Dynamic system-wide mass spectrometry based metabolomics approach for a new Era in drug research

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Chapter 10

Summary and future perspectives

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Metabolomics is a very informative and powerful strategy to find phenotypic signatures in complex biological systems. In this thesis, the focus of this 'omics' platform was directed towards the lipidome analysis in cells and in living organisms. The challenge of the work described herein was to build a solid and robust quantitative and qualitative LC/MS platform that could deal with samples from different biological matrices such as whole plasma or serum, feces, tissues, and bile. Of particular interest for lipid profiling experiments was to conduct lipid analysis in the lipoprotein particles, this was accomplished by exploring the utilization of gradient gel electrophoresis in combination with LC/MS by excising the lipoprotein bands and extracting the lipids in each band to obtain a detailed data set for lipids in the VLDL, LDL and HDL fractions. All of the above were static measurements, which are measurements at a certain moment in time. Next, particular attention was also directed towards the kinetic measurements of lipids. This was successfully applied by the use of metabolic tracers which comprised the infusion of deuterated bile acid (D_4-CA) *in-vivo* to measure the reconjugation step of bile acids following silencing of specific genes involved in the reconjugation step of BAs (Slc27a5). Flux analysis was also investigated by the use of 'heavy water' analysis *in-vivo*. The main purpose was to develop and LC/MS platform that could be more informative than current GC/MS strategy and to obtain a better understanding of lipid trackfficking in an *in-vivo* setting. In order to establish a link between metabolic signatures and perturbations occurring at the mRNA level, gene expression was successfully utilized to provide a more detailed portrait for the coupling of the transcriptome and the metabolome.

In chapter 2, the development of the analytical LC/MS platform that was used throughout the thesis is described. The analytical strategy consisted of a high resolution LC/MS approach using the so called 'MS^E' (alternating low and high energy CID collisions from a single LC injection) which is fully described in chapter 2. This platform was able to obtain in an untargeted manner quantitative and qualitative lipid information from a single injection. The application of this platform allowed the measurement of the perturbation of lipids in osteoarthritis patients suffering from different degrees of severity.

Chapter 3 described the development and application of an ion mobility-TOF platform for the localization of fatty acyl positions and double bonds in PC and LPC. The novelty of this approach resided in the fact that the data was collected without the use of alkali metal adducts ions, which has extensively been used in the past as the only way in electrospray positive ion mode to obtain fatty acyl and double bond information in PCs. The developed method has the advantage to speed up the analysis and simplify the identification process via enhanced fragmentation in combination with accurate mass.

Chapter 4, an investigation in a preclinical dislypidemic golden Syrian hamster was utilized to measure the lipid composition of the lipoprotein particles followed by inhibition of CETP. The results from this investigation resulted in

higher HDL-c levels for animals treated with anacetrapib. Overall it was observed that the changes in HDL lipid composition, taken together with increased fecal sterol excretion and increased HDL cholesterol efflux capacity, may result in promoting cholesterol excretion in this particular animal model, with the hypothesis that this may have been achieved by modulation of reverse cholesterol transport.

Chapter 5 it was investigated whether inhibition of FATP5 (Slc27a5) could offer protection from liver steatosis induced by ApoB siRNA. This study was characterized by the utilization of two siRNAs specifically targeting *Fatp5* and *ApoB* , this study was a proof-of-concept for the combination of two siRNAs *in vivo* using a single siRNA-lipid nanoparticle (LNP) platform. The results from this chapter showed that *Fatp5* knockdown did not influence the size, composition, or zonal distribution of the hepatic triglyceride pool generated by *ApoB* siRNA treatment suggesting that fatty acids are not transported to the liver from dietary uptake or from a store such as adipocytes.

