Comprehensive metabolomics of the experimental opisthorchiasis

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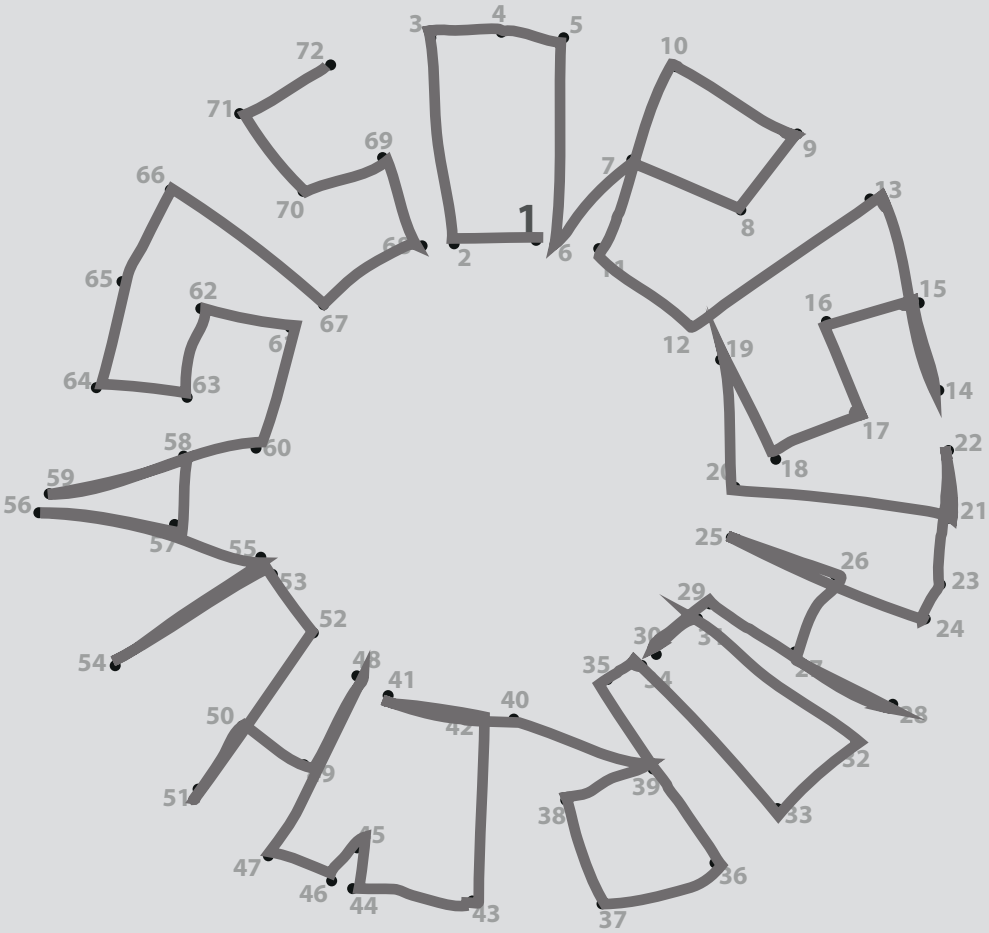
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General discussion

In this thesis, the systemic host metabolic response to experimental *Opistorchis felineus* (*O. felineus*) infection using Nuclear Magnetic Resonance (NMR) spectroscopy has been investigated. Overall, the studies examined the experimental model of opisthorchiasis, which included male and female animals, mild and severe infections, acute and chronic (up to 32 weeks) as well as biofluids and organs. Multi-block data analysis was applied to the model of chronic opisthorchiasis to delineate how infection affects the metabolic profile of infected versus uninfected animals at a detailed level, enhancing our understanding of this neglected disease affecting 56 million worldwide.

