



Universiteit
Leiden
The Netherlands

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 1

General introduction and scope

Introduction

The role of systems pharmacology in drug discovery and development

Drug discovery and the successful development of drugs towards the market have become increasingly challenging. During the clinical phase, the overall likelihood of approval for new drugs has dropped to only 7.9% between 2011 and 2020, compared with 9.6% during 2006-2015¹. The main attrition occurs in the phase II transition (during which a proof-of-concept is established) with a phase success rate of only 28.9%¹. Compared with traditional small molecular new molecular entities (NMEs)^{1,2}, biologics-based NMEs currently show higher success rates. The reason behind this low success rate is multidimensional. Notably, a better-established relationship between drug mechanism and disease dynamics in the discovery and early development phase increases the probability of clinical proof-of-concept^{1,3,4}. Therefore, improving the mechanistic understanding of disease dynamics and drug action is necessary to enhance the success rate during drug discovery, for instance using systems biology-based approaches.

Systems biology, which investigates the behavior and relationships of multiscale elements instead of individual genes, proteins, or metabolites, has shown to be the most important concept in studying complex biological systems^{5,6}. System pharmacology strategies, using theories of system biology in drug discovery and development, have shown great value in understanding the mechanisms of drug action⁴. By combining experimental and computational approaches, system pharmacology builds and analyzes multiscale networks of disease dynamics and drug action, which starts at the molecular level to cellular/tissue level, followed by organ-level, and, eventually, describes and predicts outcome of drug treatment at the level of the organism (**Figure. 1**).

The experimental approaches used to build network models at the molecular level include genomic, transcriptomic, proteomic, and metabolomic analyses⁴. These “omics” approaches enable measurements of a wide range of variables, which are related to disease dynamics and/or drug treatment. Genomics characterizes DNA sequences^{4,7}. Transcriptomics studies change of gene expression patterns⁴. Proteomics identifies, characterizes, and quantifies proteins, as well as provides information on protein

interaction^{8,9}. Metabolomics identifies and/or quantifies small molecules (i.e., mass lower than 1 kDa), such as amino acids, carbohydrates and lipids that are endogenous metabolites^{8,9}. Currently, genomic approaches are being applied throughout the whole drug development pipeline, while proteomics and metabolomics are mostly used in preclinical and early-stage clinical development phases^{4,7-9}.

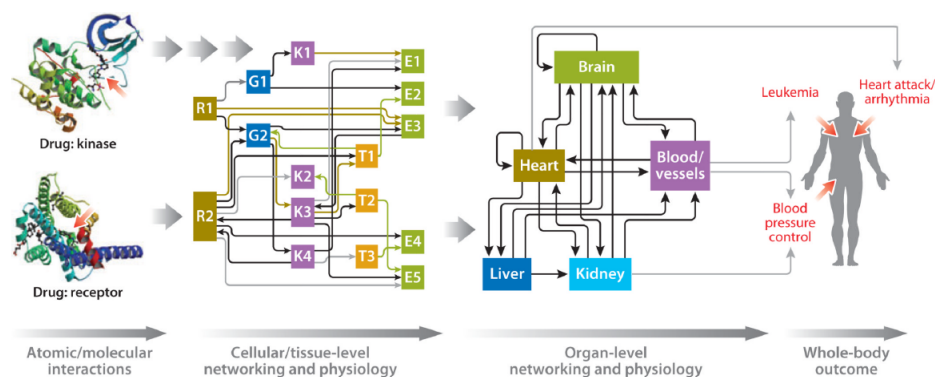


Figure 1. Representation of the multiscale networks that can be used to better understand and predict drug action. *Adapted with permission from Zhao, S. et. al. Annual review of pharmacology and toxicology, 52, 505–521⁴.*

In order to take full advantage of multi-omics data, computational models are beneficial to generate knowledge by integrating all available data into network modules¹⁰⁻¹². Frequently-used strategies include metabolism-centric methods, which connect metabolic pathways and other omics layers related to the generation and modulation of the endogenous metabolites. Since metabolic pathway analyses are supported by accumulated evidence from biochemical studies, networks based on such foundation show high credibility¹²⁻¹⁴. A general workflow for metabolism-centric methods starts with the construction of “maps” of metabolic pathways, which are generated using metabolomics-based approaches. Information of established pathways from freely-available databases such as KEGG and MetaCyc is typically used as a reference in this step^{13,14}. Next, data from proteomics, transcriptomics, and genomics are projected to the maps of metabolic pathways. The connections between regulators and metabolic pathways are drawn, which enable the identification of static and dynamic signal flows, and reveal modes of global regulation^{11,12}.

Well-constructed networks help improving the understanding of disease pathology and drug pharmacodynamics (PD). Traditional PD studies often rely on a single endpoint to evaluate drug response, which provides limited information about the potential drug effects impacting the entire system^{15,16}. With network analyses, drug responses are monitored as parts of regulatory networks and components that affect drug response can be determined^{4,10,15}. This approach may also identify targets involved in adverse effects at the early stages of drug discovery and development¹⁵. Therefore, both efficacy and safety can be significantly improved using network analyses. Moreover, when considering the omics signatures of individuals or subpopulations, the drug effects and safety can be predicted in a personalized way.

Metabolomics and pharmacometabolomics

Metabolomics is a rapidly evolving field of the omics cascade. Metabolomics uses advanced analytical techniques to enable the characterization of metabolites from cells, organs, tissues, and biofluids¹⁷. These metabolites are mostly endogenous compounds, such as lipids, amino acids, short peptides, nucleic acids, sugars, alcohols, or organic acids¹⁷ and are not only structural components of organisms, but also play important roles as signaling molecules, immune modulators, endogenous toxins, and environmental sensors in multiple biological processes^{17,18}. In recent years, metabolomics has shown to be a useful tool for the identification of novel biomarkers that can be clinically used for screening and diagnostic purposes¹⁹. Moreover, in combination with other omics layers, metabolomics has demonstrated to be a powerful tool for investigating the mechanisms of physiological and pathological processes^{17,18,20}. In this context, pharmacometabolomics, i.e., the application of metabolomics for monitoring the response of an organism to pharmacological modulation, can be used in the preclinical and early-stage clinical evaluation of efficacy and safety of drug candidates^{9,21}.

