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# Role antioxidační obrany v syntéze antidiabetických lipokinů

# Role of antioxidant defense in the synthesis of antidiabetic lipokines

Disertační práce

Školitel: RNDr. Ondřej Kuda, Ph.D.

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## ABSTRACT

Fatty acid esters of hydroxy fatty acids (FAHFAs) are a recently discovered group of lipokines consisting of a fatty acid attached to a hydroxy fatty acid with an ester bond resulting in a diverse group of compounds with many different biological activities. Antidiabetic and anti-inflammatory activities are the most studied.

The thesis aimed to study this group of bioactive lipids to elucidate the metabolism of FAHFAs and describe the role of antioxidant defense, namely peroxiredoxin 6 (Prdx6), in their biosynthesis with the help of isotopic labeling together with in vitro and in vivo experiments. All the samples, including white adipose tissue, liver, and even human breast milk, were subjected to untargeted or targeted lipidomic and metabolomics analysis using LC-MS/MS.

We used the data from isotopically labeled experiments to describe the role of 5-PAHSA in glucose uptake and to show which specific pathways are stimulated by 5-PAHSA administration and which by insulin to compare the effect of both antidiabetic agents. In our samples, we analyzed TAG estolides, which function as intracellular reservoirs of FAHFAs, and managed to describe the role of specific lipases in their metabolism. We reported the role of Prdx6 and the specific enzymatic activity involved in synthesizing precursors that could be utilized for FAHFA biosynthesis. At last, we investigated if any changes in human breast milk metabolome of FAHFA levels were caused by timing, mode of delivery, or lactation stage.

The findings mentioned in this dissertation thesis provide insight into the metabolism of FAHFAs and TAG estolides and can be used in further research.



**Key words**: antioxidant defense, FAHFAs, lipokines, metabolic pathways, peroxiredoxin 6, TAG estolides, white adipose tissue

# ABSTRAKT

Větvené estery mastných kyselin, zkráceně FAHFA, jsou poměrně nedávno objevené biologicky aktivní lipidy spadající do skupiny lipokinů. Tyto látky se skládají z mastné a hydroxy mastné kyseliny, které jsou spojené esterovou vazbou. Jedná se o poměrně velkou skupinu látek, jejichž zástupci mohou vykazovat různé účinky, nejčastěji jsou zmiňovány hlavně antidiabetické a protizánětlivé.

Cílem této práce bylo prostudovat metabolismus těchto látek a objasnit, jakou roli má v biosyntéze FAHFA antioxidační obrana, především antioxidační enzym peroxiredoxin 6 (Prdx6). K naplnění cílů práce jsme využili izotopového značení společně s in vitro a in vivo modely. Všechny vzorky, včetně bílé tukové tkáně, jater a mateřského mléka, byly podrobeny necílové i cílové lipidomické a metabolomické analýze s využitím LC-MS/MS.

Výsledky získané pomocí izotopového značení znázorňují úlohu 5-PAHSA při zpracování glukózy, metabolické dráhy aktivované po jejím podání a porovnání účinků 5-PAHSA a insulinu. Ve vzorcích jsme navíc detekovali TAG estolidy, z jejichž stuktury jsou FAHFA v případě potřeby uvolňovány působením specifických lipáz. Byla také popsána role peroxidázové aktivity Prdx6 v syntéze prekurzorů, které mohou být následně použity k biosyntéze FAHFA. Nakonec jsme se zaměřili na analýzu mateřského mléka, kde jsme sledovali, jak termín, druh porodu a laktační fáze ovlivňuje složení mateřského mléka, především hladiny lipokinů FAHFA.

Poznatky uvedené v této disertační práci poskytují nové informace o metabolismu FAHFA a TAG estolidů, na jejichž základech může stavět budoucí výzkum.



**Klíčová slova**: antioxidační obrana, bílá tuková tkáň, FAHFA, lipokiny, metabolické dráhy, peroxiredoxin 6, TAG estolidy

# CONTENTS

# ABBREVIATIONS

| | |
|---|---|
| 12,13-diHOME | 12,13-dihydroxy-9Z-octadecenoic acid |
| 4-HNE | 4-hydroxynonenal |
| ADTRP | Androgen-dependent TFPI regulating protein |
| AIG1 | Androgen-induced gene 1 |
| AKO | ATGL deficient mouse model |
| AKR | Aldo-keto reductases |
| ARE | Antioxidant response element |
| AT | Adipose tissue |
| ATGL | Adipose triglyceride lipase |
| ATX | Autotaxin |
| BAT | Brown adipose tissue |
| BHT | Butylated hydroxytoluene |
| BSA | Bovine serum albumin |
| β-Gal | β-Galactosidase |
| C18:1-LPA | Oleoyl-lysophosphatidic acid |
| C47S | Knock-in mouse model with mutating cysteine at position 47 to serine |
| CE | Cold exposure |
| CEL | Carboxyl ester lipase |
| CES3 | Carboxylesterase 3 |
| CGI-58 | Comparative gene identification 58 |
| ChREBP | Carbohydrate response element-binding protein |
| CoA | Coenzyme A |
| COX | Cyclooxygenases |
| CS | Caesarean section |
| CYP450 | Cytochrome P450 oxidases |
| D140A | Knock-in mouse model with mutating aspartate at position 140 to alanine |
| DAG | Diacylglycerol |
| *db/db* mice | Type 2 diabetic mice |
| DDHD2 | DDHD domain containing protein 2 |
| DHA | Docosahexaenoic acid |
| DHAHLA | Docosahexaenoic acid ester of hydroxy linoleic acid |
| DM | Diabetes mellitus |
| DMEM | Dulbecco’s modified eagle medium |

