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

Citation

Version: Not Applicable (or Unknown)
License: Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden
Downloaded from: https://hdl.handle.net/1887/45223

Note: To cite this publication please use the final published version (if applicable).
The handle http://hdl.handle.net/1887/45223 holds various files of this Leiden University dissertation

**Author:** Schoeman, Johannes Cornelius  
**Title:** Virus-host metabolic interactions: using metabolomics to probe oxidative stress, inflammation and systemic immunity  
**Issue Date:** 2016-12-20
Chapter 1

Introduction
Chapter 1

Background

Viral infections can be considered the perpetual thorn in the flesh of human health with “new” viral strains appearing at regular onset, each presenting with its own unique set of challenges ranging from infectiousness, transmission routes, pathogenicity, therapeutic and vaccination strategies. These challenges, among several other were evident in the recent Zaire Ebolavirus (ZEBOV) outbreak causing mass hysteria, stigmatising, pain, suffering, death and inflicting severe economic losses in the affected populations. The ZEBOV epidemic exposed the global unpreparedness, inadequate host-pathogen interaction knowledge, lack of therapeutic targets, vaccine development bottlenecks and should not be seen as an isolated incidence.

The integrated nature of host metabolism, metabolic stress and immunology is emerging as an unexplored field in studying and understanding viral infections. Individually each has been extensively studied attempting to illuminate their respective roles in host-pathogen interactions. Metabolic studies have illustrated the metabolic engineering abilities of viruses, able to effectively exploit the host metabolism during its different life stages. The field of immunology is enhancing our understanding of viral induced innate and adaptive immune responses and simultaneously revealing the craftier aspect of viruses in their immune evasive mechanisms. Still, several questions remain unanswered including: i. is prior viral infection able to induce metabolic and immune priming affecting future infections, ii. what is the role of metabolism in vaccination responses, iii. do metabolic dysregulation and metabolic stress play a role during chronic immune activation, and lastly iv. are we able to identify metabolic therapeutic targets that are able to influence the host’s immune response? Advances in nutrient sensing research have revealed links between dysregulated host metabolic homeostasis, metabolic stress and the subsequent immune and inflammatory implications thereof. As small intra- and extracellular metabolites have the ability to shape the immune response, a critical paradigm shift is needed in how we approach a more holistic/systems biology approach in studying viral infections.

Viruses as metabolic engineers

A constant within the ever evolving viruses interacting with the host, is the human metabolism and the core role it plays in host-pathogen interactions and infection. The study of this complex interplay between host metabolism and viral exploitation has been gaining momentum in recent years as an emerging hot topic in the field of virology, cell and molecular biology and more recently immunology in understanding viral infections. Host cellular metabolism can be defined as the anabolic and catabolic small molecule related biochemical processes occurring within the living cells to maintain life. These biochemical processes deliver the metabolic resources required during viral infection, with the adaptability of viruses to optimally exploit these metabolic resources truly astounding. Cell and tissue specific host metabolism has also been identified as viral tropism determinants, highlighting the intimate nature of viral requirements in dictating the specific site of infection. For example, nucleotide pools within cells provide a metabolic distinction between dividing and quiescent cells. Non-dividing macrophages have very low nucleotide levels rendering them ineffective as viral host cells, except for human immunodeficiency virus-1 (HIV-1) which contains a reverse transcriptase with a much higher nucleotide affinity compared between the retrovirus family viruses. Increased energy demand, structural and
genetic biomass, and the production, organization and maturation of viral components are all requirements needed during the viral life cycle and often supplied by metabolic processes.

