Exploration of the endocannabinoid system using metabolomics

Di, X.

Citation

Di, X. (2023, February 7). *Exploration of the endocannabinoid system using metabolomics*. Retrieved from https://hdl.handle.net/1887/3515754

Version:	Publisher's Version
License:	Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden
Downloaded from:	https://hdl.handle.net/1887/3515754

Note: To cite this publication please use the final published version (if applicable).

Chapter 7

Conclusions and perspectives

Conclusions and future perspectives

This section presents conclusions and suggestions for future improvements related to the content presented in each chapter. Furthermore, a more general perspective for optimizing and applying metabolomics-based workflows in drug development and clinical studies is discussed.

System pharmacology-based strategies, which analyze multiscale networks of disease dynamics and drug action, have shown great value in better understanding pathology and drug action mechanisms. Metabolism-centric methods are frequently used to build and analyze these networks, by connecting metabolic pathways with other omics layers related to the generation of metabolites and/or modulation of their concentrations. In this context, metabolomics-based methods are key to unravel the metabolic pathways involved in disease dynamics and drug action, which represent the foundations of these networks.

In this thesis, state-of-the-art metabolomics-based workflows were developed to enable the profiling of metabolites from a wide range of metabolic pathways related to the endocannabinoid system (ECS). The developed workflows were validated for application to a large diversity of biological matrices and used to explore the potential roles of ECS in cardiometabolic health, as well as the mechanisms underlying the positive effects of exercise for cardiometabolic health.

Chapter 2 describes the development of a liquid chromatography - mass spectrometry (LC-MS) worklfow, which enables the identification and quantitation of a large number of N-acyl-phosphatidylethanolamines (NAPEs), plasmalogen-NAPEs (pNAPEs), 2-lyso-N-acyl-phosphatidylethanolamines (lyso-NAPEs), glycerol-phospho-acylethanolamines (GP-NAEs), lyso-pNAPEs, N-acylethanolamines (NAEs), diacylglycerols (DAGs), 2-acyl-glycerols (2-AcGs), and free fatty acids (FFAs) in mice brain. Typically, due to their large difference in lipophilicity, these metabolites are measured using multiple runs, which is time consuming. The method described in Chapter 2 relies on the use of a ternary gradient during the chromatographic separation, which enables the resolution of all these metabolites and, in turn, accurate quantitation. Unlike traditional identification methods mostly guided by the fragmentation pattern of analytes, a novel approach based on the retention time (RT)

mapping was used for the identification of these metabolites. Using a limited number of (internal) standards, this strategy increased the confidence in the identification of metabolites with multiple fatty acid chains, such as NAPEs. The developed method was applied to the analysis of the eCB related metabolites in different biological matrices, such as mice brain lysates and neuron cell pellets.

Future perspective: with the method described in **Chapter 2**, the confidence in the identification of these metabolites has been improved. Nevertheless, additional work is required to further increase the confidence in identification. Higher confidence in the identification can be reached with the use of high resolution time-of-flight (TOF) or Orbitrap mass spectrometers, which isolate possible interferences from other classes of metabolites with the same molecular weight. Novel approaches can also be used to further increase the confidence, such as electron-associated dissociation (EAD), which is an alternative fragmentation technique and may help discriminating between sn-1 and sn-2 isomers by generating a fragment containing sn-1 fatty acid chain[1]. Another approach that may help increase the confidence in identification relies on the use of ion mobility mass spectrometry, which relies on a orthogonal separation mechanism and may allow for the differentiation of sn-1 and sn-2 isomers[2]. Finally, to reach the level 1 identification confidence defined by the Metabolomics Standards Initiative (MSI)[3], the specific reference standards should be synthesized for the NAPEs with biological interest. By comparing RTs and MS/MS fragmentation data with these reference standards, the analytes can be identified with best confidence.

