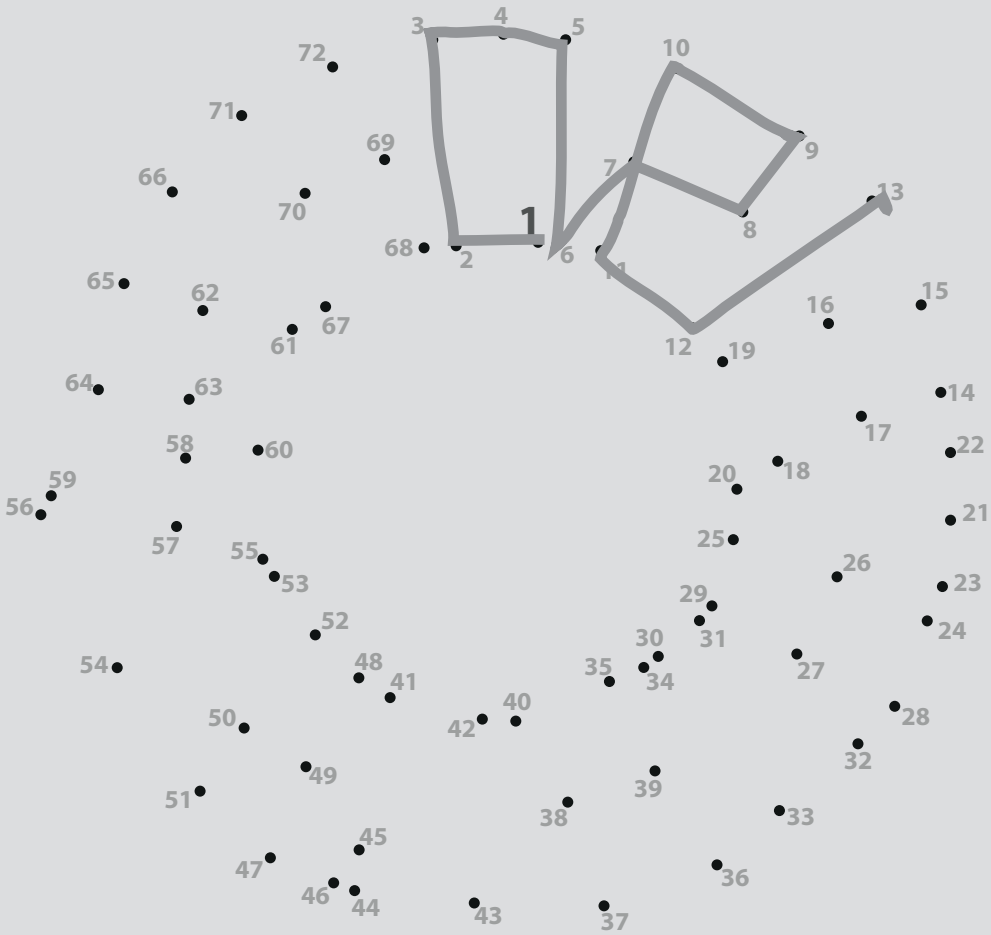
Comprehensive metabolomics of the experimental opisthorchiasis

Kokova, D.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Twenty years on: metabolomics in helminth research

D. Kokova
O.A. Mayboroda

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Abstract

This contribution makes a critical assessment of the metabolomics application to helminthic infection research. To ensure a cross-comparison of the results published by different laboratories over a period of almost two decades, we restrict the discussion to only the publications where nuclear magnetic resonance (NMR) spectroscopy is used as the analytical platform. We review the metabolites consistently reported for the body fluids of animals infected with the parasitic helminths and the characteristic metabolic patterns, arguing that the field needs a complete integration of metabolomics into research lines that examine host–helminth interactions.

Highlights

- Helminth infections were among the very first experimental models used in metabolomics; the first results were positive, and they inspired great expectations.
- Robustness of the NMR metabolomics analysis enables comparison of the data obtained for a period of two decades.
- There are three major metabolic traits which NMR metabolomics studies of the helminth infections revealed: remodeling of the amino acid metabolism, strong change in the metabolism of lipids, and dysregulation of the microbiota.
- Metabolomics research generated a number of functional hypotheses, but the follow-up studies are often missing.