Metabolomics uses both untargeted and targeted methodologies. Untargeted metabolomics analyze the global metabolomic profile without specific hypothesis²². While targeted metabolomics measures defined groups of chemically characterized and biochemically annotated metabolites²³. This thesis focuses mainly on targeted metabolomics. The frequently used targeted metabolomics workflow is shown in **Figure 2**. A targeted

metabolomics workflow starts with the creation of a target list of metabolites, based on the study aims, available information from literature, and previous preliminary data (e.g., from untargeted metabolomics experiments). The sample handling and storage conditions are essential to ensure high data quality and should be optimized based on the properties of metabolites and the composition of the biological matrix. Based on the physico-chemical properties of the metabolites, requirement for sensitivity, selectivity, and throughput, the optimal analytical techniques are selected. The entire workflow (including sample preparation and instrument settings) is optimized and the study samples can be measured using the validated workflow, together with quality control (QC) samples, which are normally pooled from study samples. They are prepared and analyzed together with study samples, and used in the data preprocessing and data quality check.

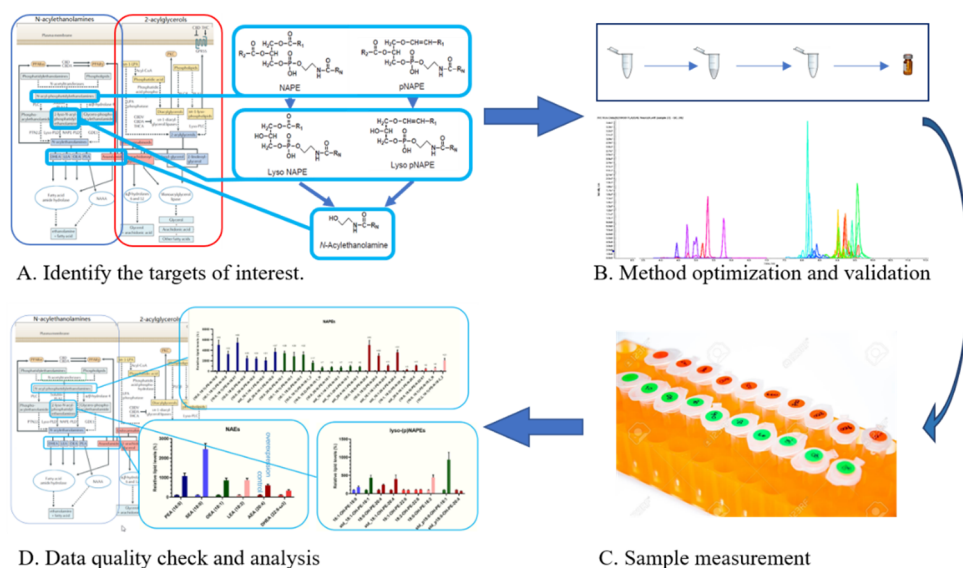


Figure 2. Typical targeted metabolomics workflow from metabolites selection towards data analysis. Step A is the creation of a target list of metabolites. Step B is the development and validation of the sample preparation method and LC-MS method for these selected metabolites. Step C is the sample measurement. Step D is the data quality check and analysis.

Common analytical techniques used in metabolomics include gas chromatography - mass spectrometry (GC-MS), liquid chromatography - mass spectrometry (LC-MS), capillary electrophoresis-mass spectrometry (CE-MS), and nuclear magnetic resonance (NMR)¹⁷.

Compared with the other techniques, LC-MS shows the advantage of wide coverage and high sensitivity^{24,25}. Moreover, it also has good throughput and the ability for the identification of metabolites. With these advantages, LC-MS is a preferred approach for metabolomic studies. The advantage of LC-MS on analytical sensitivity can be further increased using micro-flow LC, which operates at a flow rate from 1 - 10 $\mu\text{L}/\text{min}$. By lowering the flow rate, the injected sample is less diluted and the ionization efficiency is increased. Therefore, micro-flow LC-MS provides higher sensitivity using lower amounts of sample, and enables the detection of metabolites in low concentration and/or volume-limited samples. In the first part of this thesis, both conventional LC-MS and micro-flow LC-MS techniques are developed and applied in the development of methods that determine the endocannabinoids (eCBs) and related metabolites in various biological matrices.

The endocannabinoid system and related pathways

The endocannabinoid system (ECS) is a widely distributed signaling system involved in multiple physiological and pathological processes^{26,27}. The ECS is mainly composed of the cannabinoid receptor type 1 (CB1R) and type 2 (CB2R), the eCBs, as well as enzymes involved in their metabolism^{26,27}. The discovery of ECS started from the investigation of the pharmacological effects of the major psychoactive cannabinoid in marijuana (Δ^9 -tetrahydrocannabinol, THC), during which the CB1R and CB2R were identified^{28,29}. Then, two endogenous agonists of the CBRs were discovered (i.e., the so-called eCBs), first anandamide (AEA) followed by 2-arachidonoyl glycerol (2-AG)^{30,31}. Their structural analogues of AEA and 2-AG do not have affinity for CB1R or CB2R but are playing important roles in ECS signaling³². These eCB analogues include other N-acyl ethanolamines (NAEs), such as N-palmitoylethanolamine (PEA), N-oleoylethanolamine (OEA), and N-linoleylethanolamine (LEA), as well as other 2-acylglycerols (2-AcGs), such as 2-linoleoylglycerol (2-LG) and 2-oleoylglycerol (2-OG).

The metabolic pathways involving the generation and metabolism of NAEs and 2-AcGs are depicted in **Figure 3**. The synthesis of NAEs begins with the generation of N-acylphosphatidylethanolamines (NAPEs) from membrane phosphatidylethanolamines (PEs) and other phospholipids, involving N-acetyltransferases from the phospholipase A and acyltransferase (PLAAT) family and calcium-dependent phospholipase A2 (PLA2) subfamily²⁷. Then, NAEs are generated from NAPEs directly by N-acyl