Chapter 2 is dedicated to an overview of the metabolomics studies on trematode infections. No relevant publications on *Opistorchis sp*. were found, however, the review highlights the studies on other trematodes such as *Schistosoma mansoni* (*S. mansoni*) *Schistosoma japonicum* (*S. japonicum*), *Fasciola hepatica* (*F. hepatica*), and *Echinostoma caproni* (*E. caproni*), which are widespread infections worldwide. Focusing on NMR spectroscopy, known for its robustness, we tried to achieve a more reliable comparison of the results from the different laboratories. Likewise, animal studies offer a degree of control for multiple confounding factors, which is almost impossible to reach in human studies. The literature overview led us to the two conclusions relevant for the current work: a) the main features of the hosts metabolic response to the trematode infections can be described as a dysregulation of the amino acid and lipid metabolism, and changes in the microbiota related metabolites; and b) a prevailing case-control design and analysis of a single biofluid type gives only a limited overview of the possible metabolic adaptation of the host to the infection. Therefore, we adapted our experiential design to include the following features: longitudinal data sampling, two body fluids (urine and plasma); two different degrees of *O. felineus* infection (mild and severe) compared with a control group (PBS-vehicle); and the animals of the both genders were included in the experiment. The results of the time-resolved studies are presented in **Chapters 3** (urine data) and **Chapter 4** (blood plasma data).

Our data in these chapters show the strongest metabolic response in both urine and plasma biofluids is observed between the 2^{nd} until 10^{th} week post-infection, which is related to the acute stage of opisthorchiasis. During this period worms reach the bile duct where they transform into an adult form and start egg production [1]. The

maximal metabolic response to the infection was observed in 4th week post-infection; the time point when eggs are first detected in the feces. The metabolic response of the host to acute opisthorchiasis has a few general characteristics. The first one is the changes in the metabolism of the essential amino acids. The acute stage of the infection is characterized by depletion of the branched-chain amino acids (BCAAs) – valine, leucine, isoleucine in blood, but the elevation of isoleucine in urine samples, which could indicate a modulation of liver function [2]. Taurine, another essential amino acid, participating in bile formation, demonstrated a trend for a decrease in urine of the infected group. This, in turn, might relate to overproduction of bile acids as a consequence of blockage of bile ducts by worms or eggs. The increase of bile acids in urine of *O. felineus* infected group may confirm this hypothesis. Therefore, bile acids might be a prospective marker for diagnostics of opisthorchiasis at the acute phase of infection. On the other hand, taurine plays an important part in the regulation of lipid metabolism, and the level of urinary taurine can relate to changes in host lipid metabolism, which can be strongly manifested in blood plasma data [3].

A strong trend of the metabolic response to *O. felineus* infection is a shift in lipid metabolism. At the first 12 weeks of infection, there is an extreme upregulation of lipoproteins for both infected groups (mild and severe). Our results mostly agree with the study of *O. viverrini* infected hamsters, where it had been shown that changes in lipid metabolism are associated with leakage of α-tocopherol from an injured liver [4]; moreover, changes of lipoproteins were reported for other trematode infections [5].

Changes in lipid metabolism are strongly connected with another metabolic change during acute opisthorchiasis, namely in nicotinic acid metabolism. This change is unique for opisthorchiasis and has not been reported in metabolomics studies on other trematode infections. We hypothesize that in the first weeks of the infection, which is often described as a period of metabolic stress, a consumption of nicotinic acid increases as it is needed for NAD+ production. This, in turn, leads to the reduced release of its derivatives such as e.g. nicotinuric in urine of the infected group. Even though urine and plasma samples have shown similar trends, urine samples had a gender-specific response – a stronger response is seen in males than in females. The explanation for this phenomenon could be the gender specific difference of the renal system, though blood which reflects the sum of all

processes is less affected by sex.

Taken together, the metabolomics studies have highlighted that *O. felineus* infection modulates lipid, energy, and amino acid metabolism, but the response is not specific for the infection and can be mediated by common metabolic stress. By using a longitudinal study design, it was demonstrated that the strongest metabolic response was observed during the acute stage (until the 12th week of infection) with the peak corresponding to when egg production commences.