| | |
|---|---|
| DNL | De novo lipogenesis |
| EPHX | Epoxide hydrolases |
| ERAD | Endoplasmic reticulum-associated degradation |
| eWAT | Epididymal white adipose tissue |
| FA | Fatty acid |
| FAHFAs | Fatty acid esters of hydroxy fatty acids |
| FAHLAs | HLA-containing FAHFAs |
| FFA | Free fatty acid |
| GLUT-4 | Glucose transporter 4 |
| GPCR | G protein-coupled receptor |
| GPX4 | Glutathion peroxidase 4 |
| GSH | Glutathione |
| HEK293T | Human embryonic kidney (HEK) 293T cells |
| HFA | Hydroxy fatty acid |
| HFD | High-fat diet |
| HLA | Hydroxy linoleic acid |
| HpFA | Hydroperoxy fatty acid |
| HSL | Hormone-sensitive lipase |
| IgE | Immunoglobulin E |
| IL-3 | Interleukin 3 |
| IsoPs | Isoprostanes |
| ITT | Insulin tolerance test |
| Keap1 | Kelch-like ECH-associated protein 1 |
| KO | Knock-out mouse model |
| LAHLA | Linoleic acid ester of hydroxy linoleic acid |
| LC-MS | Liquid chromatography coupled to mass spectrometry |
| LC-MS/MS | Liquid chromatography coupled to tandem mass spectrometry |
| LLE | Liquid-liquid extraction |
| LOESS | Locally estimated scatterplot smoothing |
| LOX | Lipoxygenases |
| LPA | Lysophosphatidic acid |
| LPCAT | Lysophosphatidylcholine acyltransferase activity |
| MAG | Monoacylglycerol |
| MDA | Malondialdehyde |
| MJ33 | Inhibitor of $PLA_2$ activity |

| | |
|---|---|
| ML210 | Inhibitor of phospholipid peroxidase activity |
| MTBE | Methyl *tert*-butyl methyl ether |
| MUFAs | Monounsaturated fatty acids |
| NAFLD | Non-alcoholic fatty liver disease |
| NASH | Non-alcoholic steatohepatitis |
| NOXs | Nicotinamide adenine dinucleotide phosphate oxidases |
| Nrf2 | Nuclear factor erythroid 2-related factor 2 |
| OAHPA | Oleic acid ester of hydroxy palmitic acid |
| OGTT | Oral glucose and insulin tolerance tests |
| OxPLs | Oxidized phospholipids |
| PA | Phosphatidic acid |
| PAHPA | Palmitic acid ester of hydroxy palmitic acid |
| PAHSA | Palmitic acid ester of hydroxy stearic acid |
| PB | Preterm birth |
| PBS | Phosphate-buffered saline |
| PC | Phosphatidyl choline |
| $PGE_2$ | Prostaglandin $E_2$ |
| PI (18:1/18:1) | 1,2-dioleoyl-*sn*-glycero-3-phospho-(1'-myo-inositol) |
| $PLA_2$ | Phospholipase $A_2$ activity |
| PL-OOHs | Peroxidized phospholipids |
| PLs | Phospholipids |
| PM20D1 | Peptidase M20 domain containing 1 |
| PNPLA1 | Patatin-like phospholipase domain containing 1 |
| PNPLA2 | Patatin-like phospholipase domain containing 2 |
| PNPLA3 | Patatin-like phospholipase domain containing 3 |
| PO | Palmitoleic acid |
| PPARα | Peroxisome proliferator-activated receptor alpha |
| Prdx6 | Peroxiredoxin 6 |
| Prdx6 null | Mouse model with deficient Prdx6 |
| PUFAs | Polyunsaturated fatty acids |
| QC | Quality control |
| RNA | Ribonucleic acid |
| ROS | Reactive oxygen species |
| RSD | Relative standard deviation |
| SCD1 | Stearoyl-CoA desaturase 1 |

| | |
|---|---|
| scWAT | Subcutaneous white adipose tissue |
| SEM | Standard error of the mean |
| SFAs | Saturated fatty acids |
| SGLT-2i | Sodium-glucose co-transporter-2 |
| sMaf | Small musculoaponeurotic fibrosarcoma proteins |
| SPE | Solid phase extraction |
| TAG | Triacylglycerol |
| TAG EST | Triacylglycerol estolide |
| TBARS | Thiobarbituric acid reactive substances |
| TN | Thermoneutrality |
| TNP | Trinitrophenol |
| UCP1 | Uncoupling protein 1 |
| UPR | Unfolded protein response |
| VB | Vaginal birth |
| WAT | White adipose tissue |
| WT | Wild-type |

# INTRODUCTION

## 1 Oxidative Stress

Oxidative stress is an imbalance between the production of oxidants such as reactive oxygen species (ROS) and the ability of an organism to cope with them (Sies & Cadenas, 1985). It is of general knowledge that the main contributors to the endogenous production of ROS are oxidative enzymes such as cytochrome oxidases (CYP450) and nicotinamide adenine dinucleotide phosphate oxidases (NOXs), mitochondrial respiratory chain (Phaniendra et al., 2015; Zhang et al., 2022). Oxidative stress leads to lipid peroxidation and damage of nucleic acids and proteins, resulting in tissue damage or cell death (Forman & Zhang, 2021). Recently, a new type of programmed cell death called ferroptosis was described. The process of ferroptosis does not show typical characteristics of autophagy, necrosis, or apoptosis. It is dependent on iron and distinguished by accumulated peroxidized lipids (Dixon et al., 2012).

Oxidative stress has been linked with several pathological conditions, including obesity (Chattopadhyay et al., 2015; Furukawa et al., 2004), cancer (Dhupper et al., 2022; Janion et al., 2020), diabetes (Ahmed et al., 2006; Chattopadhyay et al., 2015; Jiang et al., 2019; Khand et al., 2022) and insulin resistance (Houstis et al., 2006; Pepping et al., 2013), pulmonary (Hamid et al., 2019; Kellner et al., 2017), neurodegenerative (Abd El Mohsen et al., 2005; Bosco et al., 2006; Chung et al., 2016), kidney (Khand et al., 2022), and cardiovascular diseases (Hamid et al., 2019; Zhao et al., 2008). However, reactive oxygen species also have an essential role in the immune system and cell signaling. (Mittal & Murad, 1977; Roth & Droge, 1987).