The central carbon metabolism, shown in Figure 1.1, channels carbon derived from glucose through glycolysis, the tricarboxylic acid cycle (TCA), and mitochondrial oxidative phosphorylation to form ATP. The central carbon metabolism is a primary target for viral hijacking together with interlinked metabolic pathways shown in Figure 1.1. The hijacking of glycolysis relates to its central role in i. ATP, NADH and NADH production \(^{11,12}\) (via the Warburg effect), ii. nucleotide and NADPH syntheses \(^{13,14}\) (via the pentose phosphate pathway), iii. glycosylation motifs \(^{15,16}\) (from carbohydrate and sugar synthesis), iv. lipid synthesis \(^{17,18}\) (via the glycerol-3-phosphate pathway) and lastly v. amino acid precursors required during the different viral life stages. Alterations in the glycolysis pathway reducing the carbon flux through the TCA cycle has highlighted the role of glutamine as a secondary carbon source, supplementing the TCA cycle via \(\alpha\)-ketoglutarate referred to as glutaminolysis \(^{19}\). Apart from its NADH/FADH\(_2\) synthesising role, the TCA cycle is also central in fatty acid biosynthesis via acetyl-CoA and malonyl-CoA. Fatty acid and lipid metabolism is another metabolic pathway integrated within the viral life cycle, playing important roles in viral assembly, organisation, maturation, structural envelope integrity, post translational protein modifications, lipid rafts as well as viral entry and shedding \(^{17,18}\).
The human immune response is a complex signalling cascade occurring during viral infection inducing metabolic stress which provides an interface for cellular stress and immune activation through the activation of the unfolded protein response. The interplay of the metabolism with human immunology is also a critical factor. Metabolic homeostasis is a fundamental requirement for cellular survival. Exciting research in the field of metabolic syndrome has an intimate relationship with the innate and adaptive immune response, including: determining disease severity and prognosis. The immune response is divided into the innate and adaptive immune responses for in the infected host's cellular metabolic responses for pathogens, priming the cells to alert the innate defence pathways to restore homeostasis and to reduce the cell's susceptibility to injury and/or altered metabolic pathways and then acts upon this dysregulation through activating among others the innate defence pathways to restore homeostasis and to reduce the cell's susceptibility to injury.

Even with the small compact viral genome, the array of hijacking mechanisms viruses have at their disposal to exploit host metabolic pathways are impressive. Hijacking at the protein level include the mimicry of host short linear motifs, while viral micro RNAs have been identified as the culprits in regulating host post transcriptional modifications. Viral hijacking effectively alters the host's cellular metabolic responses for its own needs, thus in essence forming a “virus factory”, consequently inducing metabolic stress in the infected cells. Metabolic stress is the physiological process whereby the cell senses either dysregulated nutrient levels and/or altered metabolic pathways and then acts upon this dysregulation through activating among others the innate defence pathways to restore homeostasis and to reduce the cell's susceptibility to injury.

Figure 1.1: Central carbon metabolism and viral needs. A simplified overview of central carbon metabolism and its interlinks with other metabolic pathways. The coloured blocks indicate the specific viral needs supplied by each specific metabolic pathway. This figure is an combined adaptation from.

Even with the small compact viral genome, the array of hijacking mechanisms viruses have at their disposal to exploit host metabolic pathways are impressive. Hijacking at the protein level include the mimicry of host short linear motifs, while viral micro RNAs have been identified as the culprits in regulating host post transcriptional modifications. Viral hijacking effectively alters the host’s cellular metabolic responses for its own needs, thus in essence forming a “virus factory”, consequently inducing metabolic stress in the infected cells. Metabolic stress is the physiological process whereby the cell senses either dysregulated nutrient levels and/or altered metabolic pathways and then acts upon this dysregulation through activating among others the innate defence pathways to restore homeostasis and to reduce the cell’s susceptibility to injury.

Figure 1.1: Central carbon metabolism and viral needs. A simplified overview of central carbon metabolism and its interlinks with other metabolic pathways. The coloured blocks indicate the specific viral needs supplied by each specific metabolic pathway. This figure is an combined adaptation from.
metabolic homeostasis is a fundamental requirement for cellular survival. Exciting research in the field
of metabolic syndrome diseases, especially type II diabetes and atherosclerosis initiated a paradigm shift in how
we integrate metabolic studies and the immunological consequences thereof. The sensing and regulation of
cellular nutrients levels has an intimate relationship with the endoplasmic reticulum (ER) which is the interface
between the highly conserved nutrient sensing pathways, maintaining metabolic homeostasis and innate
immune activation. Metabolic stressors disrupt ER homeostasis inducing ER stress characterised by the
accumulation of unfolded proteins. ER stress triggers a protective response in the ER, known as the unfolded
protein response which activates different stress-sensing pathways relaying the dysregulated homeostasis
message to the nucleus. An important implication of ER stress is the activation of nuclear factor-κB (NF-
κB) which translocates to the nucleus and initiates the activation of the innate immune response. NF-κB
regulates the expression of pro-inflammatory gene products including: TNFα, IL-1β, IL6 and COX-II.