phosphatidylethanolamine-specific phospholipase D (NAPE-PLD) or from other intermediate metabolites, such as 2-lyso-N-acyl-phosphatidylethanolamines (lyso-NAPEs) or glycerol-phospho-acylethanolamines (GP-NAEs). The NAEs are further metabolized by fatty acid amide hydrolase (FAAH) or N-acylethanolamine-hydrolyzing acid amidase (NAAA) into free fatty acids (FFAs) and ethanolamine. On the other hand, the 2-AcGs are mostly generated from diacylglycerols (DAGs) by diacylglycerol lipase (DAGL), but other precursors, such as lysophosphatidic acids (LPAs), are also involved. The degradation of 2-AcGs is mostly catalyzed by monoacylglycerol lipase (MAGL), as well as other metabolic enzymes including α,β -hydrolase 6 (ABHD6) and α,β -hydrolase 12 (ABHD 12). Glycerol and FFAs are the downstream metabolites of 2-AcGs. In neurons and other types of cells, the FFAs from 2-AcGs, specifically the poly-unsaturated fatty acids (PUFAs), may serve as the precursors of the so-called oxylipins³³. Oxylipins are oxidation products of PUFAs performed by cyclooxygenase (COX), lipoxygenase (LOX), or cytochrome P450 (CYP450) enzymes³³. Based on the double bond position of their PUFAs precursor(s), oxylipins can be classified into omega-6 and omega-3 oxylipins. Another relevant source of PUFAs comes from the hydrolyzation of membrane PEs via calcium-dependent enzymes from the PLA2 family³⁴, which is also responsible for the generation of eCBs. The production of oxylipins and eCBs, as well as eCB analogues, may be triggered by similar mechanisms. Moreover, some of their biological roles are also closely connected.

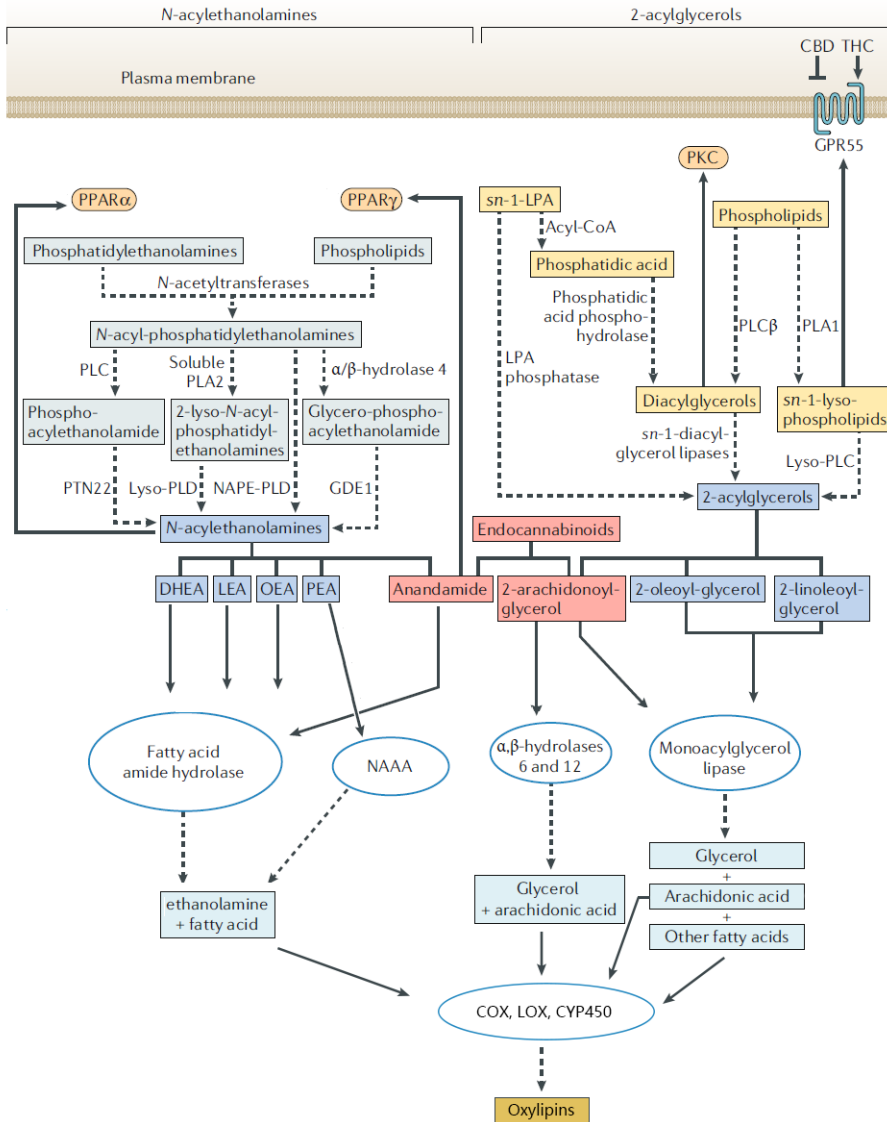


Figure 3. Metabolic pathways involved in the generation and metabolism of endocannabinoids and related compounds. CBD, cannabidiol; COX, cyclooxygenase; CYP450, cytochrome P450; DHEA, N-docosahexaenoyl-ethanolamine; GDE1, glycerophosphodiester phosphodiesterase 1; LEA, linoleylethanolamine; LOX, lipoxygenase; lyso-PLD, lysophospholipase D; NAPE-PLD, N-acyl-phosphatidylethanolamine-specific phospholipase D; OEA, oleoylethanolamine; PEA, palmitoylethanolamine; PLA, phospholipase A; PLC, phospholipase C; PTN22, tyrosine-protein phosphatase non-receptor type 22; THC, Δ^9 -tetrahydrocannabinol. Adapted with permission from Cristino, L. et al. *Nature Reviews Neurology*, 16(1), 9-29²⁷

Physiological and pathological roles of the endocannabinoid system and related pathways

The ECS is involved in multiple physiological processes, such as appetite, energy balance, pain-sensation, mood, memory, fertility, pregnancy, pre- and postnatal development, and activity of the immune system^{26,27}. ECS dysregulation has been observed in multiple diseases, including obesity and cardiometabolic diseases (CMD), pain, inflammation, neurological disorders such as stroke, multiple sclerosis, migraine, and Alzheimer's disease, eye disorders, gastrointestinal and liver disorders, as well as musculoskeletal disorders^{26,27}.