A major aim in metabolomics is to develop methods providing the largest coverage of the metabolome. In case of volume-limited samples or when the endogenous concentration of metabolites is very low, highly sensitive methods are essential to achieve a sufficient metabolome coverage. In **Chapter 3**, a highly sensitive and selective micro-LC-MS/MS method was developed to quantify eCBs and eCB analogues in human cerebral spinal fluid (CSF). The developed and validated method was successfully applied to the analysis of hundreds of human CSF samples, in which the level of eCBs, especially the clinically important anandamide (AEA), was below the limits of detection typically observed with conventional LC-MS methods. The analyses were performed at a flow rate of 4 μL/min, which is ca. 100 times lower compared with conventional LC systems. Moreover, by

increasing the polarity of the injection solution, a higher volume of sample could be injected (i.e., 3 μL), which further increased the sensitivity, while retaining adequate peak shapes, thereby overcoming the drawback of limited injection volumes typically observed with micro-LC approaches. Altogether, these optimizations led to a 20-fold increase in sensitivity compared with conventional LC-MS systems, while the run time only increased by 4 min (25% longer compared with conventional LC).

Future steps: When increasing the polarity of the injection solution in the method showed in **Chapter 3**, the recovery of some metabolites was decreased, which represents a limitation of the current method. Online pre-concentration strategies represent an interesting strategy to further enhance the overall sensitivity of the method. An example of such strategies is on-column trapping, in which a relatively high volume of sample is injected into the trapping column first, then washed out with a smaller volume of mobile phase into the separation column. The second strategy is using an hanging droplet evaporator directly incorporated in the autosampler. In such set-up, the sample is slowly delivered into a metal needle and forms a droplet at the needle tip. Heated nitrogen is used to evaporate the injection solution in the droplet, which allows for a significant concentration of the sample into the droplet prior to injection. This set-up is currently at the development stage and not yet commercially available. These strategies are showing high potential in increasing the usability of micro-LC-MS systems. Overall, the hardware of the micro systems should be further improved to reach sufficient stability, reliability, and repeatability.

The developed and validated LC-MS workflows were used to investigate the correlation of plasma levels of endocannabinoids (eCBs), eCB analogues (**Chapter 4**) and oxylipins (**Chapter 5**) with body composition and cardiometabolic risk (CMR) factors. The possibility of using these metabolites as biomarkers for CMR was further investigated. In the studied cohort of young sedentary individuals, the plasma levels of the majority of eCBs and eCB analogues, as well as several omega-6 oxylipins, were positively correlated with adiposity and traditional CMR factors, and negatively correlated with brown adipose tissue (BAT) parameters. On the other hand, plasma levels of several omega-3 oxylipins were negatively correlated with adiposity and traditional CMR factors. Specifically, plasma levels of eCBs and their analogues were higher in metabolically unhealthy overweight-obese (MUOO) participants compared to metabolically healthy overweight-obese (MHOO)

participants. To improve the predictability of these metabolites, stepwise linear regression models were used, in which some of the eCBs, eCB analogues, and oxylipins improved the prediction of the variance of different body composition parameters and CMR factors. Therefore, this set of metabolites should be further investigated to evaluate their potential as early diagnostic markers of CMR in young adults.

Future steps: The clinical applicability of the biomarker candidates identified in the studies described in **Chapter 4** and **5**, and the pathways related to such candidates, should be further evaluated and replicated in larger cohorts, and in studies specifically investigating the development of cardiometabolic disease (CMD). Notably, additional longitudinal studies that last for decades are required to unveil the causality, i.e., whether individuals with a more active ECS signaling tend to develop higher visceral adipose tissue (VAT) depots, or whether VAT is more active compared with other adipose tissues (i.e., white adipose tissue) in terms of eCBs production, secretion, and release into the circulation. Genomics, transcriptomics, and proteomics data from related tissues, especially from VAT, should also be incorporated. Such strategy may help to explain the regulation of these metabolic pathways and build regulatory networks, which can help to get a better understanding of the development of CMD, the identification of diagnostic and prognostic markers, and the discovery of potential therapeutic targets.

Chapter 6 focused on identifying the mechanisms, at a molecular level, underlying the positive effects of exercise using different exercise modalities. Both acute endurance and resistance exercises increased plasma levels of eCBs, eCB analogues, and oxylipins, whereas long-term moderate-intensity, but not vigorously-intense exercise, decreased plasma levels of eCBs, eCB analogues, and AA-derived omega-6 oxylipins in young sedentary adults. These results suggest that these metabolites are potential mediators in the inflammatory response triggered during acute exercise[4–6]. Acute exercise induces muscle damages, followed by recovery after exercise[7]. The reduction of the baseline levels of eCBs, eCB analogues, and oxylipins after long-term moderate exercise suggests a reduction of systematic inflammation status, which is in accordance with previous studies on plasma levels of IL-6[8,9]. Together with the results described in **Chapters 4 and 5**, this might explain the beneficial effects of regular exercise on cardiometabolic health.

