Metabolomics and Lipidomics applications in the context of immune and cancer cells metabolism

Alarcon-Barrera, J.C.

APPENDICES

Summary

This thesis exemplifies the application of metabolomics as a tool for the scientific exploration of molecules aimed at unravelling molecular mechanisms involved in cellular processes shaping biological phenotypes. Such approaches are already enabling us to identify molecular targets tailored to clinical applications serving as diagnostic markers as well as the identification of novel patho-physiologies and disease relevant mechanisms. The thesis is divided into five research chapters (chapters 2-6) in which different analytical technologies were used to explore metabolic alterations, i.e., liquid chromatography mass spectrometry (LC-MS), differential mobility spectroscopy (DMS) and nuclear magnetic resonance spectroscopy (NMR). To explore the applicability of metabolomics analysis as an important tool in patho-physiological research, I applied these different technologies in diverse experimental frameworks, as for example different *in vitro* and *in vivo* models, intervention studies, and the combination of multi-omics data.

Chapter 2 presents an overview of the current state of metabolomics analysis applied in drug development and discovery. The review starts with an introductory section in which I explain basic metabolomics concepts together with a brief description of the commonly used technologies and strategies in the field. The section ends with an update of the currently used methodologic strategies, such as flux analysis or the combination of multiple omics approaches. The main body of the chapter is divided in two sections. In the first section I describe the different steps of drug development and investigate in which parts metabolomics analysis already stands out as an important analytical method. My literature search led me to the conclusion that metabolomics is becoming increasingly used for the investigation of a drug candidates' mode of action, the analysis of target engagement markers as well as possible adverse effects. The second section introduces novel, future areas in which metabolomics plays an increasingly important role, i.e., pharmaco-metabolomics and drug repurposing. Both fields rely on the fact that the metabolome is the closest link to the biological phenotype, therefore, using metabolomics analysis is a crucial approach to sketch an accurate picture of the molecular effects of a new chemical entity and the potential repurposing of registered drugs including the identification of novel targets. In summary, a detailed metabolic understanding of biological phenotypes holds great promise for a refined drug development and repurposing process.

In chapter 3, I explored the correlation between cellular phenotypes and their respective lipid composition (lipidome) using a quantitative lipidomics analysis

platform called "Lipidyzer". In the first part of the chapter, the platform is analytically validated, i.e., linearity and repeatability were assessed. The platform proved to be highly robust as most of the lipid classes showed a coefficient of variation below 15% and a good linearity with regression coefficients above 0.95. Subsequently I analysed the lipidome of three different immune cell subpopulations, lymphocytes, monocytes, and neutrophils. I first compared the lipidome of these cells in the unstimulated state concluding that the lipidome of monocytes and lymphocytes showed to be comparable to each other. On other hand, the lipidome of neutrophils displayed a much higher triacyl glyceride content, making this cell population vastly different on the molecular level. These results were some of the first to show how specific immune cell phenotypes are accurately reflected in their lipid composition. Next, I investigated the possible differences of these cell population upon stimulation with either CD3/CD28 or calcium ionophore. The main differences were observed at the level of fatty acyl saturation in which lymphocytes were rendered mostly enriched in polyunsaturated fatty acyl chains, whereas monocytes showed enrichment in saturated short chain fatty acids. Interestingly, under my experimental conditions, neutrophils did not show significant changes in their lipidome upon stimulation. In summary, this chapter describes the *in vitro* lipidomics analysis of immune cells, the results derived from this analysis let us to correlate the lipid content of these cells with their phenotype and assess molecular lipid changes upon stimulation.

Chapters 4 and 5 both comprise collaborative work in which cell-based analysis and *in vivo* assays were integrated to gain a deep understanding of immune responses in the field of inflammation resolution and trained immunity.

In chapter 4 we aimed to investigate the role of the neuronal guidance protein, Semaphorin 7A (Sema7A), in the metabolic reprogramming of naive macrophages during severe inflammation. Genetic expression analysis showed that Sema7A was involved in M2 macrophage polarization, promoting a pro-resolving phenotype. To obtain a more detailed role of Sema7a, we constructed Sema7A-knockout macrophages and assessed their metabolism using the *Sea Horse* technology combined with NMR based metabolomics. We found a significant boost in glycolytic metabolism, showing increased production of lactate along with the accumulation of TCA cycle intermediates such as succinate, fumarate, and malate. Interestingly, itaconate, a metabolite typically related to M1 polarization, was also accumulated. Subsequently, protein microarray analysis let us to explore the cellular signalling pathways activated during Sema7A autocrine/paracrine stimulation. The results showed that the mTOR and AKT1 pathway were the main controllers of the

metabolic shift observed during M2 macrophage polarization. Finally, we aimed to investigate the role of lipid mediators in this process. For this I used a liquid chromatography-tandem mass spectrometry (LC-MS/MS) – based lipid mediator analysis platform. Our analysis revealed an increase of pro-resolving lipids such as LXA_4 and PDX during the Sema7A-modulated immune response. Finally, we studied plasma levels of Sema7A in a cohort of paediatric patients suffering from abdominal compartment syndrome. Interestingly, plasma levels of Sema7A showed good correlation with the overall health status of these critically ill patients and associated with the clinical outcome.