### 1.1 Lipid Peroxidation

Lipids prone to peroxidation generally contain one or more carbon-carbon double bonds, such as polyunsaturated fatty acids (PUFAs). Biological membranes consist mainly of PUFA-containing phospholipids, making them the primary target for lipid peroxidation, which may be driven enzymatically or non-enzymatically (Chng et al., 2021).

Products of non-enzymatic lipid peroxidation include lipid hydroperoxides (Fig. 1), 4-hydroxynonenal (4-HNE), malondialdehyde (MDA), and isoprostanes (IsoPs). Some of the products are toxic or mutagenic (Esterbauer et al., 1990). As they were linked with most of the above-mentioned health disorders, they are also subjects of quantification as oxidative stress biomarkers (Eskelinen et al., 2022; Jatavan et al., 2020; Vanova et al., 2018; Wang et al., 2022). Moreover, the isoprostane 15-F2t-IsoP derived from arachidonic acid is reported as the gold standard oxidative stress marker. Some long-established methods of determining oxidative

stress are inaccurate, for example, TBARS (thiobarbituric acid reactive substances) assay, which can produce false-positive results (Feillet-Coudray et al., 2019; Jung et al., 2016).

Enzymatically oxidized lipids function as signaling molecules and hormones, and their biosynthesis is controlled by lipoxygenases (LOX), cyclooxygenases (COX), cytochrome P450s (CYP450), and aldo-keto reductases (AKR), which catalyze the oxidation of PUFAs or sterols (Hajeyah et al., 2020). This group comprises several compounds, such as oxylipins, endocannabinoids, steroid hormones, and others that participate in inflammatory processes, immune response, and various diseases, including cardiovascular (Ou et al., 2021; Pinckard et al., 2021; Sido et al., 2016).

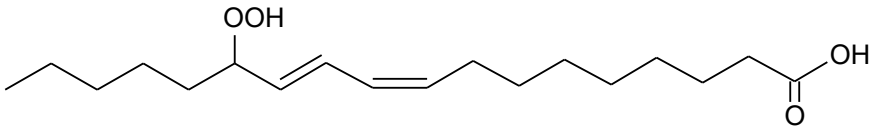



Figure 1. Linoleic acid 13-hydroperoxide

## 2 Antioxidant Defense

Living organisms have many protective mechanisms to prevent, intercept or repair oxidative damage to organs and tissues. Antioxidants can be divided into enzymatic and non-enzymatic, including vitamins, flavonoids, glutathione (GSH), and others (Gulcin, 2020). Oxidative stress has been linked with various diseases. Therefore, extensive research focused on antioxidants and antioxidant defense is of interest. Although antioxidants showed promising results in basic research and preclinical trials, the potential of antioxidant therapy has yet to be fulfilled in clinical studies (reviewed in (Forman & Zhang, 2021)).

### 2.1 Nuclear Factor Erythroid 2-Related Factor 2

The transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) is a vital regulator of the antioxidant defense system as many of its target genes ameliorate the manifestation of oxidative stress to restore redox homeostasis (Dodson et al., 2019). The Nrf2 signaling pathway is regulated by Kelch-like ECH-associated protein 1 (Keap1). During redox homeostasis, two Keap1 molecules are attached to Nrf2, causing its degradation. However, under oxidative stress conditions, Keap1 is oxidized and thus inactivated. Nrf2 is stabilized and translocated into the nucleus, forming a heterodimer with small musculoaponeurotic fibrosarcoma (sMaf) proteins (Itoh et al., 2003; Motohashi et al., 2004; Tong et al., 2006). This complex is then bound to the antioxidant response element (ARE), resulting in expressing antioxidant and cytoprotective genes such as peroxiredoxin 6 (Chhunchha et al., 2022; Itoh et al., 1997; Rushworth et al., 2008). Except for maintaining redox homeostasis, Nrf2 has been reported to play a crucial role in distinct metabolic pathways such as iron metabolism and

ferroptosis associated with it (Dong et al., 2021), apoptosis (Fan et al., 2022), xenobiotic metabolism (Forootan et al., 2017; Lubelska et al., 2016; Nishimoto et al., 2017), carbohydrate and lipid metabolism (Ding et al., 2019; Liu et al., 2021) with which biosynthesis of lipokines called FAHFAs was specifically linked (Kuda et al., 2018). This means that the disruption of the Nrf2 signaling pathway results in a wide range of health risks (W. Chen et al., 2021; Lu et al., 2022; Mancini et al., 2022).

### 2.2 Peroxiredoxin 6

Peroxiredoxin 6 (Prdx6) is the last identified enzyme of the peroxiredoxin family. It forms an important part of the antioxidant defense due to its ability to repair peroxidized phospholipids (PL-OOHs) (Fisher, 2017; Kang et al., 1998). Sometimes the enzyme is called 1-cys peroxiredoxin because its activity is based only on a single active cysteine compared to the common 2-cys mechanism in most peroxiredoxins (Rhee & Woo, 2020). Prdx6 forms a dimer (Fig. 2), which is crucial for its function and for Prdx6 regeneration. In contrast with most other peroxiredoxins, Prdx6 uses glutathione (GSH) as a reducing agent instead of thioredoxin (Kang et al., 1998; Zhou et al., 2016). This enzyme has three enzymatic activities: glutathione peroxidase activity, phospholipase $A_2$ ($PLA_2$) activity, and lysophosphatidylcholine acyltransferase (LPCAT) activity. This makes Prdx6 a unique member of the mammalian peroxiredoxin family (Feinstein, 2019).