In the second part of this thesis, the role of the ECS and related pathways in obesity and CMD has been investigated. It is well-known that the use of cannabinoids increases appetite via activation of CB1R^{35,36}. Besides appetite, the activation of ECS signaling stimulates the synthesis of fatty acids in the liver and adipocyte differentiation into white adipose tissue and, thereby, leads to fat storage³⁷⁻³⁹. In addition to the eCBs, the eCB analogues may also contribute to the energy balance of an organism. In this respect, one of the main functions of the eCB analogues is to enhance the effects of AEA and 2-AG on the cannabinoid receptors by increasing their affinity for the receptors or inhibiting their hydrolysis, the so-called entourage effect^{40,41}. Moreover, additional possible effects of the eCB analogues on obesity through the activation of peroxisome proliferator-activated receptor- α (PPAR- α) and peroxisome proliferator-activated receptor- γ (PPAR- γ) have been reported^{42,43}. Generally, enhanced ECS signaling increases energy storage and thereby contributes to obesity. This might be explained by the role the ECS was playing during evolution, where it was "designed" to maximize intake and conservation of energy, which increased the possibility of survival⁴⁴. The eCBs are secreted during exercise, inducing the so-called "runner's high", but also stimulating energy intake⁴⁵⁻⁴⁷. This reaction is vital for an organism, as it allows sufficient energy to escape from another predator or chase a prey. While the ECS mostly evolved when food was scarce, similar to other systems involved in food-seeking behavior, in modern times food has been abundant and as a consequence, obesity has become a fast-growing problem in developed countries⁴⁸.

The prevalence of obesity has, amongst others, increased the burden of CMD, which is a cluster encompassing cardiovascular disease (CVD), diabetes mellitus, chronic renal failure,

and/or non-alcoholic hepatic failure^{48,49}. The link between CMD and the ECS through the role of the latter in the energy balance is obvious and has been already reviewed elsewhere³⁶. However, preclinical and clinical evidence suggest that ECS signaling is also directly linked to the deterioration of cardiometabolic health⁵⁰⁻⁵³. This direct connection may be partly explained by the role of ECS in the regulation of inflammation⁵⁴, since an inflammatory response is typically observed during the development of CMDs^{55,56}. Meanwhile, oxylipins, which are closely linked to the ECS in metabolic pathways^{33,34}, have also shown to play important roles in inflammation^{57,58}. Indeed, the omega-6 oxylipins, especially eicosanoids such as prostaglandins, are known for their pro-inflammatory, vasoconstricting, and proliferative functions, all of which are involved in the progression of obesity and CMD⁵⁹. On the other hand, the omega-3 oxylipins show anti-inflammatory effects and are crucial in the resolution of inflammation^{57,58}. Overall, this underlines the need for further investigation of the metabolism of eCBs, eCB analogues and oxylipins in the context of CMD, focusing on the inflammatory status.

Development of drugs targeting the endocannabinoid system

Numerous drugs targeting the ECS have been developed so far, but with limited success. Drugs or drug candidates targeting the ECS can be divided into three classes, i.e., CBR agonists, CBR antagonists, and metabolic enzyme inhibitors. Most of the current CBR agonists are non-selective exogenous cannabinoids acting on both CB1R and CB2R. The CBR agonists nabilone and dronabinol are approved by FDA as third-line antiemetics for chemotherapy^{60,61}. A buccal spray containing THC and cannabidiol has been approved in Canada for the treatment of neuropathic pain associated with multiple sclerosis^{62,63}. However, these treatments have or may have neurological side effects, such as psychosis and schizophrenia, especially in long-term treatments, which limits their long-term use⁶⁴. Besides the non-selective candidates, selective CB1R or CB2R agonists, which are expected to have a better safety profile, are currently at early stage development^{65,66}.

Synthetic CBR antagonists are more selective and are further divided into CB1R antagonists and CB2R antagonists. The most well-known CB1R antagonist is rimonabant. As a first-in-class drug, rimonabant lead to weight loss and significantly improved multiple cardiometabolic parameters in overweight patients with metabolic syndrome in phase III

clinical trials^{67,68}. In 2005, it was approved by European Medicines Agency (EMA) for clinical use. However, rimonabant was withdrawn from the market in 2008 due to neuropsychiatric side effects such as anxiety, depressive mood disorders, and increased suicidal ideation⁶⁹. Due to its neuropsychiatric side effects, multiple clinical trials of similar CB1R antagonists were terminated⁷⁰. This triggered the development of second-generation peripheral-limited CB1R antagonists, which do not enter the central nervous system (CNS) and therefore have limited neuropsychiatric side effects⁷⁰. However, there has been no clinical success reported so far, due to challenges in achieving both good pharmacokinetic properties and efficacy⁷⁰. Furthermore, third-generation peripheral limited dual-target antagonists, which inhibits CB1R and an additional target (e.g., inducible nitric oxide synthase), are being developed for better therapeutic efficacy in complex disorders, such as various forms of fibrosis, for which proof-of-concept has been established pre-clinically⁷⁰⁻⁷². Compared to CB1R antagonists, there have been fewer investigations on CB2R antagonists, and mostly on treating or preventing a disease associated with immune dysfunction^{65,66}.

Metabolic enzyme inhibitors modulate enzymes responsible for the generation or degradation of eCBs and related metabolites such as NAPE-PLD, FAAH, NAAA and MAGL. Among them, FAAH inhibitors have received most interest and have been intensively studied^{65,73}. FAAH inhibitors can increase the concentration of AEA, as well as other NAEs such as PEA⁶⁵. In animal models, FAAH inhibitors showed promises in the treatment of pain and stress, without neuropsychiatric side effects⁷⁴⁻⁷⁷. Moreover, in a randomized clinical trial, FAAH inhibition has shown to be effective for the treatment of problematic cannabis use⁷⁸. However, in another clinical trial, a FAAH inhibitor failed to induce effective analgesia in patients with osteoarthritic pain^{73,79}. More recently, new concepts of dual-target inhibitors were proposed, where both FAAH and COX-2 are inhibited. Such drug candidates are currently at their preclinical stage^{80,81}.

One of the most relevant clinical failures associated with FAAH inhibition concerns the candidate BIA 10-2474. In 2016, a phase I multiple ascending dose trial of Bial's FAAH inhibitor BIA 10-2474 resulted in severe neurological adverse effects and caused one death⁸². Subsequent research revealed that these severe adverse effects were likely related to off-target effects of BIA 10-2474^{83,84}. Compared with other FAAH inhibitors that were

clinically safe, BIA 10-2474 inhibited not only FAAH, but also several additional lipases, such as ABHD6, and produced substantial alterations in lipid networks in human cortical neuron cells⁸⁴. These off-target effects likely caused metabolic dysregulation in the nervous system, which resulted in the severe neurological adverse effects⁸⁴.