Future steps: the down-regulation of these metabolites may be explained by a down-regulation of enzymes synthesizing these metabolites, or an up-regulation of the hydrolyzing enzymes eliminating the metabolites. Multi-omics approaches are needed to further unravel the associated biomolecular mechanisms. In muscle and adipose tissue biopsies collected on study participants or using mice models, transcriptomics approaches (i.e., RNAseq) could be used to determine the expression of the upstream genes of these enzymes, as well as identify more related genes, while proteomics and activity based protein profiling (ABPP) may bring additional information on the expression and activity of these enzymes. Combining these multi-omics data will possibly help in understanding the mechanisms behind the reduction of the baseline levels of these metabolites observed in this study.

Future perspectives for optimizing and applying metabolomics-based workflows

In this thesis, state-of-the-art metabolomics-based workflows targeting metabolites related to the ECS were developed and applied to the explore the roles of ECS-related pathways in cardiometabolic health and exercise. The development and implementation of metabolomics-based methods are both associated with numerous challenges. This section, discusses these challenges and proposes possible solutions not only to improve the overall quality of metabolomics-based approaches, but also their successful application to drug discovery and (pre-)clinical studies. The suggested solutions include standardization of sample collection and handling steps, automation of sample preparation and data pre-processing, using metabolomics for drug safety and efficacy evaluation, as well as the integration with other omics.

Standardize pre-analytical sample collection and handling steps

The stability of metabolites in samples remains a major challenge during bioanalytical studies, as degradation of metabolites may lead to high variability within and between groups and wrong interpretation of the results. The stability of targeted metabolites during sample collection and handling process should thus always be assessed, and experimental conditions (e.g., storage temperature, time) be consistent. The pre-analytical conditions should be discussed with partners involved in a metabolomics study, including analytical

chemists, clinical chemists, nurses, biochemists, etc., while establishing the experimental design. To optimize pre-analytical conditions, available information from literature should be used in combination with pilot experiments, such as the type of anticoagulants, sample handling temperature, number of freeze-thaw cycles, etc. In this context, the following guidelines should be respected. Firstly, the samples should be aliquoted directly after the sample collection and prior to long-term storage in accurate and adequate volumes. For human plasma samples, a volume between 50-100 μL for each aliquot is typically adequate for metabolomics purposes. Pooled samples, i.e., samples that will be used as quality controls during the actual measurements (10% of all the study samples), should also be prepared during this initial aliquoting step. Furthermore, all samples should ideally be randomized and divided into batches during the initial aliquoting step to avoid an extra free-thaw cycle. This tedious randomizing step can for instance be automated using printed barcodes on the collection and aliquoting tubes, which also decreases the risks for manual errors with mislabeled tubes. By avoiding unnecessary procedures which are prone to errors, and are usually carried out at room temperature and/or lead to additional freeze-thaw cycles, sample and metabolites degradation can be limited.

Sample preparation and data pre-processing

Currently, the preparation of most type of samples, such as plasma and tissues, is often performed manually, which is time-consuming and prone to errors. Besides, exposure to relatively large amounts of organic solvents, specially the extractants during the liquid-liquid extraction (LLE), may lead to additional health risks for the operator. Therefore, automatic robots that integrate specially-designed pipettes, blenders, centrifuges and evaporators should be designed, allowing for a fully automated LLE or solid phase extraction (SPE) step. Such robots can significantly increase the efficiency and lower the variability introduced during sample preparation, which is key in large-scale metabolomics.

During data pre-processing, using current commercial or open source software, peak integration requires many additional manual steps to ensure adequate integration. This is poorly efficient and would highly benefit from being automized. Techniques such as artificial intelligence (AI) image recognition represent an attractive alternative to improve the quality of peak integration. In combination with automatic data quality check procedures,

the development of such approaches can significantly increase the throughput and improve data quality, especially in large-scale metabolomics studies. This is for instance relevant for some of the pathways studied in this thesis, where detected signals in LC-MS were often very low.