Peroxidase activity is capable of reducing peroxidized phospholipids to the corresponding alcohols, and it is dependent on catalytic cysteine at position 47 (Fisher et al., 1999). From all Prdx members, Prdx6 is the only one to possess $PLA_2$ activity, which hydrolyzes an oxidized fatty acid (FA) bound at the *sn-2* position resulting in the formation of lysophospholipids. For proper function, $PLA_2$ activity requires a catalytic triad consisting of aspartate at position 140, histidine at position 26, and serine at position 32 (Fisher et al., 2005; Manevich et al., 2007). LPCAT activity is the last discovered activity of Prdx6. It is linked to the $PLA_2$ activity and it is responsible for the re-acylation of lysophospholipid with FA-CoA to the *sn-2* position (Fisher et al., 2016). Several mouse models with genetically modified Prdx6 have been created over the years to enable detailed investigation of Prdx6 (summarized in (Feinstein, 2019)) as Prdx6 has been linked with diabetes, acute lung injury and inflammation, and male fertility (Bumanlag et al., 2022; Fisher et al., 2021; Li et al., 2020; Novoselova et al., 2021). Moreover, it is assumed to participate in ferroptosis, associated with cancer (Lu et al., 2019; Xu et al., 2016).

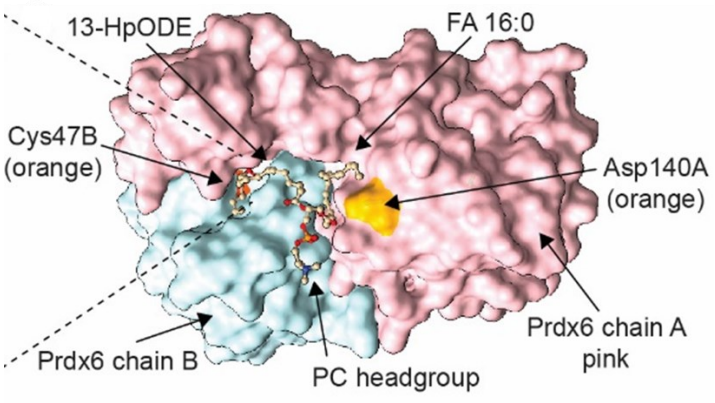



Figure 2. Model of the Prdx6 dimer (Paluchova et al., 2022)

## 3 Lipokines

Adipose tissue (AT) is a complex organ not only known for its protective and storage function. For the past few decades, the endocrine function has been of interest as adipose tissue affects metabolic homeostasis, neuroendocrine function, and immune function through the secretion of various bioactive compounds; adipokines (Scherer et al., 1995; Zhang et al., 1994), lipokines (Cao et al., 2008), and exosomal microRNAs (Ying et al., 2017).

The term lipokine was introduced in 2008 by Hotamışlıgil Lab to describe a group of adipose depot-derived bioactive lipids that connects adipose tissue with other non-adipose tissues, including liver, muscle, and pancreas and regulate systemic metabolic responses (Cao et al., 2008; Ying et al., 2017). However, not all bioactive lipids produced by adipose tissue can be called lipokines, for instance, prostaglandins. These lipid mediators were the first ones linked with AT in the late 1960s and they function as autocrine and paracrine signaling molecules (Jabbour et al., 2006; Shaw & Ramwell, 1968). In contrast, lipokines act as endocrine mediators due to their presence in blood plasma (Cao et al., 2008; Rancoule et al., 2013; Yore et al., 2014). Lipokines were initially described as compounds produced solely by adipose tissue, but according to recent studies, lipokines might also be synthesized in other metabolic tissues (Burhans et al., 2015; Liu et al., 2016).

Lipokines are a complex group of the lipid family with their members having diverse structures. The common part of the structure for all lipokines is a fatty acid, but it differs in the degree of unsaturation, oxidation, and chain length. This group includes various compounds such as lysophosphatidic acid (Bandoh et al., 2000; Boucher et al., 2005), palmitoleic acid (Bolsoni-Lopes et al., 2014; Cruz et al., 2018), 12,13-diHOME (Nieman et al., 2014; Stanford et al., 2018), and N-acyl amino acids (Gao et al., 2022; Lin et al., 2018), which are better-known representatives. Furthermore, new lipokines are being described, such as PI (18:1/18:1), which has recently joined the lipokine family (Gutierrez-Juarez et al., 2006; Thurmer et al., 2022).

Although each member exerts specific effects, all of their biological activities are based on the connection to glucose and lipid metabolism (Betz et al., 2021; Gao et al., 2022; Lynes et al., 2017; Miklankova et al., 2022; Rancoule et al., 2013).

### 3.1 FAHFAs

Fatty acid esters of hydroxy fatty acids (FAHFAs) are a group of lipokines discovered in 2014. As the name suggests, the structure of FAHFA comprises a fatty acid bound to a hydroxy fatty acid via an ester bond. Furthermore, the final number of FAHFA members is even higher when we consider the branching position of HFAs (Yore et al., 2014). Compounds varying in the branching position, so-called regioisomers, are commonly reported with the number of the branching carbon atom as regioisomers may exhibit different biological effects. An example of such a compound might be 5-PAHSA – palmitic acid ester of hydroxy stearic acid with the branching positing at the 5$^{th}$ carbon from the α-carbon atom (Fig. 3). Therefore, there is a number of possible FAHFAs structures, which makes this novel group of bioactive lipids quite extensive (Fig. 4) (Brejchova et al., 2020).