To conclude, the development of therapeutics targeting ECS has seen a lot of failures in the last decade. Such development remains challenging and requires a better understanding of the regulation of the ECS and related pathways. The concept of modulating dual-targets or even multiple targets has emerged and shows high potential, but more effort in the identification of potential off-target effects is needed. Moreover, due to the wide distribution of the ECS, undesired pharmacological modulation outside of the targeted tissue/organ may also lead to side effects. System pharmacology-based approaches, such as pharmacometabolomics, are expected to contribute to an increased understanding of the ECS and, in turn, more successful development of drugs targeting the ECS.

Aim and scope of the thesis

The aim of this thesis was to investigate the role of the ECS and related pathways in cardiometabolic health using metabolomics and explore whether the beneficial effects that exercise plays in cardiometabolic health are related to the modulation of ECS and related pathways. The underlying hypothesis was that the ECS may play a key role in cardiometabolic health and may be represent an adequate drug target for CMD. Due to the complexity of ECS, a wide range of metabolites from different pathways need to be considered when studying the ECS, showing the need for state-of-the-art metabolomics approaches targeting a large number of metabolites.

The first section of the thesis reports the development of metabolomics-based strategies for the determination of eCBs and related metabolites in various biological matrices. The second section reports the application of developed metabolomics workflows to investigate the role of the ECS and related pathways in cardiometabolic health and study whether beneficial effects of exercise are related to the modulation of ECS and related pathways.

In **Chapter 2**, the aim was to develop a method for the analysis of eCB-related metabolites, enabling the characterization of pathways responsible for the generation of eCBs and eCB analogues, since previous reported methods only enabled the analysis of limited number of classes of these lipids. Based on current knowledge about eCB metabolism, the metabolites in the target list included NAPEs, plasmalogen-NAPEs (pNAPEs), lyso-NAPEs, lyso-pNAPEs, GP-NAEs, NAEs, DAGs, 2-AcGs, and FFAs. Using commercially available and synthesized standards, fragments were identified. A screening approach based on retention time mapping and fragmentation pattern was used for the identification of the metabolites. The method was optimized for multiple types of biological matrices, such as neuro cell pellets and mice brain tissues, showing its usefulness in *in-vitro* and *in-vivo* studies investigating the roles of potential metabolic enzymes in the ECS, as well as the evaluation of new drugs.

The metabolite concentrations in cultured cells and tissues are usually high. Therefore, these metabolites are relatively easy to quantify with a conventional LC-MS setup. However, in human cerebral spinal fluid (CSF), the concentration of eCBs and their analogues are present at significantly lower concentrations (i.e., picomolar range), which makes their detection and accurate quantitation extremely challenging. Therefore, in **Chapter 3**, the aim

was to develop a highly sensitive and selective method to analyze eCBs and eCB analogues in human CSF. To improve the sensitivity, a micro-LC system with micro electro spray interface (ESI) was used instead of a conventional LC instrument. At low-flow rates, the injected sample is less diluted. Moreover, the spray droplets are smaller and more uniform, which significantly improves the ionization efficiency. However, due the use of small inner diameter tubes, micro-LC-MS systems frequently suffer from problems, such as clogging. The developed hardware and the sample preparation method were further optimized to allow the analysis of hundreds of samples with good repeatability.

In **Chapter 4 & 5**, the aim was to investigate the roles of eCBs, eCB analogues (**Chapter 4**) and oxylipins (**Chapter 5**) in cardiometabolic health, and to study whether they can be used as early biomarkers for cardiometabolic diseases. The correlations of plasma levels of eCBs, eCB analogues, oxylipins and PUFAs with body composition and traditional cardiometabolic risk (CMR) factors were studied. Specifically, the differences in the plasma levels of these metabolites between metabolically healthy overweight-obese (MHOO) individuals and metabolically unhealthy overweight-obese (MUOO) individuals were assessed. Moreover, instead of using these metabolites as individual biomarkers, we used forward stepwise regression models to integrate them and develop a biomarker fingerprint. With these models, we were able to assess if these related metabolites can be integrated and used as adequate predictors for CMR.

It is well known that cardiometabolic health benefits from regular exercise. However, it remains unclear how exercise is affecting the ECS-related metabolites, which may help to better understand the mechanism of the positive influence of exercise on cardiometabolic health. In **Chapter 6**, we studied the influence of acute endurance and strength exercises on plasma levels of eCBs, eCB analogues and oxylipins. We also investigated the effect of a 24-week supervised exercise intervention on plasma levels of these metabolites in young sedentary adults to assess the effect of exercise on a longer term. Different intensities of exercise were compared.

Lastly, in **Chapter 7**, the thesis is concluded. Additional developments and possible applications for metabolomics in drug discovery and development targeting the ECS are discussed.