In the beginning: metabolomics meets parasitology

For a parasitic worm, coexistence with its host is a "long-term project". The time of this coexistence is measured in years or decades, during which the host and the parasite are competing for the available energy resources, as well as metabolic building blocks; that profoundly affects the metabolic homeostasis of each other. Hence, it is not surprising that, with the emerging of a novel discipline, metabolomics (see Glossary), parasitic helminth infections were among the first experimental models to which it was applied. Of course, the metabolic effects of helminths on the host were studied before the "omics" era. For instance, the changes in serum lipids were described for rabbits infected with *Schistosoma japonicum* [1]. Moreover, an attenuation of the key metabolic enzymes in liver and the consequent shift in the metabolic homeostasis of the host were shown for humans infected with *Schistosoma mansoni* [2]. Yet, metabolomics boded something completely new: a combination of the advanced analytical techniques capable of simultaneously detecting multiple compounds and the multivariate modeling or machine-learning algorithms, which offered a strong alternative to the conventional biochemical experimentation [3]. New and more accurate diagnostic methods based on the multiparametric metabolic readouts, as well as novel approaches for estimating morbidity or monitoring host–parasite interaction, appeared to be within a hand's reach. The first reports on metabolomics of *S. mansoni* infection appeared promising [4]. A clear biological response to this parasitic infection was discovered that involved changes in the pattern of amino acids as well as the composition of the microbiota-related metabolites. Since then, despite the enormous progress in analytical instrumentation and, most importantly, the data analysis routines, metabolomics of the body fluids in helminth infections has not provided the field with much in-depth insight. Here, we would like to give our point of view on the disagreement between a highly promising methodology and the rather disappointing results. We have restricted our discussion to studies that: (i) involve only members of the *Trematoda*, (ii) are conducted in animal models, and (iii) use the method of nuclear magnetic resonance (NMR) spectroscopy

as the analytical platform (Box 1) to ensure a cross-comparison of the results published in different laboratories. Of course, any restriction introduces a bias, but we believe that consistency of the discussed data is more important than extensive coverage of the literature.

Box 1. NMR-Based Metabolomics NMR spectroscopy, in essence, is a physical tool for investigating matter. NMR exploits the properties of the atomic nuclei (Nuclear) in a strong magnetic field (Magnetic) using radiofrequency waves (Resonance) to gain information about the composition and quantity of the molecular entities in the complex mixtures. The first published biological application of NMR goes back to the 1950s when the method was applied to study the hydration properties of DNA in solution [32]. A proton NMR or ^{1}H-NMR used in the above-mentioned work became, after several instrumental improvements, an analytical core of NMR metabolomics. ^{1}H is the most commonly used nucleus for NMR analysis of biofluids, thanks to its natural abundance of 99.98% and its invariable presence in organic molecules. ^{1}H-NMR cannot match the sensitivity of mass spectrometric techniques, but it offers superior reproducibility and facile quantitation based on a linear relation between the peak area and the concentration of the corresponding analyte [33, 34]. A truly unique feature of NMR metabolomics is the possibility to analyze the data with an exploratory untargeted or targeted approach to the same set of raw data. Finally, the automated workflows that integrate data acquisition, preprocessing, and modeling algorithms are making the platform accessible to a broad scientific community [35].

The numbers: how many metabolites are there, and how many do we see?