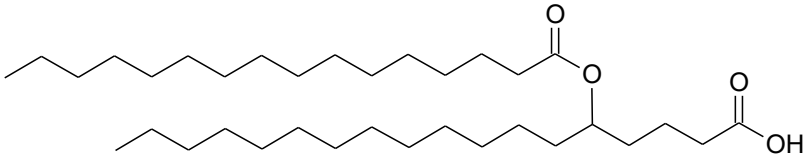



Figure 3. Structure of 5-PAHSA

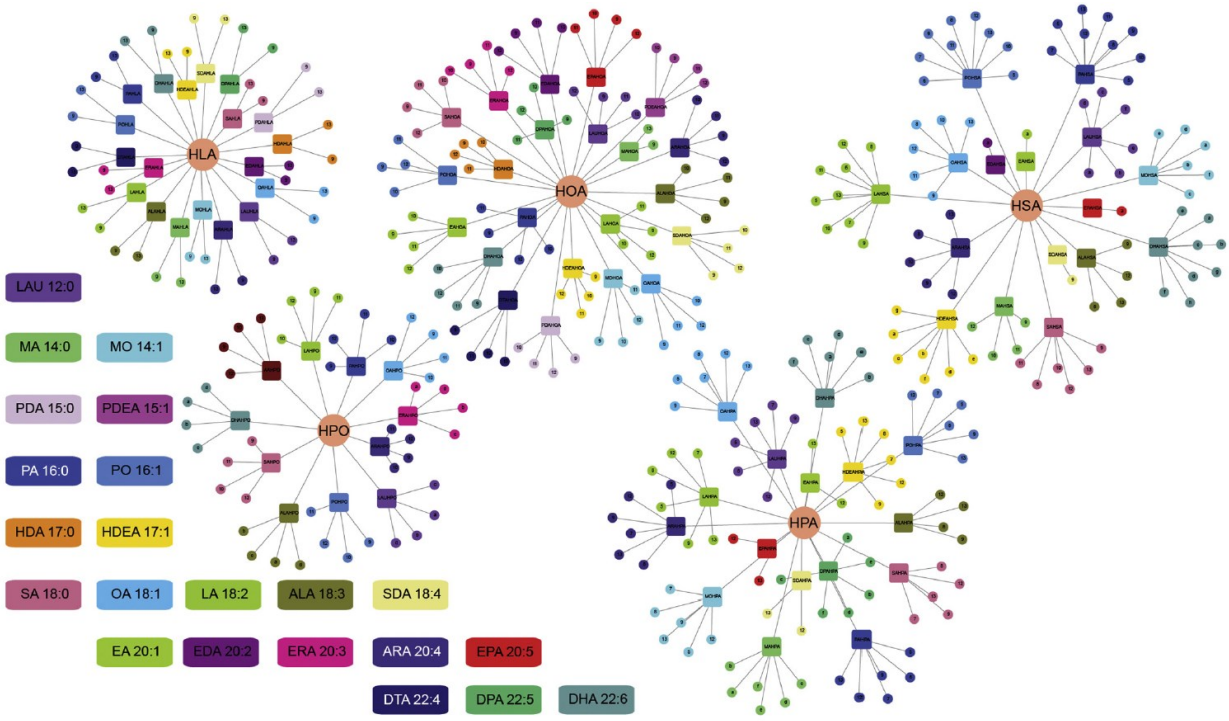



Figure 4. Illustrative scheme of most abundant FAHFA families (Brejchova et al., 2020)

### 3.1.1 Sources

The type and amount of FAHFAs vary, but FAHFA-rich food is found across animal- and plant-based products (Brezinova et al., 2018; Kolar et al., 2019; Liberati-Cizmek et al., 2019; Pham et al., 2019; Zhu et al., 2018). Although dietary intake was shown to affect levels of FAHFA in human plasma, there still needs to be more evidence for if and how FAHFAs survive the digestion process (Brezinova et al., 2018; Kellerer et al., 2021; Kolar et al., 2019; Syed et al., 2018). Since FAHFA hydrolytic enzymes have been identified, it is assumed that most FAHFAs come from endogenous sources (Kolar et al., 2016; Kuda et al., 2016; Patel et al., 2022; Yore et al., 2014). Branched FAHFAs were detected in serum, breast milk, and in a majority of tissues, including brown adipose tissue (BAT), white adipose tissue (WAT), liver, kidney, pancreas, heart, and thymus, with PAHSAs and SAHSAs (stearic acid ester of hydroxy stearic acid) prevailing in adipose tissues (BAT and WAT) (Brezinova et al., 2018; Dongoran et al., 2020; Hammarstedt et al., 2018; Kuda et al., 2016; Yore et al., 2014; Zhu et al., 2017). FAHFAs of a more linear structure (α- and ω-FAHFAs) were found in the tear film and meibum (Butovich et al., 2009; Lam et al., 2014; Wood, Donohue, et al., 2018), vernix caseosa (a protective coating on the skin of newborns) (Hancock et al., 2018; Vavrusova et al., 2020), sperm and semen (Wood et al., 2016), amniotic fluid (Wood, Ball, et al., 2018), and in the skin (Hancock et al., 2018).

### 3.1.2 Biosynthesis

Since FAHFAs consist of a fatty acid and a hydroxy fatty acid linked by an ester bond, the process of their biosynthesis is divided into three stages – synthesis of a fatty acid, synthesis of a hydroxy fatty acid, and formation of the ester bond between FA and HFA (summarized in (Riecan et al., 2021)). The process of an FA formation called de novo lipogenesis (DNL) generates palmitate in adipose tissue and the liver. The nascent palmitate molecule may then be further modified by elongation and/or desaturation (Carta et al., 2017; Eissing et al., 2013; Yilmaz et al., 2016). This pathway is regulated by a number of transcription factors, including carbohydrate response element-binding protein (ChREBP), which has been linked with insulin sensitivity and FAHFA biosynthesis (Herman et al., 2012; Vijayakumar et al., 2017; Yore et al., 2014). Fatty acids are not only produced by DNL. It was shown that fatty acid composition correlates with the diet, too (Hodson et al., 2020; Paluchova, Vik, et al., 2020; Pham et al., 2019).

