Virus-host metabolic interactions: using metabolomics to probe oxidative stress, inflammation and systemic immunity

Schoeman, J.C.

Chapter 8

Conclusions and perspectives

The persisting pathogenicity of novel and old world viruses illustrates the capability of viruses to deploy evolutionary mechanisms to ensure their survival in a host by targeting cellular and immunological responses. Since the end of the 20th century, elucidation of viral engineering of host metabolic pathways for viral genome replication, viral maturation and progeny production and survival of infected cells has made great strides as introduced in Chapter 1. In this thesis, metabolomics complemented with transcriptomics and immunology was used to study the host-virus interaction on the metabolic level with a special emphasis on the metabolic role of inflammation and oxidative stress during the innate immune response against viral infection. We have chosen respiratory syncytial virus (RSV) and hepatitis B virus (HBV) as candidate viruses to metabolically study their role in acute respiratory infection and chronic hepatitis B infection. Secondly we also investigated infant metabolic and immunological consequences of *in utero* exposure to antiretroviral intervention and human immunodeficiency virus (HIV).

Method development

The development of accurate, precise and robust analytical methods is fundamental to research in the evolving field of metabolomics. In Chapter 2 we report the development and validation of a liquid chromatography mass spectrometric method capable of providing a cellular readout of inflammation, oxidative – and nitrosative stress. Isoprostane and nitro fatty acid (NO_2-FAs) profiles are determined to assess lipid peroxidation and lipid nitration, respectively. In addition, the method provides a metabolic inflammatory readout by measuring prostaglandins, lysophosphatidic acids and lyso-sphingolipids. This method was optimised for different matrixes (serum as well as six organ tissues: heart, liver, lung, kidney, brain and spleen) to evaluate systemic as well as organ-specific (localised) metabolic responses. The application of this methodology in healthy mice demonstrated that the systemic (serum profile) readout of stress and inflammation markers was nonspecific for any of the different organ tissues investigated. In addition, we identified that each organ presented a unique homeostatic stress and inflammation profile. A limitation in this study was the lack of a suitable experimental group that would reflect a severe localised metabolic perturbation. Since it is possible that a severe organ perturbation can spill over (i.e. leakage into plasma) into the circulation, the metabolic serum profile may reflect the specific perturbed organ. Investigating the pathophysiological interactions between different organs during a chronic infection such as HBV, could aid in elucidating HBV driven pathophysiology and possibly even liver mediated pathophysiology. Applying this methodology too *in vitro* and animal models of viral infection will broaden our understanding of systemic and localised oxidative stress and inflammation on the metabolic level experienced as part of the immune response.

In Chapter 3, we investigated the suitability of urine as non-invasive sample matrix to provide insights into inflammation and oxidative stress, targeting the oxidised lipids profile. To obtain a total oxidised lipid profile, i.e. conjugated and free oxidised lipids, we investigated different ß-glucuronidase (GUS) deconjugation methods. Subsequently, we developed and validated a bovine liver-GUS hydrolysing sample preparation method coupled with LC-MS/MS. This methodology allowed for specific and sensitive measurement of more than 70 oxidized lipids, biosynthesized from two non-enzymatic (ROS, RNS) and three enzymatic pathways (COX, LOX and P450). Thus, we established a non-invasive readout for inflammation and oxidative stress in urine.

Urine as a sample matrix compared to serum is not volume limited, ideal in studies involving children, contains minimal proteins, and provides a more complete catabolic readout.