Consequently, the metabolic hijacking occurring during viral infection induces cellular stress and
disrupted cellular homeostasis. The disrupted cellular homeostasis is sensed through the ER which provides an
interface for cellular stress and immune activation through the activation of the unfolded protein response.

The human immune response

The human immune response, consisting of the innate as well as adaptive response, in principal is
similar between individuals, nevertheless each individual has a unique immunological response to stimuli with
respect to duration and strength. Due to this the immune response has been identified as a critical factor in
determining disease severity and prognosis. Various factors have been identified as critical in influencing the
immune response, including: psychology, environment, diet, internal microbiota, concurrent infections or
diseases and age. The interplay of the metabolism with human immunology is also a critical factor as the
initiation and maintenance of immune activity is a metabolically costly process. The experienced metabolic
dysregulation, as evident during viral infections, could have detrimental effects in adequate host responses
influencing disease severity and prognosis. The immune response is divided into the innate and adaptive
immune response; we will focus primarily on the innate immune response.

The innate immune response

The innate immune response is the non-specific, first line of defence in the body, and it is responsible
for pathogen recognition (for example a virus) in the host and subsequent immune activation. The primary
function of the innate immune response is to alert the infected cell and surrounding cells of the invading
pathogen, priming the cells into defence mode and subsequently ensuring activation of a robust adaptive
immune response to follow. The cell-intrinsic innate immune response is a complex signalling cascade, with
intracellular sensors detecting pathogen associated molecular patterns (PAMPs) in infected cells. During acute
respiratory viral infection, airway epithelial cells will mediate this initial intrinsic innate immune response. The

innate defence pathways to restore homeostasis and to reduce the cell’s susceptibility to injury. As the host
is not a passive bystander, but a dynamic system with highly conserved, intrinsic metabolic sensing networks,
cell-intrinsic innate immune response is mediated via plasma membrane expressed Toll-like receptors (TLRs) and cytoplasmic sensors, including NOD-like receptors (NRLs), RIG-I-like receptors (RLRs) and cyclic GMP-AMP synthase (cGAS), which recognize the PAMPs (viral proteins and/or viral DNA/RNA). Pathogen recognition leads to the activation of an array of transcription factors among the most important being NF-κB, interferon regulatory factors (IRF) and activator protein 1 (AP-1). These transcription factors regulate an important immunological process and activate pro-inflammatory cytokines (IL-1, TNFα, IL-12, IL-6, interferons(IFNs)), chemokines (MCP-1, RANTES, IP-10), signalling lipid mediators (eicosanoids, lysosphingolipids), proteases, reactive oxygen species and co-stimulatory molecules. Important physiological processes resulting from innate immune activation are inflammation and oxidative stress which will be briefly reviewed below.

**Inflammation and the innate immune response**

Inflammation of the infected area is a direct consequence of the innate immune response, via the activation of NF-κB and IRFs (among others) which are responsible for the release of pro-inflammatory cytokines creating a pro-inflammatory milieu at the site of infection. The classical clinical signs of inflammation include: swelling, redness, pain, fever and a loss of function of the affected area. The induced inflammation leads to the development of the acute phase response (APR) which is an early complex reaction of the host defence system against various stimuli, such as viral infection. Physiologically inflammation (the APR) is responsible for: i. protecting the host from extensive injury caused by the pathogen and facilitating in its elimination, ii. promoting tissue repair, iii. initiating the adaptive immune response, and lastly iv. establishing an immunological memory, enabling faster future response upon exposure. During host control and regulated immunological responses, acute inflammation is resolved between 4 to 7 days after infection and results in minimal tissue damage. In the case of immunological dysregulation or pathogen mediated pathology, acute inflammation can be left unresolved resulting in chronic inflammation leading to considerable tissue damage.