Application of metabolomics-based tools to drug discovery and development

The main applications of the developed metabolomics-based approach described in **Chapter 2** are the discovery and evaluation of potential biomarkers and drug targets, as well as early-stage evaluation of drug efficacy and safety. It is also envisioned that such metabolomics-based tools can be applied in multiple stages of drug discovery and development[10]. Firstly, during the high throughput screening (HTS) step, very high throughput MS-based metabolomics tools may be useful for the optimization of lead compounds[11]. During *in vitro* studies, methods covering the metabolic pathways that are modulated by the drug action can be used for the evaluation of target engagement and down-stream signaling. Moreover, high-coverage untargeted and/or targeted metabolomic methods can then already be used for the screening of possible off-target effects.

Next, an advantage of MS-based metabolomics in animal studies is that drug and drug metabolite concentrations can be measured simultaneously together with the metabolome in the same plasma or tissue sample, which increases efficiency, saves costs, and decreases the number of animals needed. This strategy is also applicable in clinical studies, as metabolites levels related to target engagement, down-stream signaling, and/or off-target effects can be measured in plasma samples from pharmacokinetic studies, which may provide additional information on the efficacy and safety of drugs.

During the preclinical and clinical stages of drug development, metabolomics can play an important role in the evaluation of off-target effects and safety. An example here is the BIA 10-2474 trial[12,13]. If the metabolism of lipids had been assessed using metabolomics during preclinical trials, off-target effects may have been observed before the dose escalation study. When using metabolomics for identification of off-target effects, the coverage of the method should be as wide as possible. Hydrophilic interaction chromatography (HILIC), which uses hydrophilic stationary phases with apolar eluents, could be a good option for achieving high

coverage of metabolites[14]. Since in HILIC, lipids are separated based on their headgroup, multiple metabolite groups can be quantified in one analysis[14]. Nevertheless, one single method is not sufficient to reach an exhaustive coverage of the metabolome, which exceeds 200,000 in total, a number that is still increasing[15]. Therefore, multiple separation methods, including HILIC, reversed-phase (RP) chromatography and supercritical fluid chromatography (SFC), should be combined to reach both desired coverage and confidence of identification. By combining these chromatographic approaches, a panel of metabolites belonging to key metabolic pathways could be identified and used in the clinic, similar to, for example, the liver function panel, a routine test for assessing risks of liver failure. A similar panel of metabolites related to the function of important organs/systems could be established, and a normal range for the concentration of these metabolites should be defined. If a drug causes out-of-range changes in the metabolites levels in the panel, then off-target effects that may cause severe side effects are likely and continuation to the next phase of development should only occur once this potential risk has been assessed and treated.

Integrate metabolomics data with other omics data

A limitation of the workflows described in the current thesis is the focus on metabolomics only. Similarly, most current studies only report one type of omics data, which limits the understanding of causal relationships and mechanisms behind pathophysiological processes and/or drug action[16,17]. Multi-omics approaches that integrate multi-layer omics data based on system thinking have emerged and been recognized to be useful[16–19]. Therefore, in future studies of the ECS, biopsies from specific tissues or animal models should be made available. In these samples, transcriptomics approaches can be used to determine the RNA expressing these enzymes and other regulators, while proteomics and ABPP can be used for the determination of the amount/activity of metabolic enzymes. With advanced data analysis techniques that combine these omics data with metabolomics data, a more complete ECS-related network of (patho)physiological interactions can be established, which allows for a better understanding of disease and drug action mechanisms, as well as the assessment of potential side-effects.