In summary, the biological questions we address, for example during viral infection, will determine the ideal experimental design. i. Where is the main site of infection and what is the best compartment to sample? ii. What data do I need and want to integrate (i.e. "omics" levels to target and physiology)? And if the ideal design is not possible (e.g. sampling a certain organ), especially in the case of human studies, what is the best alternative approach able to address the biological question? Different biological sample types present with different challenges, each requiring optimised intervention and quantification strategies. Tissue samples require homogenisation and adequate protein removal, whereas urine contains high salt concentrations and conjugated metabolites, thus sample preparation is of critical importance. Across compartment comparisons and integration require accurate quantitative protocols; once available, they will enhance our modelling and biological elucidation capabilities. In this thesis, we found different matrix effects between different sample types and explored semi-quantitative approaches to compare concentration levels of metabolites. The next step would be incorporating absolute quantification which would require robust academic calibration curves, using both endogenous as well as labelled internal standards. Absolute quantification would allow better to compare and integrate different studies, or data from different sample compartments. Chapter 2 and 3 show that with the proper optimization our novel oxidative/nitrosative stress platform can be applied to all (tested) tissues, blood and urine samples. Next, application of this methodology to an in vitro model of RSV infection will contribute to elucidate the metabolic role of oxidative stress and inflammation during acute respiratory infection.

In vitro based metabolomics

In vitro models are excellent starting points for proof of concept studies such as to explore the usefulness of metabolomics to study metabolic innate immune responses, and for subsequent mechanistic validation/verification. We used the well-established A549 - adenocarcinoma human alveolar basal epithelial - cell model as host cells during a three-day time course of respiratory syncytial virus (RSV) infection to probe the metabolic response during the innate response. Recently, the appreciation of lipid mediators in cellular signalling in the field of immunology and inflammation has been gaining momentum and integration with more established "omics" data, such as transcriptomics and/or proteomics, presents us with new ways to study viral infections. In Chapter 4 we identified that A549 cells, upon RSV infection, display a calcium-independent upregulated phospholipase signature necessary for the release of lipid mediator substrates, concurrently with an upregulated cyclooxygenase (COX-II) and reduced cytochrome P450 pathway in the first 24h. This natural pro-inflammatory host innate immune response is characterised by high levels of prostaglandin E_2 and prostaglandin $F_{2\alpha}$ within the first 24h. In addition, RSV has developed the ability to interfere with and modulate the host response even on the metabolic level with reduced rates of enzymatic prostaglandin catabolism. The resulting high PGE_2 levels present RSV with several immune evasive strategies including attenuating the macrophage mediated T_H1 - IFN-γ immune response and skewing it towards the less favoured T_H2 regulatory T-cell response. Prostaglandin E_2 also suppress apoptosis, thus hindering the formation of antigen containing apoptotic vesicles necessary for the switch between innate and adaptive immune responses. Although the resulting increase in PGA_2 levels, a non-enzymatic PGE_2 metabolite, eliciting multiple anti-viral properties

reflects the co-evolution of the RSV-host interaction. The late onset of LOX and P450 metabolites may highlight these pathways as anti-inflammatory regulators opposing the role of COX derived prostaglandins.

Furthermore, in Chapter 5 we used metabolomics and transcriptomics to explore the metabolic relationship between RSV and the host pertaining to oxidative stress and the cellular metabolic anti-oxidant capacity. We found increased levels of lipid peroxidation metabolites within 24 hours of infection revealing the capacity of RSV to induce oxidative stress in A549 cells upon infection. Furthermore, metabolomics revealed an impaired glutathione metabolism and compromised antioxidant capacity during RSV infection in accordance with literature. Although low levels of ROS are necessary for host anti-viral defences, RSV's modulation of the host antioxidant response increases ROS levels to induce oxidative stress which contributes to cell death, loss of immune function, uncontrolled inflammatory response and increased viral replication. In addition, we found dysregulation to a much larger extent in the host's sulphur metabolism with increased cysteine and cystathionine levels and decreased levels of taurine following a similar trend as glutathione. Transcriptomics supported our metabolic findings and also alluded to the pathogenic role of sulfide:quinone oxidoreductase in further dampening anti-viral host responses in rapidly metabolising H_2S derived from increased cysteine. The host appears not to be a passive bystander as we identified the upregulation of alternative antioxidant pathways resulting in increased levels of proline and spermidine. The host's biogenic amine resilience and compensatory mechanism (<24h) was unsustainable against RSV after 48 and especially 72 hours. This study is a further proof of the exquisite ability of RSV to modulate host responses resulting in pathogenic mechanisms on the metabolic level.