Inflammation has also been identified to interplay with host metabolism, especially influencing the host lipid metabolism and β-oxidation. During the early stages of an infection plasma triglycerides (TG) levels increase due to increased VLDL secretion as a result of adipose tissue lipolysis. A variety of factors and cytokines have been identified as being responsible for the increased VLDL levels. Fatty acid β-oxidation is also suppressed due to down regulation of hepatic carnitine palmitoyltransferase (CPT)-I and CPT-II, the rate limiting enzymes during β-oxidation. Concurrently with these lipid changes, phospholipase enzymes are upregulated and mediate the release of free poly unsaturated fatty acids such as arachidonic acid (AA), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) from the membrane bound phospholipids. Arachidonic acid is enzymatically oxidised to form pro-inflammatory signalling lipids called the prostaglandins (PGs) and leukotrienes (LTs), governing early host defence response on the metabolic level introduced in section 4.1.

Furthermore, lipid signalling mediators have also been implicated in the resolution of inflammation. Resolution involves various cellular and tissue processes, including apoptosis, phagocytosis,
cytokine/chemokine profiles and their scavenging mechanisms, as well as lymphatic drainage. The classes of lipid mediators important in resolution include: lipoxins, resolvens, protectins and maresins derived mainly from w-3 essential fatty acids. These specialised fatty acids have potent anti-inflammatory properties, and are only synthesised temporarily in cells in response to the resolution of inflammation. They have a direct impact on leukocyte migration, macrophage clearance mechanisms which disproves the old beliefs that inflammation resolution is a passive process.

**Redox biology, oxidative stress and the innate immune response**

Cellular redox biology concerns signalling transduced via oxidation and reduction reactions catalysed through the actions of hydrogen peroxide (H$_2$O$_2$). Healthy living cells contain low levels of oxidants such as H$_2$O$_2$, enzymatically derived from oxygen, which is necessary for normal redox controlled physiological processes like cell division and for killing micro-organisms. These cellular oxidant levels are well regulated by the cellular antioxidant capacity, mediated primarily by glutathione and antioxidant enzymes: catalyses and peroxiredoxins. Oxidative stress is a condition characterised with uncontrollably high levels of reactive oxygen species (ROS) derived from H$_2$O$_2$, leading to macromolecular damage.

In the case of a viral infection, pathogen recognition receptors utilise H$_2$O$_2$, among other signalling pathways, derived from the mitochondria to activate downstream signalling cascades initiating the innate immune response and subsequent inflammation. These H$_2$O$_2$ mediated signalling pathways reveal the interlinked nature of redox biology, innate immune activation, inflammation and oxidative stress (Figure 1.2). It has been shown that viruses have the ability to modulate the cellular redox capacity for its own selfish needs, inadvertently leading to high levels of oxidative stress during infection. The cellular membrane bound glycerophospholipids are reservoirs for unsaturated fatty acids, vulnerable to intracellular free radical attacks during conditions of oxidative stress. Oxidation of these unsaturated fatty acids affects and impairs membrane integrity and function, leading to dysregulated homeostasis and cell stress. Isoprostanes are stable lipid peroxidation markers, with their readout able to inform about the experienced oxidative stress in vitro and in vivo. Similarly, nitrosylated lipids (NO$_2$-FAs) synthesised via reactive nitrogen species (RNS) can also be formed within the cell.

Understanding the intricate (cause and effect) relationship between oxidative stress and inflammation is a hot research topic, as elucidating these mechanisms will improve our understanding of disease pathology and therapeutic approaches. Only recently has this intricate relationship been approached from a metabolic perspective with bioactive signalling lipids playing an integral role within.
Figure 1.2: Redox biology, innate immune activation and oxidative stress. Pathogen recognition receptors sensing PAMPs/DAMPs uses amongst other pathways hydrogen peroxide (H$_2$O$_2$) to facilitate immunological signalling. NADPH oxidases and the mitochondria contribute to the production of superoxide resulting in H$_2$O$_2$ via the actions of superoxide dismutase. Redox biology involves the H$_2$O$_2$ catalysed hydroxylation and activation of protein motifs signalling in the innate immune cascade. Oxidative stress is the macromolecular damage in the cell. Blue block processes occur in a regulated fashion whereas red block processes are the result of uncontrollable levels of H$_2$O$_2$. PAMPs – Pathogen associated molecular patterns; DAMPs – Damage associated molecular patterns; TLR – Toll-like receptor; NLR – NOD-like receptors; RLR – RIG-I-like receptors; NOX – NADPH oxidases; SOD – Superoxide dismutase; H$_2$O$_2$ – Hydrogen peroxide; PRx - Peroxiredoxin; GPx – Glutathione peroxidase; CAT – Catalyses; ROS – Reactive oxygen species; RNS – Reactive nitrogen species; S – Cysteine residue.
Signalling lipid mediators and their role in host immunity