References

1. Biotechnology Innovation Organization. Clinical development success rates and contributing factors 2011-2020. *Infoma report*. Published online 2021:1-34.
2. Kinch MS, Griesenauer RH. 2017 in review: FDA approvals of new molecular entities. *Drug Discovery Today*. 2018;23(8):1469-1473. doi:10.1016/j.drudis.2018.05.011
3. Kinch MS. An overview of FDA-approved biologics medicines. *Drug Discovery Today*. 2015;20(4):393-398. doi:10.1016/j.drudis.2014.09.003
4. Zhao S, Iyengar R. Systems pharmacology: network analysis to identify multiscale mechanisms of drug action. *Annu Rev Pharmacol Toxicol*. 2012;52:505-521. doi:10.1146/annurev-pharmtox-010611-134520
5. Ideker T, Galitski T, Hood L. A new approach to decoding life: systems biology. <http://dx.doi.org/101146/annurev.genom21343>. 2003;2:343-372. doi:10.1146/ANNUREV.GENOM.2.1.343
6. Tavassoly I, Goldfarb J, Iyengar R. Systems biology primer: the basic methods and approaches. *Essays in Biochemistry*. 2018;62(4):487-500. doi:10.1042/EBC20180003
7. Putu D, Shoveller J, Montaner J, Feng C, Nicoletti R, Shannon K. Pharmacogenomics in early-phase clinical development. *Tal. Physiol Behav*. 2016;176(1):139-148. doi:10.2217/pgs.13.81.Pharmacogenomics
8. Nandal S, Burt T. Integrating pharmacoproteomics into early-phase clinical development: State-of-the-art, challenges, and recommendations. *International Journal of Molecular Sciences*. 2017;18(2). doi:10.3390/ijms18020448
9. Burt T, Nandal S. Pharmacometabolomics in early-phase Clinical Development. *Clinical and Translational Science*. 2016;9(3):128-138. doi:10.1111/cts.12396
10. Olivier M, Asmis R, Hawkins GA, Howard TD, Cox LA. The need for multi-omics biomarker signatures in precision medicine. *International Journal of Molecular Sciences*. 2019;20(19). doi:10.3390/ijms20194781
11. Hasin Y, Seldin M, Lusic A. Multi-omics approaches to disease. *Genome Biology*. 2017;18(1):1-15. doi:10.1186/s13059-017-1215-1
12. Yugi K, Kubota H, Hatano A, Kuroda S. Trans-Omics: How to reconstruct biochemical networks across multiple “omic” layers. *Trends in Biotechnology*. 2016;34(4):276-290. doi:10.1016/j.tibtech.2015.12.013
13. Karp PD, Riley M, Paley SM, Pellegrini-Toole A. The metacyc database. *Nucleic Acids Research*. 2002;30(1):59-61. doi:10.1093/NAR/30.1.59
14. Kanehisa M, Goto S. KEGG: Kyoto encyclopedia of genes and genomes. *Nucleic Acids Research*. 2000;28(1):27-30. doi:10.1093/NAR/28.1.27
15. Iyengar R, Zhao S, Chung SW, Mager DE, Gallo JM. Merging systems biology with pharmacodynamics. *Science Translational Medicine*. 2012;4(126). doi:10.1126/SCITRANSLMED.3003563/SUPPL_FILE/4126PS7_SM.PDF
16. Kirouac DC, Onsum MD. Using network biology to bridge pharmacokinetics and pharmacodynamics in oncology. *CPT: Pharmacometrics & Systems Pharmacology*. 2013;2(9):1-7. doi:10.1038/PSP.2013.38
17. Wishart DS. Metabolomics for investigating physiological and pathophysiological processes. *Physiol Rev*. 2019;99(4):1819-1875. doi:10.1152/PHYSREV.00035.2018
18. Rinschen MM, Ivanisevic J, Giera M, Siuzdak G. Identification of bioactive metabolites using activity metabolomics. doi:10.1038/s41580-019-0108-4
19. Odom JD, Sutton VR. Metabolomics in clinical practice: improving diagnosis and informing management. *Clinical Chemistry*. 2021;67(12):1606-1617. doi:10.1093/CLINCHEM/HVAB184
20. Johnson CH, Ivanisevic J, Siuzdak G. Metabolomics: beyond biomarkers and towards

- mechanisms. *Nature Reviews Molecular Cell Biology* 2016 17:7. 2016;17(7):451-459. doi:10.1038/nrm.2016.25
21. Clayton TA, Baker D, Lindon JC, Everett JR, Nicholson JK. Pharmacometabonomic identification of a significant host-microbiome metabolic interaction affecting human drug metabolism. *Proc Natl Acad Sci U S A*. 2009;106(34):14728-14733. doi:10.1073/PNAS.0904489106
 22. Schrimpe-Rutledge AC, Codreanu SG, Sherrod SD, McLean JA. Untargeted metabolomics strategies – Challenges and Emerging Directions. *J Am Soc Mass Spectrom*. 2016;27(12):1897. doi:10.1007/S13361-016-1469-Y
 23. Roberts LD, Souza AL, Gerszten RE, Clish CB. Targeted Metabolomics. *Current Protocols in Molecular Biology*. 2012;CHAPTER(SUPPL.98):Unit30.2. doi:10.1002/0471142727.MB3002S98
 24. Zhou B, Xiao JF, Tuli L, Ressom HW. LC-MS-based metabolomics. *Mol Biosyst*. 2012;8(2):470. doi:10.1039/C1MB05350G
 25. Emwas AH, Roy R, McKay RT, et al. NMR spectroscopy for metabolomics research. *Metabolites*. 2019;9(7). doi:10.3390/METABO9070123
 26. Pacher P, Bátkai S, Kunos G. The endocannabinoid system as an emerging target of pharmacotherapy. *Pharmacol Rev*. 2006 Sep;58(3):389-462. doi: 10.1124/pr.58.3.2.
 27. Cristino L, Bisogno T, di Marzo V. Cannabinoids and the expanded endocannabinoid system in neurological disorders. *Nature Reviews Neurology*. 2020;16(1):9-29. doi:10.1038/s41582-019-0284-z
 28. Munro S, Thomas KL, Abu-Shaar M. Molecular characterization of a peripheral receptor for cannabinoids. *Nature*. 1993 365:6441. doi:10.1038/365061a0
 29. Matsuda LA, Lolait SJ, Brownstein MJ, Young AC, Bonner TI. Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature* 1990 346:6284. doi:10.1038/346561a0
 30. Devane WA, Hanuš L, Breuer A, et al. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science*. 1992;258(5090):1946-1949. doi:10.1126/SCIENCE.1470919
 31. el Mechoulam R., Ben-Shabat S, Hanus L, et al. Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochem Pharmacol*. 1995 Jun 29;50(1):83-90. doi: 10.1016/0006-2952(95)00109-d.
 32. Tsuboi K, Uyama T, Okamoto Y, Ueda N. Endocannabinoids and related N-acylethanolamines: Biological activities and metabolism. *Inflammation and Regeneration*. 2018;38(1). doi:10.1186/s41232-018-0086-5
 33. Smith WL, Urade Y, Jakobsson PJ. Enzymes of the cyclooxygenase pathways of prostanoid biosynthesis. *Chemical Reviews*. 2011;111(10):5821-5865. doi:10.1021/cr2002992
 34. Bingham CO, Frank Austen K. Phospholipase A2 enzymes in eicosanoid generation. *Proc Assoc Am Physicians*. 1999;111(6):516-524. doi:10.1046/J.1525-1381.1999.99321.X
 35. Bermudez-Silva FJ, Cardinal P, Cota D. The role of the endocannabinoid system in the neuroendocrine regulation of energy balance. *Journal of Psychopharmacology*. 2012;26(1):114-124. doi:10.1177/0269881111408458
 36. Silvestri C, di Marzo V. The endocannabinoid system in energy homeostasis and the etiopathology of metabolic disorders. *Cell Metabolism*. 2013;17(4):475-490. doi:10.1016/J.CMET.2013.03.001
 37. Shelke, A. R. , Roscoe, J. A. , Morrow, G. R. , Colman, L. K. , Banerjee, T. K. , & Kirshner JJ. Activation of the peripheral endocannabinoid system in human obesity. *Bone*. 2008;23(1):1-7. doi:10.1038/jid.2014.371
 38. Osei-Hyiaman D, DePetrillo M, Pacher P, et al. Endocannabinoid activation at hepatic CB1 receptors stimulates fatty acid synthesis and contributes to diet-induced obesity. *Journal of*