In summary, metabolomics is a powerful tool to elucidate viral-host interactions on the metabolic level, and far more informative compared to single metabolite assays. Complementing metabolomics with targeted transcriptomics analyses, contributes to a deeper appreciation of the metabolic relationship between pathogen and host. In both Chapters 4 and 5, we showed changes on the metabolic level upon RSV infection in the inflammatory metabolism as well as the redox metabolism of the host. Understanding the metabolic role of the host's innate immune response with regards to inflammation and redox biology during viral infection, as well as viral exploitation strategies of these metabolic processes, could fundamentally change how we could therapeutically target the viruses. The defined nature of metabolic pathways and functions does pose an interesting conundrum: can we identify virus specific metabolic changes and phenotypes, enabling us to differentiate between viruses and possibly even other pathogenic insults? In addition, understanding the fine balance between signalling lipid mediators' biological role in the immune response and pathogens' ability to exploit the host innate response may contribute in determining disease severity, prognosis, risk stratification and effective therapeutic intervention strategies. Addressing the above mention ambitions would require translational studies from *in vitro* models to patients. Using patient material offers valuable insight into the naturally derived infection and human specific disease pathophysiology. In this theses we have studied two different patient populations in order to investigate whether our ambition to use metabolomics to understand immune response and virus-host interactions as well as the effect of therapeutic interventions; we investigated the metabolic progression of chronic HBV over its clinical phases as well as the metabolic consequences of *in utero* cART and HIV exposure.

Patient based metabolomics

Explorative and hypothesis testing using *in vitro* and *in vivo* animal models cannot always mimic the host's complex physiological responses to an insult. The value and insights derived from patient material is of exceptional significance, as it is the result of natural, real-life host-pathogen interactions. Hence validation of *in vitro* and animal model findings in patient cohorts is of critical importance and vice versa. In Chapter 6 we present the first characterisation of the natural metabolic progression of chronic hepatitis B virus (HBV) infection through its distinct clinical phases using targeted and biology driven metabolomics profiling. We identified liver related metabolic – and liver injury - perturbations, which reflect the natural progression of the disease using serum of HBV infected patients. With the liver being the central organ in nutritional regulation and metabolism, it is not surprising that chronic HBV infections induce multiple metabolic alterations in host lipid metabolism via viral hijacking. The altered glycerophospholipid metabolism in the first immune tolerant (IT) phase was attributed to the HBV hijacking of the G3P-NADH shuttle which also has an intimate relationship with the persisting lipid dysregulation observed in the subsequent immune active (IA), immune control (IC) and HBV surface antigen negative (ENEG) clinical phases. Increased levels of the very long chain triglycerides in the IA phase and urea cycle intermediates in the IC phase highlights the causal metabolic risk for developing secondary liver complications such as steatosis and non-alcoholic fatty liver disease during chronic HBV. These metabolites might prove useful as markers of disease progression and severity. The presented study also provided many leads and insights to design follow-up studies and at the same time highlights the need of a complementary systems biology approach to better understand chronic HBV. As discussed in Chapter 2, here we used serum and we were able to measure spilled over liver specific metabolic alterations such as lipid metabolism and urea cycle metabolism during chronic HBV in serum. It is interesting to note that while we were able to measure an altered lipid metabolism, we were unable to find a systemic lipid mediator inflammatory signature from the chronically infected and inflamed liver, which might be due to tight local regulation of these de novo synthesised bioactive lipid mediators.

Chapter 7 focused on a vulnerable but often overlooked population of human immunodeficiency virus (HIV) and combination antiretroviral therapy (cART) exposed infants. Although HIV-negative, these infants present higher mobility and mortality compared to their HIV and cART unexposed counterparts. Our results demonstrate extensive metabolic dysregulation of several pathways in infants with direct and indirect *in utero* exposure to cART and HIV, respectively, providing the underlying conditions for a pro-inflammatory milieu in the infants. More specifically, the lipid profile was significantly altered in cART-HIV-exposed infants, with increased triglyceride and decreased phospholipid species compared to control infants without *in utero* HIV and cART exposure. Furthermore, we detected an increase in oxidized lipids, which are generated upon reactive oxygen species (ROS) production by dysregulated mitochondria. Finally, integration between metabolites and classical immunological markers revealed that alterations in lipid metabolites and lipid peroxidation products were associated with increased levels of pro-inflammatory lysophospholipids as well as the pro-inflammatory cytokines IL-1β and IP-10, respectively. In summary, these data reveal disturbances of cell homeostasis ensuing *in utero* cART and HIV exposure leading to increased immune activation in the infant and may have long-lasting adverse effects.