Underlying host immunity is a cascade of signalling mediators governing the associated physiological processes such as inflammation and oxidative stress. These signalling mediators are responsible for ensuring intracellular host defence activation, signalling to nearby cells alerting them of imminent danger and furthermore signalling for the activation of the adaptive immune response. Immunological studies mostly focus on: i. characterising the classical immune signalling pathways, ii. investigating the functions and interaction of cytokines and chemokines with their respective receptors and targets, while simultaneously trying to iii. identify new pathways and targets. Cytokines and chemokines are de novo synthesised proteins resulting from immune activation, orchestrating broad immunological responses. They have also been extensively studied and reviewed based on their structure and function within the (innate) immune response. Cytokines and chemokines are considered as the principal regulators of the immune response, but recently the importance of metabolism and lipid signalling mediators has been discovered to play an active role in also modulating the systemic and cellular immune responses.

Lipid signalling refers to a signalling event where a specialised lipid mediator binds to a protein target, such as a receptor, kinase or phosphatase, which in turn activates their signalling cascade, eliciting a specific cellular response. Specialised signalling lipid mediators interact with cellular and membrane receptors activating discrete signalling pathways, controlling cellular processes including: cell proliferation, metabolism, chemotaxis, differentiation, apoptosis, as well as stress and inflammatory responses. Just like cytokines and chemokines, lipid mediators are de novo biosynthesised where needed and act locally in the affected area. The synthesis, regulation, functions, transport, accurate measurements and subsequent biological interpretation of these lipid mediators gives way to new areas of research supporting the need for interdisciplinary metabolic and immunological studies. Below is an overview of four different signalling lipid classes including: prostaglandins, lyso-sphingolipids, lysophosphatidic acids, and nitro-fatty acids and their reported functions during viral infections.

Prostaglandins

The prostaglandins are a family of enzymatically oxidised bioactive lipids known for their prominent role in initiating and maintaining inflammation and lesser role in resolving inflammation. Lately, prostaglandins have also been implicated as important signalling lipid mediators in immunomodulation. Activation of the innate immune responses upon the recognition of pathogens via the pattern recognition receptor stimulates the release and translocation of NF-κB to the nucleus. Both cytosolic phospholipase A2 (cPLA2) and cyclooxygenase-II (COX-II) are gene products of NF-κB and pivotal in prostaglandin synthesis. The synthesised cPLA2 migrates to the cellular membrane via a Ca^2+ influx induced gradient, and subsequently catalysing the release of poly unsaturated fatty acids (PUFAs) like arachidonic acid (AA, C20:4 ω6) from the phospholipid bilayer. These free PUFAs are the substrate for COX-II oxidation, initiating the start of prostaglandin synthesis. Cellular prostaglandin secretion is mediated through the multidrug-resistance-associated protein 4 (MRP4), while absorption is done via the prostaglandin transporter (PGT) a family member of the organic anion transporters. Due to the prostaglandin signalling capacity they are short lived in
Chapter 1

circulation, metabolised either enzymatically 69 or via dehydration reactions 70,71 and excreted in the urine and faeces. The prostaglandin E2 (PGE2) is the most extensively studied family member followed by PGF2α and PGD2, while eliciting their biological functions via prostaglandin specific dedicated g-coupled cellular receptors 72.

Prostaglandins have also been identified as important determinants during viral infection, with prostaglandins responses unique to different viruses. Prostaglandins elicit anti-viral activity, influencing the viral life cycle and inhibiting replication of a number of viruses, including retroviruses, poxviruses, and some herpesviruses 73,74. However, some viruses are able to exploit the host prostaglandin network, with both Influenza A virus and human cytomegalic virus (HCMV) able to induce and upregulate the expression of COX-II 75,76. Influenza A virus upregulates PGE2 during infection, and exploits its immunomodulation properties which down-regulate interferon production thus hampering antigen presentation and T cell-mediated immunity 75. With the growing body of literature supporting the role of prostaglandin metabolism and signalling during health and disease, their specific contributions to viral infection and host immunity are only just emerging. When also considering the safety and efficacy of approved nonsteroidal anti-inflammatory drugs, targeting the prostaglandin biosynthesis pathway is promising as affordable and attractive therapeutic intervention strategies are in principle available.