In Chapter 6, was a continuation of the previous chapter in which silencing of the Slc27a5 gene in mice was further explored to obtain a lipid phenotype and proof-of-concept regarding atherosclerosis and possible application to diet-induced obesity. Loss of function of the Slc27a5 gene resulted in altered bile acid (BA) conjugation and concentration in plasma and bile. In turn, this resulted in positive effects on lipid and lipoprotein profiles and diet-induced obesity.

The main aim of chapter 7 was to focus on the use of metabolic tracers. In this chapter an in-depth description was given on how it was possible to measure biomarkers *in vitro* and *in vivo* with the use of a metabolic tracer. The model investigated was the Slc27a5-cKD mice and the tracer D_4-Cholic acid was administered via an intravenous tail injection. It was found that these cKD mice had reduced D_4-conjugated BAs in the plasma as compared to WT mice. BA profiles from the bile showed that there were an increased number of unconjugated BA metabolites in the Slc27a5-cKD mice, specifically tetrahydroxy cholanoyl metabolites. Even though this metabolite of CA has been previously reported, the BA LC/MS method developed specifically for this application provided a greater number of tetrahydroxy cholanoyl metabolites and corresponding taurine conjugated tetrahydroxy cholanoyl metabolites. In summary, stable isotope metabolic tracers in combination with high resolution mass spectrometry provided valuable information about BA biology.

Chapter 8 described the utilization of 'heavy water' or D_2O for the measurement by LC/MS of cholesterol and cholesterol ester synthesis *in-vivo* using C57Bl/6 background mice. *De-novo* lipogenesis was markedly increased in the high carbohydrate diet with a concomitant increase in the expression of Scd1, which resulted in an increment in the enrichment of the deuterium label incorporated in the palmitate and stearate FA pools. Cholesterol synthesis was up-regulated in the high fat diet animal cohort. Without D_2O labeling this finding was not immediately obvious.

Chapter 9 combined a metabolomics and fluxomics approach to measure the static and kinetic effects on lipids on C57Bl/6 background mice as a result of dietary alterations using a high carbohydrate diet and a high fat diet. Lipid flux measurement was conducted by intra-peritoneal injection of D_2O followed by drinking water mixed with D_2O during the

course of the study. Isotopic ratios between detected M0 and M1 minus the natural background were used to gauge the kinetic lipid measurements. The data revealed that it was possible to extract lipid phenotypes based on static measurements with the use of multivariate statistical analysis, but a more comprehensive examination was achieved when this data output was coupled together with lipid flux measurements. A further advantage was that it was possible to measure individual synthesis rates for specific triglycerides and therefore tracking the kinetics of these lipids following dietary perturbations.

In conclusion, this thesis described and demonstrated the development of a comprehensive quantitative and qualitative high resolution LC/MS approach and its application to lipid profiling either in a single compartment or multiple compartment approach in different animal models to study treatment options for atherosclerosis. LC/MS as a front-line analytical platform provided very useful information. Where necessary, coupling with other platforms such as gradient gel electrophoresis, ion mobility, fast protein liquid chromatography and histology analysis offered complimentary data which facilitated the interpretation of the observations. The utilization of metabolic tracers and lipid flux analysis in this thesis has proven to be a very powerful analytical tool which can tease apart modulation of synthesis for specific metabolites involved in a single or multiple metabolic pathways/networks. These kinetic measurements are very complementary to single time points or static metabolite measurements. In addition to this, since most of the research conducted in the thesis targeted genes which were mainly expressed in the liver, mRNA measurements provided vital information which helped to guide the targeted metabolomic/lipidomic approach. Ensuring that perturbations were monitored not just at the transcriptome level but its translatability in the 'omics' cascade with metabolites as the end-points where decisions could be made. Finally, the tools developed in this thesis have already been successfully applied in decision-making in drug research. Therefore, the asset of metabolomics taken together with the other 'omics' will continue to play a pivotal role in systems biology as it will help to provide biological rationale and better understanding of lipid disorders in humans. This benefit offers the potential to develop new and novel therapies in drug research for atherosclerosis.