In chapter 5 we explored metabolic changes during the initiation of trained immunity. In this chapter we aimed to determine the role of lipid mediators in the induction of a trained immunity phenotype in monocytes. To investigate this process we used both, *in vitro* and *in vivo* models, accompanied by lipidomics and genetic analysis. The *in vitro* model using BG or BCG-stimulated monocytes showed increased levels of poly unsaturated fatty acids (PUFAs) and their derivatives that were primarily associated with the actions of lipoxygenases 12 and 15 (LOX-12/15), namely, 15-HETE, 5-HETE and 12-HETE (AA-pathway), 15-HEPE, 12-HEPE (EPA pathway), 17-HDHA, 14-HDHA (DHA pathway). Genetic analysis of BCG-trained macrophages showed that the enzyme fatty acyl desaturase (FADS 1/2) displayed differential expression in those cells along with an association of the FAD1/2 and the LOX-12 genes with cytokines such as TNFα, IL-6, and IL-1β. To validate these findings, we mainly used pharmacological approaches, inhibiting lipoxygenase enzymes, or directly stimulating cells with specific PUFAs and their derivatives. Our experiments revealed that lipid mediators were indeed involved in gene expression and cellular production of TNFα and IL-6 proving important for the trained immunity phenotype. In an *in vivo* setting, isolated monocytes from BCG-vaccinated donors were analyzed using the same lipidomics workflow. Remarkably, the LOX-12 products: 12-HETE, 12-HEPE, and 14-HDHA were increased after vaccination and thereby confirmed our previous in vitro findings. Taken together, this chapter highlights the potential role of lipid mediators, most specifically LOX-12 derived mediators, during initiation of a trained immunity phenotype and shows how lipidomics technology assists in obtaining detailed molecular insights.

In summary, chapter 4 and 5 are examples for the complementary and integrated role metabolomics analysis plays in interdisciplinary research. Both studies used metabolomics and lipidomics analysis to pave the way for a better understanding of immune mechanisms related to the actions of Sema7a and trained immunity.

The aim of the last chapter (chapter 6) of this thesis was to combine my knowledge on metabolomics and lipidomics, investigating a metabolic phenotype sustainably imprinted in cells. On this regard the tumor microenvironment, and hypoxic conditions, appeared as a good model to me. Aiming for a comprehensive analysis of a hypoxia imprinted metabolic phenotype, I set up a two-stage experiment investigating metabolic and lipidomic changes after long term (24 hours) and short term (1 hour) hypoxia/reoxygenation. HT29 cells were incubated in a hypoxic stove for a period of 24 hours whereafter cells were reoxygenated for 1 or 24 hours. Using a metabolomics/lipidomics workflow in which I combined the outputs from NMR based metabolomics and comprehensive lipidomics analysis I investigated metabolic alterations induced by this treatment. As expected, hypoxia induced a shift of cellular metabolism, increasing glucose uptake and lactate production, whereas most of the TCA cycles intermediates, along with NAD^+/NADH were reduced. While the overall lipid composition of these cells did not show significant changes, I did observe an interesting shift of the saturation index induced by hypoxia. Acute reoxygenation, curiously, was not sufficient to reverse all observed changes and mainly increased TCA intermediates and enrichment in monounsaturated fatty acids. After 24 hours of reoxygenation, however, the hypoxic metabolic shift was mostly reversed. Remarkably though, lactate levels remained increased even during long term reoxygenation as were the oxidized forms of betaine and glutathione pinpointing to an oxidative boost as a possible consequence of kick-starting TCA activity. Long term reoxygenation also caused decrease in the saturation index of triglyceride, diglyceride, phosphatidylcholine and cholesteryl ester lipids most probably by restoring lipid desaturases activity. Remarkably, sphingomyelin and ceramide lipids exhibited a divergent saturation index behavior which may suggest different metabolic and signaling regulation for sphingolipids. In summary, the results of this chapter show that metabolic changes induced by hypoxia take significant time for restoration and that metabolic phenotypes can become long term imprinted as shown by lactate levels that did not normalize even after 24 hours of reoxygenation.

Altogether, the chapters of this thesis show the application of metabolomics in different research settings, playing an important role in deciphering molecular mechanisms and the integration of interdisciplinary approaches. In each chapter metabolomics analysis proved instrumental to a better understanding of biochemical processes. Finally, it might be said, that indeed, the metabolome is the closest link to the phenotype and metabolomics is the method of choice comprehending this connection.