Lyso-sphingolipids

The sphingolipid class of lipids contain the unique amino-alcohol acyl backbone and comprises the ceramides, sphingomyelins, numerous glycosphingolipids, and the bioactive lyso-sphingolipids. The lyso-sphingolipids include the single acyl moiety sphingosine and sphingosine-1-phosphate (S1P) the latter which elicits important biological functions. Sphingosine is formed via the action of alkaline ceramidase 1 on membrane bound ceramide and is an important facilitator in sphingolipid homeostasis. Phosphorylation of sphingosine by either of the two sphingosine kinases: i) SphK1 - at the cell membrane and ii) SphK2 - in the mitochondria, ER and nucleus, produces S1P. The locally synthesised intracellular S1P is transported out of the cell via the S1P transporter (SPNS2) where it can interact with five dedicated g-coupled cellular receptors - S1PR1,5 77-79. S1P is emerging as an important signalling lipid mediator that regulates cell growth, survival, immune cell trafficking, as well as vascular and epithelial barrier integrity. S1P has a rapid turnover rate and its levels are tightly regulated by the balance between SphK1/2 synthesis and phosphatase degradation in circulation and tissues. The many diverse roles of S1P in innate and adaptive immunity include: immune-surveillance, immune cell trafficking and differentiation, immune responses and endothelial barrier integrity all being mediated by its dedicated cellular receptors 78,79. S1P stimulates the differentiation of many types of immune cells, inducing changes in their functional phenotypes and regulating production of pro-inflammatory cytokines and eicosanoids. As S1P is important in regulating the vascular endothelial barrier it also plays an important role in controlling the trafficking of leukocytes into and out of the circulation.

Furthermore, the role of S1P during viral infection is slowly being elucidated, with reports on hepatitis C virus (HCV), HCMV, and West Nile virus targeting sphingolipid metabolism during infection 80-82. During Measles virus (MV) infection SphK1 is an important viral replication determinant. Overexpression of SphK1
enhances MV replication, whereas inhibition of SphK1 impairs viral protein expression and progeny production via inhibition of NF-κB activation. S1P has also been flagged as a marker of disease severity during Dengue virus infection, with reduced circulating levels of S1P associated with plasma leakage into the circulating tissues. Utilisation of sphingolipid metabolism during viral infection has the potential to aid infection through upregulating cell proliferation and survival pathways, while also providing essential lipid components for replication compartments and assembly.

**Lysophosphatidic acids**

Lysophosphatidic acids (LPAs) are the simplest glycerophospholipids consisting of an acyl chain esterified to a glycerol backbone, and are the building blocks for the glycerophospholipids and triglycerides. Since the LPAs are intermediate metabolites in the synthesis of more complex structural lipid metabolism, their role as signalling lipids can easily be overlooked. Alternative LPA synthesis is dependent on the catabolism of more complex glycerophospholipids and lysophospholipids via the enzyme phospholipase D also known as autotaxin. LPA mediates its potent signalling ability via six different G-protein-coupled receptors (LPAR1-6) expressed on the cellular surface. LPAs are potent mitogens inducing cell proliferation, while their other functions include: cell differentiation, platelet aggregation, cell to cell interactions and tumorigenesis.

Currently the role of LPAs during viral infection has received very little attention compared to cancer research where LPA is a robust biomarker for tumorigenesis, stimulating cell division and metastases. It is thus comprehensible to imagine an important role for LPA during viral infection, as viral replication is dependent on host machinery used during cell division. Patients suffering from a chronic hepatitis C infection have shown to have higher serum LPA and autotaxin levels, providing a possible link between chronic HVC infection and the development of hepatocellular carcinoma. Since parallels have been drawn between cancer and viral metabolic perturbations the area of lysophosphatidic acid research during viral infection is a burgeoning field.