We start with the numbers. The last version of the human metabolome database (HMDB 4.0) includes over 100 000 structures [5]. Yet, by searching the database for body-fluid-specific metabolites, we get only 2025 features for urine and 3189 for blood (the search was performed using the filters "detected and quantified" and "detected but not quantified", thus limiting it only to the metabolites that were in fact measured and excluding the predicted ones). Furthermore, the diversity of the physicochemical properties of the metabolites makes it impossible for a single analytical method to provide a full coverage of the metabolome within a given biological sample. Consequently, a choice of the analytical method will always skew the analysis towards one or another group of metabolites, for example, polar (e.g., amino acids, sugars, nucleosides, nucleobases) or nonpolar (e.g., fatty acids, lipids). NMR is not an exception, though the limitations are due to the sensitivity rather than chemical selectivity (as it is in the case of mass spectrometry analysis). For instance, the introductory paper of the human urine metabolome database, published in 2013, included only 200 structures detectable by NMR [6], while the serum database published 2 years earlier included just 59 [7]. An open-access resource comparable to HMDB is not available for the rodents, but the numbers reported for human body fluids can be safely taken as a first approximation of the metabolome coverage offered by NMR for the animal rodent models. An analysis of the preselected literature revealed a set of 67 metabolites reported in the context of the trematode infections [4, 8–19]. The metabolites are summarized in Figure 1 (Key Figure). At a first glance, the number might appear low. But considering the restrictions discussed above, we can conclude that the number is just within a possible range. An additional explanation for a limited number of the reported metabolites could be due to the data analysis strategies routinely used in the exploratory metabolomics studies (Figure 2) – only the metabolites relevant to the study design that passed the selection criteria (e.g., variable ranking or variable importance for the multivariate methods or P value cut-off for the univariate ones) are reported and discussed. Thus, a typical output would have something

like 10–20 structures per report.

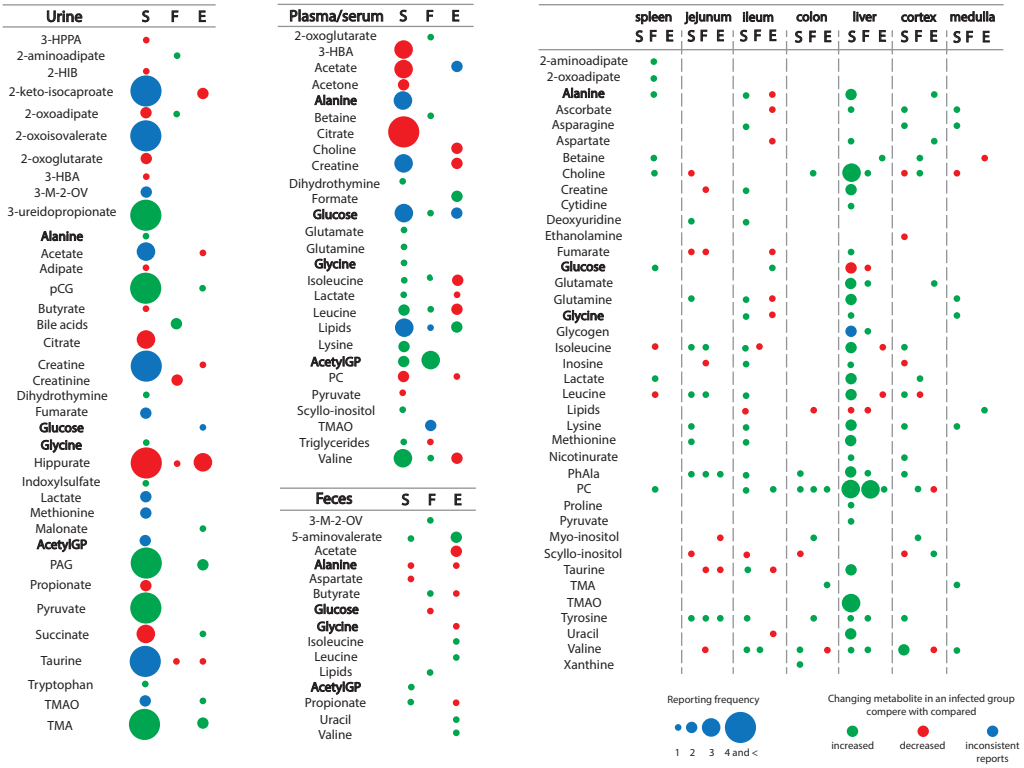

Figure 1. The included metabolites were reported as the ones with the highest contribution to multivariate models using case–control (infected versus noninfected) as the class ID [3–15]. The entries are organized according to the biological material used in the experiments and the frequency of reporting. There are 67 metabolites in total, but only 3 (highlighted in bold) are reported for all types of biomaterial; urine has the largest number (17) of the 'unique' metabolites. S, Schistosoma mansoni and Schistosoma japonicum; F, Fasciola hepatica; E, Echinostoma caproni. The size of the dots indicates reporting frequency. The colour indicates the concentration change: green, consistently increased; red, consistently decreased; blue, inconsistent data. Metabolites abbreviations: 3-HPPA, 3-hydroxyphenylpropionic acid; 2- HIB, 2-hydroxyisobutyrate; 3-HBA, 3-hydroxybutyrate; 3-M-2-OV, 3-methyl-2-oxovalerate; pCG, 4-cresol glucuronide; acetylGP, acetyl glycoproteins; PAG, phenylacetylglycine; TMAO, trimethylamine-N-oxide; TMA, trimethylamine; PC, phosphocholine; PhAla, phenylalanine.