In the two presented studies, we illustrate the power of metabolomics to reveal the perturbations of metabolic pathways during viral infection and therapeutic intervention. Human metabolism is essential to support life as we know it, but the role of metabolism in pathology of infectious disease has only recently started gaining appreciation. The role of metabolism in supporting survival of both host and pathogen and its influence in shaping the immune responses is a critical paradigm shift that is needed when studying infectious diseases such as viral infections.

Concluding discussion

The virus-host interaction on the metabolic level

Investigating two model viruses' RSV and HBV in different models and in patients, and at different levels of the metabolism, has provided insights in virus specific changes and possibly commonly shared metabolic alterations.

Investigating RSV has revealed a unique relationship with oxidative stress and the host's inflammatory and antioxidant capacity. RSV not only up-regulates prostaglandin synthesis via COX-II, which is a glutathione dependent enzyme, but it also compromises the host's glutathione metabolism leading to a significant impairment, indicating exceptional metabolic rewiring. Thus, inflammation and oxidative stress are two core RSV induced pathogenic mechanisms at play during infection. It is possible to speculate that due to the possible severity of these pathogenic mechanisms the human host has evolved a very effective anti-viral response against RSV infection leading to only a mild disease phenotype in the vast majority of cases. The situation during chronic HBV infection is significant different, actually, the inflammatory and oxidative stress status measured during a chronic HBV infection revealed only subtle changes on the metabolic level. This is somewhat counter-intuitive as chronic-HBV is characterised with a clinical inflamed liver profile. No significant differences were observed in the prostaglandin profiles over the different clinical phases of chronic HBV and when compared to healthy controls, although elevated levels of lipid peroxidation markers across all four phases were detected when compared to healthy controls. We did however discover significant impairment in the glycerophospholipid metabolism with increased plasmalogen phospholipids and decreased phospholipids, lysophospholipids and triglycerides. The initial severity of the hijacking of these lipid metabolic pathways are debatable, but since they are not fatal to hepatocytes, it reveals the evolutionary ability of HBV to co-exist with the host and establishing itself in the case of chronic infection, before alerting the host immune responses. Incorporation of specifically, phosphatidylcholine plasmalogens, rather than conventional glycerophosphatidylcholine into lipoprotein particles, will significantly enhance its oxidation potential leading to increased levels of oxidised LDL, HDL and VLDL in the circulation. Thus, the characteristic inflammatory and oxidative stress experienced during chronic HBV, coinciding with the progressive phenotypes of fibrosis, steatosis, cirrhosis and finally hepatocellular carcinoma, possibly results as a secondary consequence of HBV infection and its modulation of the hepatocytes' lipid metabolism.

Drug targets and off-target effects during viral infection

In Chapter 5 we studied RSV's interaction with the host, mainly focused on the host's compensatory responses against the RSV induced oxidative stress. The significant metabolic changes related to glutathione, cysteine, taurine and proline, and especially their ratios, might proof as useful disease severity markers for clinical decision support and are therefore worth to be clinically further investigated. Ultimately, enhancing these for the host beneficial metabolic compensatory mechanisms can be i. exploited for drug development, ii. monitoring *in vitro/in vivo* the effect of novel therapeutic interventions, and iii. maybe even be possible targets for drug development. The therapeutic benefit of antioxidant supplementation during RSV infection has been explored, and was found to only treat the side-effects of dysregulated redox biology. Taurine has the biological capacity to inhibit ROS production, thus targeting taurine could target more the cause. Furthermore, exploring the role of H_2S during RSV infection, as well as its therapeutic intervention options presents an exciting opportunity.

