabolites are known inhibitors of succinate dehydrogenase (SDH) [24], thus providing a link to host metabolic alterations of the TCA cycle that we detected here. SDH has a central role on controlling mitochondrial oxidative metabolism, driving macrophage polarization during inflammatory response to infection [25]. A hallmark of *S. mansoni* pathogenesis is the development of a classical type 2 granulomatous inflammation in the liver with infiltration of M2-polarized macrophages [26]. Therefore, these metabolic alterations might help us explain the pathogenesis of schistosomiasis.

In conclusion, our study showed that metabolic profiling using GC/MS can reveal subtle changes of urines metabolites due to *S. mansoni* infection in mice. Metabolic changes between lightly- and heavily-infected animals might be helpful to understand the immunopathogenesis of schistosomiasis. Extending from previous studies, hippurate was found to be a promising urine metabolic indicator for schistosomiasis that can also be useful to differentiate infection intensity. As metabolomics technologies develop and become more accessible, further studies will help reveal the specificity of metabolic alterations to human schistosomiasis and decipher functional correlations between metabolites and this medically-important parasitic disease.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.parint.2020.102239.

## Declaration of Competing Interest

The authors have no conflicts of interest to declare in association with this study.

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