While FA synthesis is well described, the formation of HFAs especially saturated HFAs, is yet to be elucidated. Although non-enzymatic oxidation and antioxidant defense have been linked with the synthesis of saturated FAHFAs (Kuda et al., 2018; Spickett, 2020), more is known

about unsaturated HFAs, which can be formed through enzymatically driven processes. Enzymes that could be involved in this pathway include lipoxygenases, cyclooxygenases, and CYP450, which are involved in the metabolism of eicosanoids (Christie & Harwood, 2020; Hajeyah et al., 2020; Ohno et al., 2015; Vik et al., 2019). HFA biosynthesis is also affected by the branching position. For α- and ω-HFAs that eventually form linear FAHFAs, synthetic pathways differ compared to branched HFA with a hydroxyl group located in the middle of the fatty acyl chain (Butovich et al., 2016; Hirabayashi et al., 2017; Vavrusova et al., 2020; Vik et al., 2019; H. Wang et al., 2019).

Regarding the formation of the ester bond between an FA and an HFA, the enzyme catalyzing this reaction was unknown for a long time. However, recently published work revealed the adipose triglyceride lipase (ATGL) known as patatin-like phospholipase domain containing 2 as FAHFA biosynthetic enzyme (Fig. 5). The reaction is carried out by its transacylation and lipase activities. Moreover, according to the published data, not FAs but triacylglycerols (TAGs) or diacylglycerols (DAGs) are direct precursors of FAHFAs. In the biosynthesis of FAHFAs, two precursor molecules are needed – one being an acyl donor and the other an acceptor with acyl chains 16:0, 16:1, and 18:1 as the preferred substrates. Then FA is transferred to HFA by ATGL; thus, a new molecule of FAHFA is created. As HFA is still attached to the precursor molecule (e.g., TAG), a new molecule FAHFA-containing TAG or so-called TAG estolide, is formed (Patel et al., 2022).

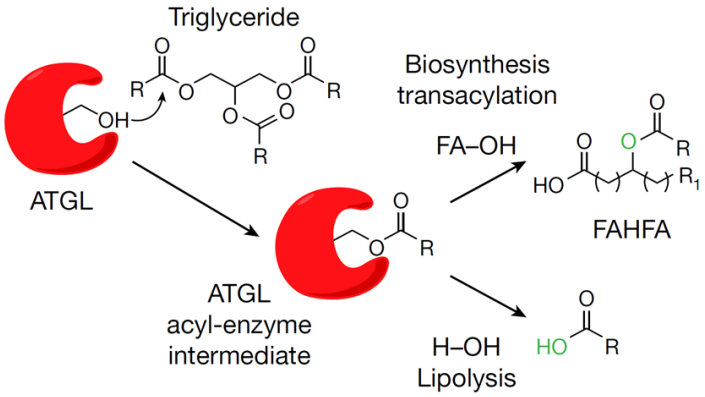



Figure 5. Scheme for FAHFA biosynthesis catalyzed by ATGL transacylation activity (Patel et al., 2022)

TAG estolide is a TAG-like compound with a FAHFA group attached to the glycerol backbone (Fig. 6). TAG estolides serve as a FAHFA reservoir in adipose tissue. Upon physiological stimuli such as fasting, FAHFAs can be released from the TAG estolide structure with the help of lipases, including ATGL and hormone-sensitive lipase (HSL). Besides FAHFA biosynthesis,

ATGL also participates in TAG estolide remodeling (Brejchova et al., 2021; Paluchova, Oseeva, et al., 2020; Tan et al., 2019).

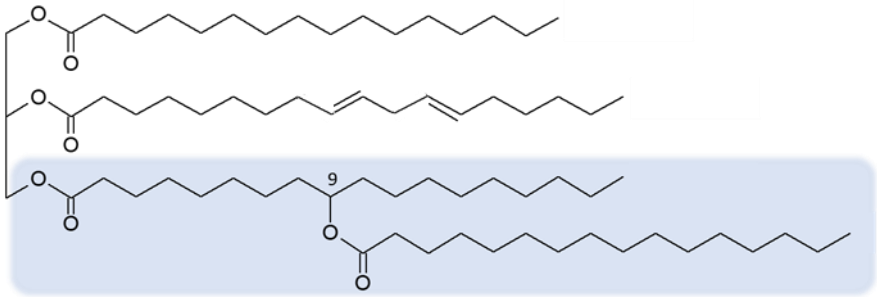



Figure 6. TAG estolide (9-PAHSA attached to the glycerol backbone is highlighted in blue)

### 3.1.3 Degradation

Various hydrolytic enzymes have been linked with the degradation of FAHFAs, including ATGL, HSL, carboxyl ester lipase (CEL), androgen-induced gene 1 (AIG1), and androgen-dependent TFPI regulating protein (ADTRP) (Brejchova et al., 2021; Erikci Ertunc et al., 2020; Patel et al., 2022). ADTRP and AIG1 were detected in the liver and brown adipose tissue, where they control the hydrolysis of FAHFAs (Erikci Ertunc et al., 2020; Parsons et al., 2016). CEL secreted by the pancreas or mammary gland is mainly found in the intestine, as its primary purpose is to hydrolyze lipids to facilitate their digestion. Interestingly, CEL prefers to hydrolyze FAHFAs over other substrates, such as TAGs or phospholipids (Brejchova et al., 2022; Brezinova et al., 2018; Patel et al., 2022). ATGL and HSL can hydrolyze FAHFA-glycerol ester bonds in TAG estolide structure, but they were also shown to degrade free FAHFAs (Brejchova et al., 2021).