**Nitro-fatty acids**

Nitro fatty acids (NO2-FAs) are the resulting products of the reactive nitrogen species (RNS) catalysed nitration of free our bound poly unsaturated fatty acids (PUFAs), and can be used as a readout for nitrosative stress. Nitrosative stress is a condition interlinked with inflammation, characterised with elevated levels of nitric oxide synthase (NOS) activity resulting in increased NO radical levels. The reaction between superoxide (O2·−) with NO forms peroxynitrite (ONOO−) which decomposes to form nitrogen dioxide (NO2) radicals. These NO2 radicals are able to nitrate the double bonds present in membrane bound phospholipids or free PUFAs catalysing the de novo synthesis of NO2-FAs. A couple of NO2-FAs have been detected and studied in serum/plasma, urine and cell culture including: nitro-arachidonic acid (NO2-AA), nitro-linoleic acid (NO2-LA) and nitro-oleic acid (NO2-OA). Subsequent transport, metabolism and primary excretion metabolites still needs to be elucidated for the NO2-FAs.

The NO2-FAs has interesting functions opposing their synthesis route in a negative feedback loop and include: anti-inflammatory actions, activation of the peroxisome proliferator-activated receptor (PPAR-γ).
inhibition of chemokine and cytokine release, relaxation of smooth muscle cells, and the inhibition of NF-κB gene expression, inhibition of prostaglandin synthesis, and NADPH oxidase inhibition together with upregulation of anti-oxidant enzymes. Currently there is no reported literature on the role of NO2-FAs during viral infections. Nitrosative stress is closely linked to oxidative stress and inflammation and the immune response. Thus, studying these signalling lipids with regards to viral infections presents an unexplored route in understanding these physiological responses during viral infection.

**Metabolism and metabolomics**

The host metabolism covers a diverse set of compound classes acting in concert via integrated biochemical reactions essential to support life as we know it. Throughout this introduction we have tried to illustrate the hijacking abilities that viruses possess to alter and re-direct host metabolism to support the viral life cycle: nucleotides are necessary for viral replication, amino acids are needed during viral protein synthesis, lipids play an integral role in viral assembly, maturation, and shedding, all while central carbon metabolism has to provide cellular energy in the form of ATP. Furthermore, we introduced special bioactive lipid mediators, integral in shaping the immunological response during viral infection, and how viruses have been shown to exploit some of their functionalities. All these different metabolite classes are quite diverse in their chemical properties (functional groups), concentrations, and chemical stability which complicates the comprehensive and reliable measurement of metabolites. Metabolomics is a continuously improving technology which aims to measure all these metabolites enabling us to elucidated the functionality and dynamics of metabolite pathways and networks from a systems biology point of view.

Metabolomics, a multidisciplinary approach integrating chemistry, biology and statistics, aims to provide a readout for small metabolites functioning within a dynamic system under homeostatic constraint. Using metabolomics to investigate health and disease aids us in elucidating pathogenic metabolism related to the phenotype of the disease. Subsequently, these studies contribute to finding biomarkers useful in: diagnosis, predicting disease severity, predicting and measuring therapeutic intervention and success thereof, identifying therapeutic targets, and even risk stratification. Continuous accurate and comprehensive metabolic measurements in a personalized medicine setting will enable us to differentiate between homeostasis, allostasis and disease, equipping us with the tools for early detection of health perturbations. As mentioned above, comprehensive human metabolic studies are hampered by the sheer diversity of the human metabolome, thus requiring us to develop tailor-made metabolomics approaches targeting specific compound classes to achieve robust metabolic readouts and which collectively can provide a comprehensive readout.

Targeted metabolomics focuses on a predefined set of metabolites covering specific metabolic pathways, and analyses these using optimized sample preparation methods in tandem with cutting-edge analytical technology. For example, measuring the above introduced signalling lipids the sensitivity provided by targeted metabolomics is ideal as these metabolites are usually found at much lower levels compared to structural and energy orientated metabolites. In addition, these metabolites require specific sample preparation to, e.g., prevent suppression of their ionization by other lipids present at much higher concentrations. When prior biological knowledge is available targeted metabolomics enables us to target specific pathways and to
study their response within the biological question. Since 2006 the use of metabolomics in viral infection related studies has been utilised to create a deeper understanding of the virus-host interaction on the metabolic level. Furthermore, the recent appreciation for the interlinked nature of host metabolism and their immunological responses will position metabolomics as an ideal tool to study this association through integrative systems biology approaches.