The numbers: how many metabolites are there, and how many do we see?

Thus, what can we extract from the metabolomics studies of helminth infections? In fact, there are several consistent trends reported from different studies. The first one is the change in the metabolism of amino acids observed in helminth-infected animals (mouse, *Mus musculus*, and Syrian hamster, *Mesocricetus auratus*, are the most used models). For some amino acids, the reported changes are helminth-specific; an example is taurine, a key metabolite for bile formation and one of the frequently mentioned amino acids affected by these infections [4, 10, 11, 15, 17]. In the *Schistosoma mansoni* infection model, the change in direction (whether the concentration of a metabolite increases or decreases in the infected animals in comparison to the noninfected ones) of urinary taurine varies, butitis consistently elevated in the liver [4, 10, 18]. In *Fasciola hepatica* infection, a decreased concentration of urinary taurine in combination with an increase in urinary bile acids [11, 16] fits nicely into a physiological paradigm which explains urinary excretion of the bile acids as a consequence of a duodenal obstruction [20]. This, in turn, may lead to the increased production of the bile acids and depletion of the taurine pool. As is the case with many other hypotheses derived from metabolomics data, this one is waiting for experimental confirmation. Another interesting example was reported for the first time in the seminal manuscript of Wang et al. [4]. The authors, as pioneers in the field, made a few interesting observations. One of them is a distinct pattern characterized by increased concentration of urinary pyruvate alongside a simultaneous decrease in citrate, succinate, and 2-oxoglutarate, which was associated with *S. mansoni* infection. The pattern proved to be consistent with further reports [4, 10]. Moreover, an original interpretation of the observation as a consequence of a systemic impairment of the pyruvate dehydrogenase was supported by an independent proteomics study [21]. Some changes in the urinary amino acid patterns, such as the decreased levels of 2-oxoisovalerate and 2-oxoisocaproate [4], are interpreted as a sign of impaired liver function, suggesting that one could estimate morbidity from a single urine sample. Yet, despite the fact that the amino acids always appear on the list of the metabolites affected by an infection, the variability

in the reported patterns remains high. With a single but notable exception: the concentration of essential amino acids, such as valine, is lower in the experimental models of *Echinostoma caproni* and is increased in the *Schistosoma* and *Fasciola* models [9–11, 15, 17, 19]. The first could be explained by the intestinal location of the parasite and as a consequence of impaired absorption of the dietary material. The second could suggest that the parasite is capable of impairing the insulin-stimulated absorption of the essential amino acids by the tissues (and muscle in the first place) to benefit from an increased pool of this important carbon and energy source in blood.

The second general observation is the infection-driven shift in lipid metabolism. The critical changes in the entire system of lipid homeostasis during the acute phase of infection were known for a long time, and the differences between rodents and primates are well documented. For example, it has been shown that, during the acute phase of an infection, the cholesterol increases in the serum of animals with low baseline values of low-density lipoprotein cholesterol (rodents) but remains stable in primates where the baseline values are higher [22]. Moreover, next to their role as the lipid transporters and regulators of cholesterol bioavailability, the lipoproteins are playing a part in the innate immune response. Thus, in the context of helminth infections, the observation that resistance to *Schis-*

Glossary

Biocomplexity: complexity as exhibited by living organisms in their structure, composition, function, and interactions, or the study of complex structures and behaviors that arise from nonlinear interactions of the biological systems.

Biofluid: any bio-organic fluid produced by an organism, such as blood, urine, saliva, cerebrospinal fluid, sperm etc.

Mass spectrometry: an analytic technique by which chemical substances are identified by the sorting of ions in electric and magnetic fields according to their mass-to-charge ratios.