### 3.1.4 Biological Activities

FAHFA structure can be built up basically of any FA and HFA. This class of bioactive lipids, therefore, includes many distinct compounds that differ in biological activities. The type of FA and HFA and the branching position affects the beneficial effects of FAHFAs (Aryal et al., 2021; Riecan et al., 2021). FAHFAs, especially those containing saturated hydroxy fatty acids such as 5-PAHSA, 9-PAHSA, 9-PAHPA, and 9-OAHSA, exert an antidiabetic effect as they stimulate glucose uptake, increase insulin sensitivity, and enhance glucose-stimulated insulin secretion (Bandak et al., 2018; Benlebna et al., 2020; Syed et al., 2018; Yore et al., 2014). Apart from that, the PAHSA family was also shown to improve adipocyte differentiation, 5-PAHSA positively affects DNL, lipolysis, and TAG remodeling, and 9-PAHSA promotes WAT browning (Hammarstedt et al., 2018; Paluchova, Oseeva, et al., 2020; Y. M. Wang et al., 2018a). Although PAHSAs belong among the most studied FAHFAs (Bandak et al., 2018;

Schultz Moreira et al., 2020; Syed et al., 2018; Vijayakumar et al., 2017; Y. M. Wang et al., 2018b; Zhou et al., 2019) a study investigating the effects of long-term intake of 9-PAHPA (palmitic acid ester of hydroxy palmitic acid) and 9-OAHPA (oleic acid ester of hydroxy palmitic acid) was published recently showing insulin-sensitizing effects in obese mice (Benlebna et al., 2020).

Other FAHFAs, including certain regioisomers from DHAHLA, LAHLA, OAHSA, POHSA, and PAHSA families, were reported to have anti-inflammatory effects (Kolar et al., 2019; Kuda et al., 2016; Paluchova, Vik, et al., 2020; Syed et al., 2019; H. Wang et al., 2019). For example, the beneficial effects of PAHSAs were linked with the intestinal tract in the prevention of inflammatory disease of the colon or with the maturation of the intestine in newborns (Brezinova et al., 2018; Lee et al., 2016). Moreover, long-term PAHSA administration diminishes pro-inflammatory macrophages in WAT (Syed et al., 2018). Other FAHFAs formed from polyunsaturated FAs or HFAs, such as 13-DHAHLA and 13-LAHLA, exert not only anti-inflammatory but also pro-resolving effects (Kolar et al., 2019; Kuda et al., 2016; H. Wang et al., 2019). Anti-inflammatory effects of FAHFAs seem to be mediated through G-protein coupled receptors such as (GPR 120 and GPR 40) and the inactivation of the NF-κB (nuclear factor kappa light chain enhancer of activated B cells) pathway (Syed et al., 2018; Y. M. Wang et al., 2018a; Zhou et al., 2019).

Although FAHFAs are known to have a range of positive biological effects on living organisms, recent investigations linked FAHFAs with health disorders, for instance, colorectal cancer and kidney disease, where FAHFA levels correlated with the disease progress (Hu et al., 2021; Liu et al., 2020).

### 3.2 Type 2 Diabetes

Type 2 diabetes mellitus (DM) is a chronic metabolic disorder. Type 2 DM is prevalent in adults and is known to be affected by genetic predisposition and a sedentary, unhealthy lifestyle (Hu et al., 2001; Ripsin et al., 2009). Obesity was proved to be closely related to type 2 diabetes as more than half of the patients were obese (Centers for Disease & Prevention, 2004). In nowadays population, the risk factors are so widespread that the number of type 2 DM cases has become epidemic in certain countries. International Diabetes Federation reported that more than 537 million people with type 2 DM had almost 7 million deaths, more than three times more than twenty years ago. In addition, they are predicting this number to increase by nearly 50% in another twenty years (Magliano & Boyko, 2021). As diabetes affects a significant number of people worldwide, new therapeutic approaches and antidiabetic compounds are being investigated.

Type 2 diabetic patients have impaired regulation and usage of glucose, leading to high blood glucose levels and insulin resistance (reviewed in (Reed et al., 2021)). Therefore, adipose tissue is being extensively studied as it secretes several insulin-resistant hormones but also several insulin-sensitizing compounds, including adiponectin (Frankenberg et al., 2017) and FAHFAs (Paluchova, Oseeva, et al., 2020; Yore et al., 2014). There are several approaches in antidiabetic therapy, such as drug monotherapy with an example of well-known metformin, incretin-based treatment, or therapy that targets other systems, such as the excretion system (Avogaro et al., 2018; Irwin & Flatt, 2015; Masarwa et al., 2021). However, each of them has its pros and cons. Metformin decreases blood glucose level, and its insulin-sensitizing effect is effective in many tissues. Although it has side effects, it is still the first-choice diabetes therapeutics (DeFronzo et al., 2016; Masarwa et al., 2021). The biggest advantage of incretin-based therapy are its several beneficial effects on the pancreas, β-cells, and insulin. Despite having great potential in treating diabetes, their fast degradation is significantly limiting for therapeutic use (Drucker et al., 2008; Nauck et al., 1993). Inhibitors of glucose reabsorption with sodium-glucose co-transporter-2 (SGLT-2i) showed to decrease glucose level and cardio-protective effects, but there is a risk of SGLT-2-associated ketoacidosis (Avogaro et al., 2018; Pasternak et al., 2019).