Feces is the sample routinely used for diagnosis of helminth infections by for example, Kato-Katz tests [6]. However, feces, as a product of the gastrointestinal tract, can have essential information about exogenous metabolites and microbiota. Therefore, it would be useful to be able to analyze feces metabolomics during opisthorchiasis but there are no standardized protocols for measurement of metabolites in feces samples. Therefore, **Chapter 5** was dedicated to the development of the NMR-based metabolomics workflow for feces. Human feces samples were chosen for the development, because the amounts available exceed that from hamsters. After tuning the method, final protocol involved few steps for sample preparation: homogenization, water extraction, and double centrifugation. Using the workflow, 62 fecal metabolites were identified in a pooled human sample. The protocol was tested on a small subset of infected and non-infected samples from *O. felineus*-infected people. The method was used in **Chapter 6.**

As it was indicated above, the differences in the metabolic composition of the body fluids between the infected and non-infected animals are becoming negligible after the 12th week of infection. We speculate that after the initial metabolic stress caused by the worm intervention and starting egg production, the host reaches metabolic homeostasis which indicates the beginning of the chronic infection. Yet, the question how and at which cost the host metabolism reaches this condition remains unanswered. **Chapter 6** presents an integrative metabolomics approach which was focused on how different organs and functional systems of a host are affected by the infection and how the host adapts to the chronic infection. Six relevant body compartments, blood serum, urine, and stool samples were included in the study, but 26 metabolites of liver, spleen, and jejunum and 6 serum fatty acids were relevant to a map of metabolic signature for chronic opisthorchiasis. Therefore, the metabolic homeostasis depends on system lipid metabolism and local metabolic changes in the liver, where

the fluke is located; the spleen, which is central to the immune system; and jejunum, the closest intestinal segment to the liver.

The elevated fatty acids belonged to linoleic acid metabolism and odd-chain fatty acids are strong in driving the data. It has been shown that those structures have a reverse association with a risk of type 2 diabetes [7-9]. Furthermore, arachidonic acid (the end product of linoleic acid pathway) is an important mediator of inflammation. It can affect the functioning of several organs and systems either directly or upon its conversion into eicosanoids including prostaglandins, thromboxanes, and leukotrienes [10].

The metabolic changes at the level of the individual organs could be explained from point of view of the regulation of the immune response. For instance, it has been shown that leucine plays a role in regulation of intestinal immunity and the entire mTOR signaling system [11,12]. An activation of the metabolic mechanisms involving leucine was also shown for Shistosoma mansoni and Fasciola hepatica infection [13,14].

Finally, our analysis shows that metabolically the spleen was the most affected organ, which could be interpreted as an indication of its key role in long-term metabolic response to *O. felineus* infection. The glutamine/glutamate and glutamine/alanine ratios in the spleen of the infected animals indicate lower cell proliferation rates [15]. At the same time, low liver glutamine/glutamate ratio showed a higher metabolic activity of the liver during chronic opisthorchiasis which can be related to adaptations to the presence of the parasite (inflammation, eosinophilia and periductal fibrosis at the chronic stage) [16,17].

To summarize, while the acute infection represents a state of temporary metabolic stress which is resolved in a new homeostasis, the new "status-quo" is achieved at the cost of the prolonged changes in the utilization of the main metabolic fuel components and the local depletion of the amino acids pool. We can tentatively describe this combination of the metabolic changes as a "metabolically mediated hybernative state of the organism" which develops during the chronic infection.

Conclusions

For the first time, the comprehensive host metabolic response to *Opistorchis felineus* (*O. felineus*) infection has been explored and is presented in this thesis. Using a longitudinal study design, it was possible to show that the metabolic response to the opisthorchiasis has two clear stages – acute and chronic. The acute stage characterizes the changes in the host metabolism of lipids, energy and essential amino acids which corresponds to common metabolic stress caused by an invasion and onset of egg production. After the period of an adaptation, at the chronic stage, the metabolic changes in the biofluids (blood and urine) are flattened. However, multicompartment metabolomics study showed that chronic opisthorchiasis suppresses the metabolic activity of jejunum, liver and spleen that can increase the risk of development of the associated pathologies. Thus, acute and chronical opisthorchiasis characterizes different metabolic conditions, at the beginning, *O. felineus* infection provokes metabolic stress which shifts to a metabolic homeostasis which is driven by systemic changes in lipid metabolism and organ-specific amino acid metabolism.

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