Metabolite: a chemical substance that is a product of metabolic action or that is involved in a metabolic process.

Metabolome: the complete set of the metabolites (such as metabolic intermediates, hormones and other signaling molecules, and secondary metabolites) to be found within a biological sample.

Metabolomics: a postgenomic discipline studying multiparametric metabolic responses of living systems to the internal and environmental stimuli.

Multivariate modeling: an application of multivariate statistics that encompasses the simultaneous observation and analysis of more than one outcome variable.

Nuclear magnetic resonance (NMR): spectroscopy is an analytic technique which measures local magnetic fields around atomic nuclei to obtain information about the molecular entities in the complex mixtures.

tosoma in rodents (rats) could be mediated by lipoproteins [23] makes the infection-related changes in lipid metabolism an important trend. Although ^{1}H NMR provides only an overview of the major lipids (fatty acids, glycerolipids, phospholipids, sterols) and lipoprotein classes, detailed analysis of the structurally diverse lipometabolites is beyond the reach of the method. Recently, quantitative analysis of more than 100 lipoprotein subclasses directly from ^{1}H NMR of serum/plasma samples has been made possible [24, 25]. Application of the standardized and quantitative method to the helminth infection models could help to resolve the existing inconsistencies in the reported changes in the lipid-related resonance regions. Finally, practically all metabolomics studies on helminth infections published so far show changes in metabolites associated with the gut microbiota. Today, it is widely accepted that the microbiota plays an essential role in the control of host physiological processes, forming a symbiotic "super-organism" [26]. One of the most pronounced metabolic traits is indirect urinary depletion of gut microbiota metabolites, namely hippurate (a conjugate of the benzoic acid and glycine), propionate, and butyrate [4]. The effect appears so consistent that it is reported not only for the rodent models of helminth infections but also in human studies in endemic regions [8, 27]. The exact physiological consequences of this depletion remain unexplored. However, considering the common symptoms of intestinal schistosomiasis (abdominal pain, diarrhea, and blood in the stool), a suppressed metabolic activity of the microbiota could be expected as a result, leading to a depletion of the main intermediates of the microbiota. However, this purely casual reasoning gives no insight into the mechanism of interaction between the intestinal metabolic pool and the excretion of the microbiotic metabolites in urine. Another interesting microbiota-related metabolic trait is the inverse relationship between the concentrations of two glycine conjugates: hippurate and phenylacetylglycine (PAG, a conjugate of phenylacetic acid and glycine) [8, 10–19]. The effect appears to be common to all helminth infections studied to date and is confirmed by independent analytical approaches [13, 14, 17, 28]. Both compounds depend on a pool of free glycine and could be synthesized by the microorganisms as well as by the host. To explain whether the observed dependency is a spurious correlation or an effect pointing to the mechanisms of response/adaptation to helminth infection, a properly designed follow-up study is required.