Chapter 6 reveals the power and potential of metabolomics, since we were not only able to identify a HBV specific metabolic signature, but we also identified a possible metabolic drug target. The hypothesis of the HBV hijacking of the glycerol-3-phosphate shuttle, modulating/directing the lipid metabolism to plasmalogen species could be targeted during therapeutic intervention. Metformin, an FDA approved drug in type II Diabetes, targets amongst others the glycerol-3-phosphate and has shown a beneficial response in HBV therapeutics. The attractiveness of host metabolic therapeutic targets stems from the evolutionary ability of viruses to acquire resistance against drugs designed to target them, although the potential for host toxicity and off-target effects should not be overlooked.

In Chapter 7 studying the infant metabolic consequences of direct and indirect *in utero* cART and HIV exposure, respectively, led us to discover the off-target effects and immunological consequences of cART. Distinguishing a direct HIV effect from the infant metabolic profile was difficult to substantiate, since cART suppresses the maternal viral load to undetectable levels. We were however able to identify significant metabolic dysregulation present at birth in the systemic cord blood of these exposed infants which we could best explain through the off-target effects of cART. Thus, just as viruses can modulate host metabolic responses, likewise can the drugs targeting viral proteins and enzyme complexes, impact the host metabolism. These phenomena have become the core aspect of pharmacometabolomics, which is to study the effects of drugs on the host metabolism, and to predict and study efficacy and adverse side effects of drug interventions. The discovery and consequences of these off-target effects of cART in infants, provides a metabolic framework for the immune disturbances observed in cART and HIV-exposed uninfected infants, which is urgently needed to justify future and follow-up studies. Improving the care for this vulnerable and neglected pediatric patient group while preventing HIV transmission with the least toxic regimen is definitely the way forward.

Both these studies reveal the possible benefit in therapeutically targeting the host metabolism to inhibit the viral lifecycle. It has to be conceded that for most retroviruses where incorporation of their genomic material into the host genome takes place, metabolic therapy does not present a cure but possibly a replication inhibitor, where in the case of HBV infection a metabolic therapeutic cure is more conceivable.

Systemic immunometabolism

Originally, host cell metabolism has been viewed as a static process that simply fuels biological processes through anabolism and catabolism, but has since also been recognised as a core process regulating cellular development, proliferation and differentiation. Recently, a novel paradigm shift has occurred with the discovery that immune cell functions are governed by cellular metabolism and their metabolic intermediates [1,2]. The new view of cellular metabolism encompasses highly dynamic processes which caters to the unique needs of different cell types while regulating its functions. Examples of this included activated B-cells generate ATP via the Warburg effect and use glutamine to supplement the TCA cycle to produce metabolic biomass and effector intermediaries, whereas resting B-cells utilise fatty acids via β-oxidation to supplement the TCA cycle for ATP and biomass production. Researchers in the field of immunology have shown that metabolic rewiring in immune cells is necessary to provide a sufficient amount of energy in the form of ATP, while also ensuring metabolic intermediates for the cell to perform their effector functions as cells transition between quiescence and activated forms. This has led to the new emerging field of "immunometabolism", in which the influence and control of metabolism on the host immune responses is studied.

In this new field of immunometabolism a further paradigm shift from cellular metabolism to systemic metabolic factors as an active immunological player is still required. Systemic metabolic factors can be defined as the metabolic status derived from the excretion of metabolic intermediates from all living cells (immune and especially non-immune cells) into the plasma. The immunological capacity of these systemic metabolic factors is derived from the circulation of these intermediates and innate nutrient sensing pathways affected during metabolic dysregulation. Furthermore, the discovery of specialised lipid signalling mediators has significantly redefined how metabolism and its effector functions are viewed. In this thesis, it is illustrated that lung epithelium cells are capable of synthesis of signalling lipids mediators as part of their innate immune response upon viral infection, thus enabling them to elicit a range of immunological functions. In addition, we also found drug and pathogen induced systemic metabolic alterations showing strong associations with important immunological markers.