References

1. Campbell JL, Baba T. Near-complete structural characterization of phosphatidylcholines using electron impact excitation of ions from organics. *Anal Chem*. 2015;87(11):5837-5845. doi:10.1021/acs.analchem.5b01460
2. Wu Q, Wang JY, Han DQ, Yao ZP. Recent advances in differentiation of isomers by ion mobility mass spectrometry. *TrAC Trends in Analytical Chemistry*. 2020;124:115801. doi:10.1016/J.TRAC.2019.115801
3. Sumner LW, Amberg A, Barrett D, et al. Proposed minimum reporting standards for chemical analysis Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI). *Metabolomics*. 2007;3(3):211. doi:10.1007/S11306-007-0082-2
4. Raichlen DA, Foster AD, Gerdeman GL, Seillier A, Giuffrida A. Wired to run: exercise-induced endocannabinoid signaling in humans and cursorial mammals with implications for the “runner’s high.” *J Exp Biol*. 2012;215(Pt 8):1331-1336. doi:10.1242/JEB.063677
5. Raichlen DA, Foster AD, Seillier A, Giuffrida A, Gerdeman GL. Exercise-induced endocannabinoid signaling is modulated by intensity. *Eur J Appl Physiol*. 2013;113(4):869-875. doi:10.1007/S00421-012-2495-5
6. Signini ÉF, Nieman DC, Silva CD, Sakaguchi CA, Catai AM. Oxylipin response to acute and chronic exercise: A systematic review. *Metabolites*. 2020;10(6):1-18. doi:10.3390/METABO10060264
7. Markworth J, Maddipati K, Cameron-Smith D. Emerging roles of pro-resolving lipid mediators in immunological and adaptive responses to exercise-induced muscle injury. *undefined*. Published online 2016.
8. Cronin O, Keohane DM, Molloy MG, Shanahan F. The effect of exercise interventions on inflammatory biomarkers in healthy, physically inactive subjects: a systematic review. *QJM*. 2017;110(10):629-637. doi:10.1093/QJMED/HCX091
9. Nicklas BJ, Hsu FC, Brinkley TJ, et al. Exercise training and plasma C-reactive protein and interleukin-6 in elderly people. *J Am Geriatr Soc*. 2008;56(11):2045-2052. doi:10.1111/J.1532-5415.2008.01994.X
10. Corona G, Rizzolio F, Giordano A, Toffoli G. Pharmaco-metabolomics: An emerging “omics” tool for the personalization of anticancer treatments and identification of new valuable therapeutic targets The application of Nanotechnology in Oncology View project Tumor Markers View project. *Article in Journal of Cellular Physiology*. Published online 2012. doi:10.1002/jcp.24003
11. Guo Y, Forbush M, Covey TR, Ghislain L, Liu C. High-throughput analysis from complex matrices: acoustic ejection mass spectrometry from phase-separated fluid samples. *Metabolites*. 2021;11(11). doi:10.3390/METABO11110789
12. Kerbrat A, Ferré JC, Fillatre P, et al. Acute Neurologic Disorder from an inhibitor of fatty acid amide hydrolase. *N Engl J Med*. 2016;375(18):1717-1725. doi:10.1056/NEJMOA1604221
13. van Esbroeck ACM, Janssen APA, Cognetta AB, et al. Activity-based protein profiling reveals off-target proteins of the FAAH inhibitor BIA 10-2474. *Science*. 2017;356(6342):1084-1087. doi:10.1126/SCIENCE.AAF7497
14. Li A, Hines KM, Xu L. Lipidomics by hilic-ion mobility-mass spectrometry. *Methods Mol Biol*. 2020;2084:119. doi:10.1007/978-1-0716-0030-6_7
15. Wishart DS, Guo AC, Oler E, et al. HMDB 5.0: the Human Metabolome Database for 2022. *Nucleic Acids Res*. 2022;50(D1):D622-D631. doi:10.1093/NAR/GKAB1062
16. Olivier M, Asmis R, Hawkins GA, Howard TD, Cox LA. The need for multi-omics biomarker signatures in precision medicine. *Int J Mol Sci*. 2019;20(19). doi:10.3390/ijms20194781
17. Hasin Y, Seldin M, Lusis A. Multi-omics approaches to disease. *Genome Biol*. 2017;18(1):1-15. doi:10.1186/s13059-017-1215-1
18. Turanli B, Karagoz K, Gulfidan G, Sinha R, Mardinoglu A, Arga KY. A network-based cancer drug discovery: From integrated multi-omics approaches to precision medicine. *Curr Pharm Des*. 2019;24(32):3778-3790. doi:10.2174/1381612824666181106095959
19. Yugi K, Kubota H, Hatano A, Kuroda S. Trans-Omics: How to reconstruct biochemical networks across multiple “omic” layers. *Trends Biotechnol*. 2016;34(4):276-290. doi:10.1016/j.tibtech.2015.12.013