- Clinical Investigation*. 2005;115(5):1298-1305. doi:10.1172/JCI200523057
39. Silvestri C, Ligresti A, di Marzo V. Peripheral effects of the endocannabinoid system in energy homeostasis: Adipose tissue, liver and skeletal muscle. *Reviews in Endocrine and Metabolic Disorders*. 2011;12(3):153-162. doi:10.1007/s11154-011-9167-3
 40. Ben-Shabat S, Fride E, Sheskin T, et al. An entourage effect: inactive endogenous fatty acid glycerol esters enhance 2-arachidonoyl-glycerol cannabinoid activity. *European Journal of Pharmacology*. 1998;353(1):23-31. doi:10.1016/S0014-2999(98)00392-6
 41. Ho WSV, Barrett DA, Randall MD. “Entourage” effects of N-palmitoylethanolamide and N-oleoylethanolamide on vasorelaxation to anandamide occur through TRPV1 receptors. *British Journal of Pharmacology*. 2008;155(6):837-846. doi:10.1038/bjp.2008.324
 42. Laleh P, Yaser K, Abolfazl B, et al. Oleoylethanolamide increases the expression of PPAR-A and reduces appetite and body weight in obese people: A clinical trial. *Appetite*. 2018;128:44-49. doi:10.1016/j.appet.2018.05.129
 43. Fu J, Oveisi F, Gaetani S, Lin E, Piomelli D. Oleoylethanolamide, an endogenous PPAR- α agonist, lowers body weight and hyperlipidemia in obese rats. *Neuropharmacology*. 2005;48(8 SPEC. ISS.):1147-1153. doi:10.1016/j.neuropharm.2005.02.013
 44. Joshi N, Onaivi ES. The evolution and comparative neurobiology of endocannabinoid signalling. *Recent Advances in Cannabinoid Research*. Published online 2019. doi:10.5772/intechopen.73801
 45. Feurecker M, Hauer D, Toth R, et al. Effects of exercise stress on the endocannabinoid system in humans under field conditions. *European Journal of Applied Physiology*. 2012;112(7):2777-2781. doi:10.1007/s00421-011-2237-0
 46. 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.” *Journal of Experimental Biology*. 2012;215(8):1331-1336. doi:10.1242/jeb.063677
 47. Sparling PB, Giuffrida A, Piomelli D, Rosskopf L, Dietrich A. Exercise activates the endocannabinoid system. *NeuroReport*. 2003;14(17):256-277. doi:10.1097/00001756-200312020-00015
 48. Ng M, Fleming T, Robinson M, et al. Global, regional, and national prevalence of overweight and obesity in children and adults during 1980-2013: a systematic analysis for the Global Burden of Disease Study 2013. *Lancet*. 2014;384(9945):766-781. doi:10.1016/S0140-6736(14)60460-8
 49. Després JP, Lemieux I. Abdominal obesity and metabolic syndrome. *Nature*. 2006;444(7121):881-887. doi:10.1038/nature05488
 50. Schloss MJ, Horckmans M, Guillaumat-Prats R, et al. 2-Arachidonoylglycerol mobilizes myeloid cells and worsens heart function after acute myocardial infarction. *Cardiovascular Research*. 2019;115(3):602-613. doi:10.1093/cvr/cvy242
 51. Melgar-Lesmes P, Perramon M, Jiménez W. Roles of the Hepatic Endocannabinoid and Apelin Systems in the Pathogenesis of Liver Fibrosis. *Cells*. 2019;8(11). doi:10.3390/cells8111311
 52. van Esbroeck ACM, Varga Z v., Di X, et al. Activity-based protein profiling of the human failing ischemic heart reveals alterations in hydrolase activities involving the endocannabinoid system. *Pharmacological Research*. 2020;151(September 2019). doi:10.1016/j.phrs.2019.104578
 53. Zhou S, Wu Q, Lin X, et al. Cannabinoid receptor type 2 promotes kidney fibrosis through orchestrating β -catenin signaling. *Kidney International*. 2021;99(2):364-381. doi:10.1016/j.kint.2020.09.025
 54. Oláh A, Szekanecz Z, Bíró T. Targeting cannabinoid signaling in the immune system: “High”-ly exciting questions, possibilities, and challenges. *Frontiers in Immunology*.