This new paradigm shift is supported by the identification of the "metabolic bridge" between the host and its internal microbiota [3,4], functioning via a comparable mechanism as introduced above: The microbiota, defined as the diverse microbial life populating the gut and intestines, has evolved an enduring mutualistic partnership with its human host. The importance of the microbiota as a link in immune homeostasis and determining host susceptibility to many immune-mediated diseases and disorders has identified a critical metabolic link. Secreted microbial metabolites including short chain fatty acids (acetic acid, butyric acid and propionic acid) and polyamines (putrescine and spermidine) have been identified as a complex communication network mediating responses within the host. From this vantage point it becomes more comprehensible for all somatic cells to participate in immunological responses during homeostasis, as well as upon insult such as contact with a pathogen inducing systemic metabolic dysregulation. Using an immunometabolism approach in investigating diseases on the cellular and systemic metabolic level integrated with immunological studies, could lead to more robust and holistic disease phenotyping. Insights into disease phenotypes will improve therapeutic approaches targeting both the host metabolic and immunological response.

In conclusion, as illustrated in this thesis, metabolomics methodologies were developed to measure important signalling metabolites in a wide variety of biological matrixes. Subsequently with these and earlier established metabolomics platforms the host-virus interaction on the systemic metabolic level was characterised. The results have revealed the opportunistic nature of viruses in exploiting host metabolism to ensure their own survival. Concurrently, as the host is not a passive bystander, host metabolic pathways attempting to correct these viral induced imbalances were found.

Future perspectives

The emerging field of immunometabolism offers many opportunities and will need the collaboration between immunologist, biochemist, endocrinologist, analytical chemist, computational modellers, and molecular biologist to each bring their area of expertise in studying the "bigger picture". Future questions to ponder over include: Does cellular metabolism contain a memory response after an insult, with potential priming consequences upon future exposure? What is the role and influence of cellular and systemic metabolism during autoimmune disease? Do systemic metabolic factors play a role during vaccination and determining its efficacy? Are metabolic pathways possible targets to combat viral infections through modulating the host response? What are the underlying mechanisms to the different responses of a host to a viral infection?

In future, metabolomics technologies should be able to provide cost effective, comprehensive metabolic coverage ensuring quantitative, accurate and sensitive metabolic readouts through innovative combination of sample preparation, chromatography, mass spectrometry and nuclear magnetic resonance spectroscopy. Optimising and developing micro-sampling procedures would enable us to accurately sample locally (within harvested tissue samples) as well as systemically (blood/urine/CSF), for improved metabolic disease characterisation and inter-compartment metabolic flux modelling. The design of tracer based studies in both metabolomics and proteomics could reveal the causative link between metabolic dysregulation and immunological activation. Whole body wide tracer based studies could also be used to explore the consequences and fate of a localised metabolic perturbation (characterised by a tracer) on the systemic factors and organs. Concurrently, the role of the metabolism during disease needs to be prioritised and scrutinised for its ability to influence pathophysiological processes, through re-evaluating current available literature dating back till the early 20th century, and studying diseases from a immunometabolic perspective. Studying the nuances of the metabolism integrated with their immunological markers within a large diverse patient population will help us in identifying immunometabolic disease phenotypes aiding us in stratifying patients for optimal therapeutic intervention. With the emphasis on personalised medicine, a deeper understanding of metabolic signalling pathways is required to improve the ability to characterise diseases as well as the immunological consequences, while also identifying possible metabolic therapeutic targets.

References

1. Lochner, M., Berod, L. & Sparwasser, T. Fatty acid metabolism in the regulation of T cell function. *Trends Immunol.* **36,** 81–91 (2015).
2. O'Neill, L. A. J. & Pearce, E. J. Immunometabolism governs dendritic cell and macrophage function. *J. Exp. Med.* **213,** 15–23 (2016).

3. Rooks, M. G. & Garrett, W. S. Gut microbiota, metabolites and host immunity. *Nat. Rev. Immunol.* **16,** 341–52 (2016).
4. McPhee, J. B. & Schertzer, J. D. Immunometabolism of obesity and diabetes: microbiota link compartmentalized immunity in the gut to metabolic tissue inflammation. *Clin. Sci.* **129,** 1083–1096 (2015).

8