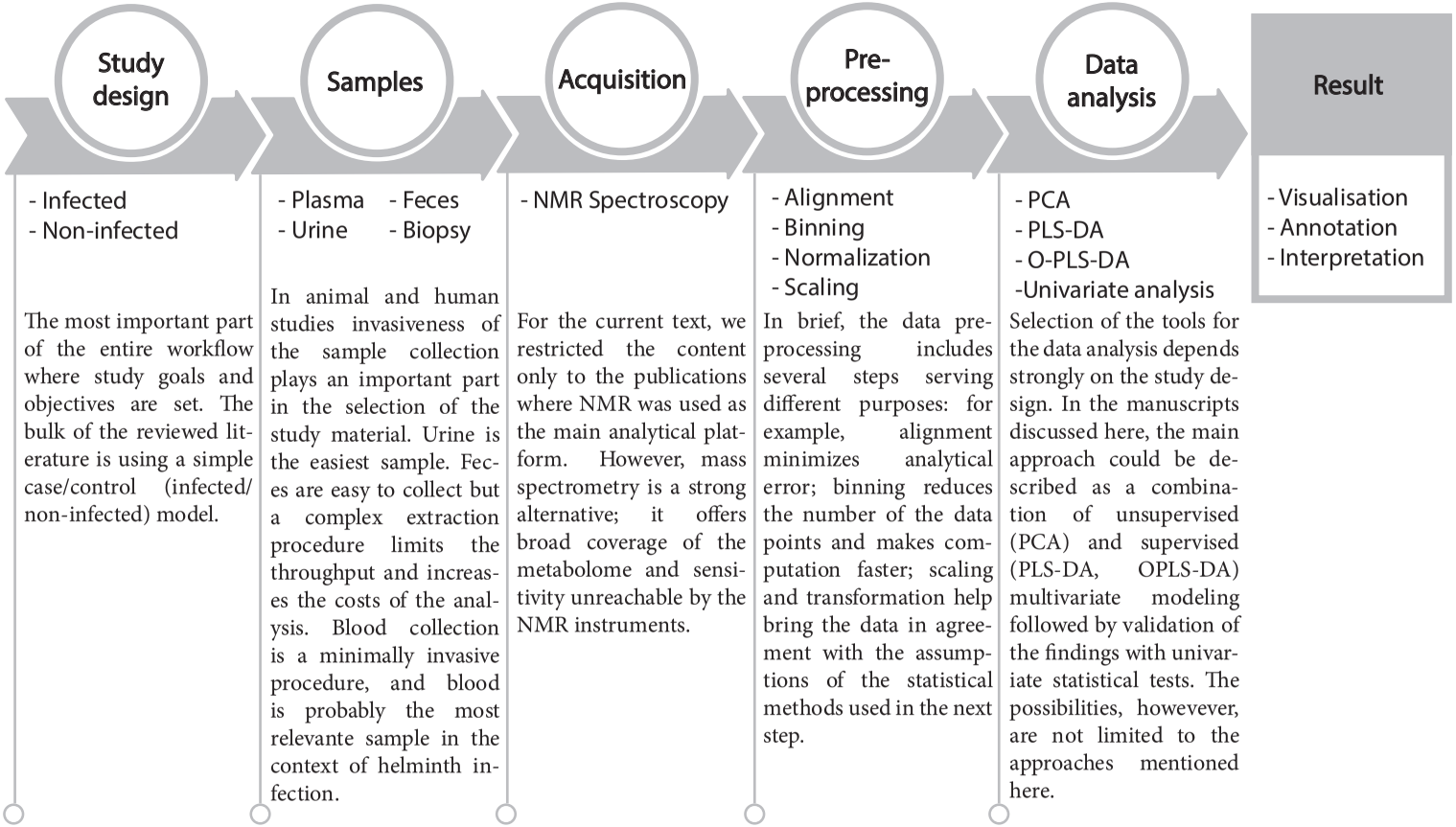

Figure 2. A generic workflow for a metabolomics study. A generic experimental workflow outlines the main steps of a metabolomic study and summarizes the especial points for every step: types of sample, possible analytical solution, essential steps in the data preprocessing and analysis.

The numbers: how many metabolites are there, and how many do we see?

One rationale for using animal models of human pathologies is the possibility to work in a highly controlled manner, minimizing the sources of the variance common for the human studies, reducing unwanted complexity, and revealing otherwise blurred patterns. Our approach to writing this text was similar: define the area of the analysis and try to grasp a general pattern. Hence, what do we have at the end? A limited set of the discriminative metabolites (Figure 1), many of which belong to a notorious list of the 'usual suspects' (a set of metabolites which appears on the top of the classification lists in any metabolomics study), and a few consistent metabolic trends. Does this imply that the discussed methodological approach leads to a dead end? We do not believe that there are grounds for such a conclusion. Not yet, at least. Firstly, the bulk of the metabolomics projects was designed and executed as exploratory studies and, as such, should generate a number of hypotheses for further testing, thus it is mainly a hypothesis-generating tool. Indeed, some interesting hypotheses (for example, a hypothesis on a systemic impairment of the pyruvate dehydrogenase during infection) have been generated. What is missing is a strategy for the follow-up studies. Secondly, the exploratory metabolomics studies used a rather limited set of data-modeling tools (see the data analysis section of Figure 2), which often address only case–control (infected versus noninfected) differences. Integration of the metabolomic data with clinical chemistry laboratory tests, demographic data, and genomic traits can broaden the data analysis repertoire. The positive examples from other fields of research (cardiology, metabolic syndrome) encourage such an approach [29, 30]. The manuscripts of Kettunen et al. [29] and Sliz et al. [30] are good examples showing that a combination of NMR metabolomics with genomic traits and an advanced data modeling made possible an accurate estimation of the heritability and risk of cardiovascular disorders. Thirdly, the host–parasite interaction is a complex phenomenon affecting all physio-logical systems of the host. Using a single biofluid to study it leads to an incomplete view of the problem. This issue was recognized from the very early days of metabolomics when parasitic