- 2017;8(NOV):1-14. doi:10.3389/fimmu.2017.01487
55. Jaiswal S, Libby P. Clonal haematopoiesis: connecting ageing and inflammation in cardiovascular disease. *Nature Reviews Cardiology*. 2020;17(3):137-144. doi:10.1038/s41569-019-0247-5
 56. Donath MY, Meier DT, Böni-Schnetzler M. Inflammation in the Pathophysiology and Therapy of Cardiometabolic Disease. *Endocrine Reviews*. 2019;40(4):1080-1091. doi:10.1210/er.2019-00002
 57. Spite M, Clària J, Serhan CN. Resolvins, specialized proresolving lipid mediators, and their potential roles in metabolic diseases. *Cell Metabolism*. 2014;19(1):21-36. doi:10.1016/j.cmet.2013.10.006
 58. Serhan CN, Chiang N, van Dyke TE. Resolving inflammation: Dual anti-inflammatory and pro-resolution lipid mediators. *Nature Reviews Immunology*. 2008;8(5):349-361. doi:10.1038/nri2294
 59. Melissa Gabbs, Leng S, Devassy JG, Monirujjaman M, Aukema HM. Advances in Our Understanding of Oxylipins Derived from Dietary PUFAs. *Adv Nutr*. 2015;6(5):513-540. doi:10.3945/AN.114.007732
 60. Wissel J, Haydn T, Müller J, et al. Low dose treatment with the synthetic cannabinoid Nabilone significantly reduces spasticity-related pain. *Journal of Neurology* 2006 253:10. 2006;253(10):1337-1341. doi:10.1007/S00415-006-0218-8
 61. Plasse TF, Gorter RW, Krasnow SH, Lane M, Shepard K v., Wadleigh RG. Recent clinical experience with dronabinol. *Pharmacology Biochemistry and Behavior*. 1991;40(3):695-700. doi:10.1016/0091-3057(91)90385-F
 62. Schoedel KA, Chen N, Hilliard A, et al. A randomized, double-blind, placebo-controlled, crossover study to evaluate the subjective abuse potential and cognitive effects of nabiximols oromucosal spray in subjects with a history of recreational cannabis use. *Human Psychopharmacology: Clinical and Experimental*. 2011;26(3):224-236. doi:10.1002/HUP.1196
 63. Karschner EL, Darwin WD, McMahon RP, et al. Subjective and physiological effects after controlled sativex and oral THC administration. *Clinical Pharmacology & Therapeutics*. 2011;89(3):400-407. doi:10.1038/CLPT.2010.318
 64. Rice ASC. Should cannabinoids be used as analgesics for neuropathic pain? *Nature Clinical Practice Neurology* 2008 4:12. 2008;4(12):654-655. doi:10.1038/ncpneuro0949
 65. Fowler CJ. The endocannabinoid system – current implications for drug development. *Journal of Internal Medicine*. 2021;290(1):2. doi:10.1111/JOIM.13229
 66. Morales P, Hernandez-Folgado L, Goya P, Jagerovic N. Cannabinoid receptor 2 (CB2) agonists and antagonists: a patent update. <https://doi.org/101080/1354377620161193157>. 2016;26(7):843-856. doi:10.1080/13543776.2016.1193157
 67. Després JP, Golay A, Sjöström L. Effects of rimonabant on metabolic risk factors in overweight patients with dyslipidemia. <http://dx.doi.org/101056/NEJMoa044537>. 2009;353(20):2121-2134. doi:10.1056/NEJMoa044537
 68. van Gaal LF, Scheen AJ, Rissanen AM, Rössner S, Hanotin C, Ziegler O. Long-term effect of CB1 blockade with rimonabant on cardiometabolic risk factors: two year results from the RIO-Europe Study. *European Heart Journal*. 2008;29(14):1761-1771. doi:10.1093/EURHEARTJ/EHN076
 69. Christensen R, Kristensen PK, Bartels EM, Bliddal H, Astrup A. Efficacy and safety of the weight-loss drug rimonabant: a meta-analysis of randomised trials. *The Lancet*. 2007;370(9600):1706-1713. doi:10.1016/S0140-6736(07)61721-8
 70. Cinar R, Iyer MR, Kunos G. The therapeutic potential of second and third generation CB1R antagonists. *Pharmacology & Therapeutics*. 2020;208:107477. doi:10.1016/J.PHARMTHERA.2020.107477

71. Cinar R, Iyer MR, Liu Z, et al. Hybrid inhibitor of peripheral cannabinoid-1 receptors and inducible nitric oxide synthase mitigates liver fibrosis. *JCI Insight*. 2016;1(11). doi:10.1172/JCI.INSIGHT.87336
72. Cinar R, Gochuico BR, Iyer MR, et al. Cannabinoid CB1 receptor overactivity contributes to the pathogenesis of idiopathic pulmonary fibrosis. *JCI Insight*. 2017;2(8). doi:10.1172/JCI.INSIGHT.92281
73. Fowler CJ. The potential of inhibitors of endocannabinoid metabolism for drug development: A critical review. *Handbook of Experimental Pharmacology*. 2015;231:95-128. doi:10.1007/978-3-319-20825-1_4
74. Griebel G, Stemmelin J, Lopez-Grancha M, et al. The selective reversible FAAH inhibitor, SSR411298, restores the development of maladaptive behaviors to acute and chronic stress in rodents. *Scientific Reports 2018 8:1*. 2018;8(1):1-25. doi:10.1038/s41598-018-20895-z
75. Naidu PS, Booker L, Cravatt BF, Lichtman AH. Synergy between enzyme inhibitors of fatty acid amide hydrolase and cyclooxygenase in visceral nociception. *The Journal of Pharmacology and Experimental Therapeutics*. 2009;329(1):48. doi:10.1124/JPET.108.143487
76. Karbarz MJ, Luo L, Chang L, et al. Biochemical and biological properties of 4-(3-phenyl-[1,2,4] thiadiazol-5-yl)-piperazine-1-carboxylic acid phenylamide, a mechanism-based inhibitor of fatty acid amide hydrolase. *Anesthesia and Analgesia*. 2009;108(1):316-329. doi:10.1213/ANE.0B013E31818C7CBD
77. Ahn K, Johnson DS, Mileni M, et al. Discovery and characterization of a highly selective FAAH inhibitor that reduces inflammatory pain. *Chem Biol*. 2009;16(4):411. doi:10.1016/J.CHEMBIOL.2009.02.013
78. George TP. FAAH inhibition for treatment of problematic cannabis use. *The Lancet Psychiatry*. 2019;6(1):3-4. doi:10.1016/S2215-0366(18)30462-0
79. Huggins JP, Smart TS, Langman S, Taylor L, Young T. An efficient randomised, placebo-controlled clinical trial with the irreversible fatty acid amide hydrolase-1 inhibitor PF-04457845, which modulates endocannabinoids but fails to induce effective analgesia in patients with pain due to osteoarthritis of the knee. *Pain*. 2012;153(9):1837-1846. doi:10.1016/J.PAIN.2012.04.020
80. Gouveia-Figueira S, Karlsson J, Deplano A, et al. Characterisation of (R)-2-(2-fluorobiphenyl-4-yl)-N-(3-methylpyridin-2-yl)propanamide as a dual fatty acid amide hydrolase: Cyclooxygenase inhibitor. *PLOS ONE*. 2015;10(9):e0139212. doi:10.1371/JOURNAL.PONE.0139212
81. Sasso O, Migliore M, Habrant D, et al. Multitarget fatty acid amide hydrolase/cyclooxygenase blockade suppresses intestinal inflammation and protects against nonsteroidal anti-inflammatory drug-dependent gastrointestinal damage. *The FASEB Journal*. 2015;29(6):2616. doi:10.1096/FJ.15-270637
82. 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
83. Huang Z, Ogasawara D, Seneviratne UI, et al. A global portrait of protein targets of metabolites of the neurotoxic compound BIA 10-2474. *ACS Chem Biol*. 2019;14(2):192. doi:10.1021/ACSCHEMBIO.8B01097
84. 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