**Outline of this thesis**

With the assault of new viral strains threatening the human population on a continuous basis, such as in the case of the 2016 Zika virus outbreak, more needs to be done to adequately prepare ourselves. The complex biochemistry and molecular biology which governs the interactions of viruses and hosts on the metabolic level has until present not received the attention it deserves, as the host's metabolism has been overlooked until recently as a possible disease determinant. The paradigm shift of host metabolism shaping and modulating immune responses have created the need for integration between the fields of immunology and metabolism, but also creates new opportunities to fight viral threats.

In this thesis, after an introduction into the field and state-of-the-art of viral infection and metabolism research, the first research part of the thesis deals with metabolomics method development. Chapter 2 contains the development of a method able to measure oxidative stress, nitrosative stress and inflammatory mediators using bio-fluids as well as tissue samples. This targeted metabolomics platform can analyse different lipid classes including: isoprostanes, prostaglandins, nitro-fatty acids, lysosphingolipids and lysophosphatidic acids metabolites. The method uses a liquid-liquid extraction sample-preparation followed by liquid chromatography and a mass spectrometric analysis.

Chapter 3 entails the development of a β-glucuronides hydrolysis method for measuring oxidised lipids within urine as a biological matrix. Urine is a non-invasive sample material containing the excreted metabolic compounds reflective of the system as a whole. Three different commercially available enzymes derived from *H. pomatia*, *E. coli* and bovine liver were tested in the development of a method to measure the total oxidised lipid profile.

The next part of the thesis focusses on *in vitro* based metabolomics work, using the A549 lung epithelial cell line and respiratory syncytial virus (RSV) as model system. In Chapter 4 the metabolic inflammatory response as induced by the innate immune response upon RSV infection was evaluated. The experimental design consisted of a three-day time course RSV infection of A549 cells. Metabolomics was used to elucidate the cellular oxylipin profile generated in response to RSV infection. The oxylipins are specialised signalling lipid mediators, playing an active role in the initiation, maintenance and resolution of inflammation. Complementary to this, a targeted transcriptomic approach focusing on the genes encoding for the oxylipin related enzymes and receptors was also carried out and an integrative data analysis approach developed.

In Chapter 5 we seek to provide novel insights into the perturbed redox biology during an A549 *in vitro* RSV infection time course study. Through the use of metabolomics and targeted transcriptomics we explore the metabolic host-RSV relationship relating to redox biology, cellular anti-oxidant capacity and oxidative stress.
Chapter 1

Finally, this thesis deals with two patient based metabolomics studies. Chapter 6 aims to characterise the altered metabolic state during the progression over the four clinical phases of a natural chronic Hepatitis B infection. Targeted metabolomics platforms were used to characterise the phospholipid, triglyceride, sphingomyelin, amino acid, acyl-carnitine, and signalling lipid profiles over the progressive nature of chronic hepatitis B, and to identify potential diagnostic disease severity markers and potential novel treatment options.

Chapter 7 entails a study of the vulnerable growing population of in utero exposed HIV and combination-antiretroviral therapy (cART) infants. Targeted metabolomics platforms were used to measure the metabolite profile in cord blood obtained from infants who were exposed in utero to cART and born to HIV-infected women and from control infants born to healthy women. To determine the interaction between metabolites and immune responses we also quantified levels of classical cytokines and chemokines in the same cord blood samples and integrated the data with those generated by the metabolomics platforms.

The thesis concludes in Chapter 8 with general conclusions and perspectives of the reported research. A critical evaluation of the research within the thesis is presented together with a discussion about the future implications and directions of the field of metabolomics to study immunological consequences related to viral infections.

References

15. Bryant, J. E. et al. Glycosylation of the dengue 2 virus E protein at N67 is critical for virus growth in


Chapter 1

50. Spiteller, G. Are lipid peroxidation processes induced by changes in the cell wall structure and how are these processes connected with diseases? Med. Hypothesis 60, 69–83 (2003).
93. Tsikas, D., Zoerner, A. a & Jordan, J. Oxidized and nitrated oleic acid in biological systems: analysis


Development and application of a UPLC-MS/MS metabolomics-based comprehensive systemic and tissue specific screening method for inflammatory, oxidative and nitrosative stress

Johannes C. Schoeman, Amy C. Harms, M. van Weeghel, Rob J. Vreeken, Ruud Berger, and Thomas Hankemeier. Manuscript submitted for publication