infections were considered as special cases of the increased biocomplexity [26]. Attempts at integrative analysis, using multiple biofluids, were made [9], but the available data are still too fragmentary to reveal the full advantage of such an approach. One could propose here a need for a more systematic, "multi-omics" approach; we recommend a recent review on this topic for a detailed overview [31]. Finally, in the early days of metabolomics, the discipline was presented as a solution to the open questions in biomedical sciences that conventional hypothesis-testing experimental models failed to solve. It is true that a holistic nature of the methodology is indeed its main 'added value'. What is missing here is a real 'embedding' of the methodology into a problem-solving process in the field. Or, in other words, the method has to earn its place in the 'toolbox' of contemporary parasitology research. Thus, in our opinion, the future of metabolomics in helminth research is to be integrated with the standard physiological readouts routinely used in parasitological studies. The detailed process of such integration is difficult to predict. From the perspective of those who practice metabolomics research, we could mention a current trend towards automation and standardization of the complex metabolomics workflows as a practical way to make the technology and the data more accessible [32]. Furthermore, the current field of metabolomics is not limited to ^{1}H NMR spectroscopy. Mass spectrometry is a strong alternative; it offers broad coverage of the metabolome and the sensitivity unreachable by NMR instruments. A balanced combination of the methodologies, as well as a knowledge of their advantages and limitations, would help us to make a right choice of the method, best fitted to a biological question or to the metabolites of interest (see Outstanding Questions).

Outstanding Questions

- Given that metabolomics offers a number of methodologies, including both the well-known ones (such as NMR or mass spectrometry) and the exotic ones (e.g., Raman spectroscopy), how does one find a realistically simple set of rules that facilitate a choice of the analytical platforms that, meanwhile, optimally fit a given study design?
- How many metabolites need to be measured to make a meaningful physiological/pathophysiological description of the studied organism?
- Is the host's metabolic response to helminth parasites specific, or does it represent the systemic response to infection? What does medical helminthology need from metabolomics to address the current challenges?

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References

1. Nishina, M. et al. (1994) Nuclear magnetic resonance (NMR) analysis on the serum-lipids of rabbits infected with Schistosoma japonicum – oxidative modifications of Diene system in fatty chains. Int. J. Parasitol. 24, 417–419
2. Sheweita, S.A. et al. (1998) Different levels of Schistosoma mansoni infection induce changes in drug-metabolizing enzymes. J. Helminthol. 72, 71–77
3. Holmes, E. and Nicholson, J.K. (2007) Human metabolic phenotyping and metabolome wide association studies. Ernst Schering Found. Symp. Proc. 4, 227–249
4. Wang, Y. et al. (2004) Metabonomic investigations in mice infected with Schistosoma mansoni: an approach for biomarker identification. Proc. Natl. Acad. Sci. U. S. A. 101, 12676–12681
5. Wishart, D.S. et al. (2018) HMDB 4.0: the human metabolome database for 2018. Nucleic Acids Res. 46, D608–D617
6. Bouatra, S. et al. (2013) The human urine metabolome. PLoS One 8, e73076 7. Psychogios, N. et al. (2011) The human serum metabolome. PLoS One 6, e16957
7. Psychogios, N. et al. (2011) The human serum metabolome. PLoS One 6, e16957
8. Balog, C.I.A. et al. (2011) Metabonomic investigation of human Schistosoma mansoni infection. Mol. Biosyst. 7, 1473–1480
9. Saric, J. et al. (2009) Panorganismal metabolic response modeling of an experimental Echinostoma caproni infection in the mouse. J. Proteome Res. 8, 3899–38911
10. Li, J.V. et al. (2011) Chemometric analysis of biofluids from mice experimentally infected with Schistosoma mansoni. Parasites Vectors 4, 179
11. Saric, J. et al. (2010) Integrated cytokine and metabolic analysis of pathological responses to parasite exposure in rodents. J. Pro-teome Res. 9, 2255–2264
12. Wang, Y. et al. (2006) System level metabolic effects of a Schistosoma japonicum infection in the Syrian hamster. Mol. Biochem. Parasitol. 146, 1–9
13. Wang, Y. et al. (2009) Systems

metabolic effects of a Necator americanus infection in Syrian hamster. J. Proteome Res. 8, 5442–5450
14. Wu, J.F. et al. (2010) Metabolic alterations in the hamster co-infected with Schistosoma japonicum and Necator americanus. Int. J. Parasitol. 40, 695–703
15. Wu, J. et al. (2010) Metabolic changes reveal the development of schistosomiasis in mice. PLoS Negl. Trop. Dis. 4, 8
16. Saric, J. et al. (2010) Systems parasitology: effects of Fasciola hepatica on the neurochemical profile in the rat brain. Mol. Syst. Biol. 6, 396
17. Saric, J. et al. (2008) Metabolic profiling of an Echinostoma caproni infection in the mouse for biomarker discovery. PLoS Negl. Trop. Dis. 2, e254
18. Li, J.V. et al. (2009) Metabolic profiling of a Schistosoma mansoni infection in mouse tissues using magic angle spinning-nuclear magnetic resonance spectroscopy. Int. J. Parasitol. 39, 547–558
19. Zhu, X. et al. (2017) Salmonella typhimurium infection reduces Schistosoma japonicum worm burden in mice. Sci. Rep. 7, 1349
20. Corbett, C.L. et al. (1981) Urinary excretion of bile acids in cholestasis – evidence for renal tubular secretion in man. Clin. Sci. 61, 773–780
21. Harvie, M. et al. (2007) Differential liver protein expression during schistosomiasis. Infect. Immun. 75, 736–744
22. Hardardottir, I. et al. (1994) Effects of endotoxin and cytokines on lipid metabolism. Curr. Opin. Lipidol. 5, 207–215
23. Bout, D. et al. (1986) Rat resistance to schistosomiasis: platelet-mediated cytotoxicity induced by C-reactive protein. Science 231, 153–156
24. Jimenez, B. et al. (2018) Quantitative lipoprotein subclass and low molecular weight metabolite analysis in human serum and plasma by (1)H NMR spectroscopy in a multilaboratory trial. Anal. Chem. 90, 11962–11971
25. Barrilero, R. et al. (2018) LipSpin: a new bioinformatics tool for quantitative (1)H NMR lipid profiling. Anal. Chem. 90, 2031–2040
26. Nicholson, J.K. et al. (2004) The challenges of modeling mammalian biocomplexity. Nat. Biotechnol. 22, 1268–1274
27. Singer, B.H. et al. (2007) Exploiting the potential of metabonomics in large population studies: three venues. In The Handbook of Metabonomics and Metabolomics (Lindon,

J.C. et al., eds), pp. 289–325, Elsevier
28. Garcia-Perez, I. et al. (2008) Metabolic fingerprinting of Schistosoma mansoni infection in mice urine with capillary electrophoresis. Electrophoresis 29, 3201–3206
29. Kettunen, J. et al. (2012) Genome-wide association study identifies multiple loci influencing human serum metabolite levels. Nat. Genet. 44, 269–276
30. Sliz, E. et al. (2018) NAFLD risk alleles in PNPLA3, TM6SF2, GCKR and LYPLAL1 show divergent metabolic effects. Hum. Mol. Genet. 27, 2214–2223
31. Sotillo, J. et al. (2017) Exploiting helminth–host interactomes through big data. Trends Parasitol. 33, 875–888
32. Jacobson, B. et al. (1954) A proton magnetic resonance study of the hydration of deoxyribonucleic acid. Nature 173, 772–773
33. Verhoeven, A. et al. (2017) Automated quantification of metabolites in blood-derived samples by NMR. Anal. Chim. Acta 976, 52–62
34. Lenz, E.M. and Wilson, I.D. (2007) Analytical strategies in metabonomics. J. Proteome Res. 6, 443–458
35. Nicholson, J.K. et al. (1999) 'Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. Xenobiotica 29, 1181–1189