### 3.3 Cold Exposure

Cold exposure is associated with a short-term and long-term stay in an environment of 4°C. According to the time spent in a cold environment, we distinguish two types of cold exposure – acute and chronic. The acute response is recognized by shivering, whereas during chronic exposure, shivering disappears and is replaced by other mechanisms such as adaptive thermogenesis, which occurs mainly in brown adipose tissue, where cold acclimation stimulates the expression of uncoupling protein 1 (UCP1) (Castellani & Young, 2016; Lowell & Spiegelman, 2000; Wang et al., 2020). UCP1 is a protein localized in the inner mitochondrial membrane. Therefore, it is very abundant in mitochondria-rich BAT. UCP1 is a crucial protein for energy dissipation to produce heat in BAT (Enerback et al., 1997)[149]. However, cold exposure is used for the investigation of glucose (Vallerand et al., 1983; Wang et al., 2020) and lipid metabolism (Hiroshima et al., 2018; Nie et al., 2015) as it has beneficial effects on both of them. During cold acclimation, the whole body weight, together with the mass of WAT, decreases. TAG lipolysis is stimulated in reaction to low temperatures due to the upregulation of ATGL, which catalyzes the initial step of lipolysis. Interestingly, DNL is stimulated during chronic cold, and genes involved in TAG/FA cycling were upregulated (Flachs et al., 2017; Sepa-Kishi et al., 2019; Xu et al., 2019).

# HYPOTHESIS AND OBJECTIVES

The dissertation thesis focuses on the novel class of lipokines with beneficial biological activities. The main aim of the thesis was to elucidate the metabolic and synthetic pathways of these bioactive lipids, particularly the involvement of antioxidant defense. For this purpose, we used isotopic labeling techniques. Therefore, the related objective of the work was to optimize currently used LC-MS methods to analyze metabolites labeled with $^{13}C$ and $^{2}H$ isotopic tracers, which we used to study in vitro and in vivo models. As FAHFAs are known to exert various biological effects, a part of the research was dedicated to this topic too.

To fulfill the objectives of the thesis, the following hypotheses were applied:

- Cold exposure increases PAHSA levels, which consequently influence both glucose and lipid metabolism in white adipose tissue.
- Adipose triglyceride lipase and hormone-sensitive lipase are lipases that participate in the catabolism of triacylglycerol estolides.
- Antioxidant enzyme peroxiredoxin 6, especially its peroxidase activity, affects levels of FAHFA.
- FAHFA levels in human breast milk are affected by timing, mode of delivery, and lactation stage.

# MATERIAL AND METHODS

## 4 Standards and Chemicals

All used LC-MS solvents and chemicals were ordered from VWR International, Merck, and J. T. Baker (Czech Republic). Most FAHFA standards were obtained from Cayman Europe (Estonia). Still, the specific FAHFAs and TAG estolides were synthesized by our collaborators at the institute of Institut des Biomolecules Max Mousseron in France.

## 5 Samples

We used different cell lines, mouse models, and human samples to investigate FAHFA metabolism, synthesis, and their levels in various matrices.

### 5.1 Cell Cultures

In the experiments, we used two different cell lines – 3T3-L1 adipocytes and transfected HEK293T cells.

#### 5.1.1 3T3-L1

Differentiation of 3T3-L1 murine adipocytes was performed according to the standard protocol (Kuda et al., 2016) and then maintained in DMEM complete medium consisting of 25 mmol/L glucose, 10% FBS, 850 nM insulin, penicillin/streptomycin). The following procedure was adjusted according to the particular experiment.

To investigate lipid synthesis (**Publication I**), cells were kept in full DMEM with $^{2}H_2O$ and water (50/50, v/v) for three days and then extracted and analyzed. To test the effect of PAHSA treatment on glucose uptake, mature adipocytes were incubated in complete DMEM with or without 5-PAHSA for three days. Cells were then serum-starved in DMEM with 0.1% (w/v) BSA for 15 hours, followed by washing and incubation in DMEM without glucose and glutamine for 30 min. To follow the carbon flux during glucose uptake, cells were labeled with $^{13}C_6$-glucose (5.5 mmol/L) in the presence or absence of 5-PAHSA (40 μmol/L) at different time points. Cells were quenched in an ice-cold water bath and washed with ice-cold PBS, and the metabolism was lysed in a mixture of methanol/water and frozen in liquid nitrogen. The modified version of the protocol was used to treat adipocytes with insulin (10 nmol/L). The $^{13}C_5$-glutamine tracer was used to explore the utilization of glutamine carbon atoms. This cell line was also used to reveal the participation of Prdx6 enzymatic activities in FAHFA synthesis (**Publication III**). Adipocytes were treated as described above, but full DMEM was supplemented with a mixture of polyunsaturated fatty acids complexed to BSA in the ratio 3:1 for four days to provide building blocks for complex FAHFAs. Inhibitors

of specific Prdx6 activities were added alone or in combination for the last 24 hours. Cells were then rinsed and frozen on liquid nitrogen, as previously described.

### 5.1.2 HEK293T

In the study of the role of ATGL and HSL in the catabolism of TAG estolides (**Publication II**), we used a human embryonic kidney cell line (HEK293T) overexpressing these two proteins together with β-galactosidase (β-Gal). They were collected 24 hours after transfection and lysed with buffer A and sonicated on ice. The homogenates were centrifuged, and the protein concentration of the supernatant was determined to be used in the following experiment.

## 5.2 Animal Studies

All the animal studies were carried out with C57BL/6J or C57BL/6N mice and with the approval of the local ethical committee in the country where the experiment was conducted. Mice were generally kept in a stable environment of 20-22°C, constant humidity, a standard light-dark cycle, and with water and a standard chow diet ad libitum unless stated otherwise. In certain studies, we also used genetically modified mice – ATGL knock-out mice (**Publication I, II**), HSL knock-out mice (**Publication II**), and mouse models with genetically altered Prdx6 (**Publication III**).

To investigate the effect of 5-PAHSA on TAG/FA metabolism in WAT (**Publication I**), mice were kept at 30°C (thermoneutrality) or 6°C (cold exposure) for one week. After three days, mice received treatment – 5-PAHSA (445 mg/kg) or vehicle gavage, and two days before sacrifice, they were injected a dose of 3,5 mL/100 g body weight of solution consisting of 0.9% NaCl w/v in 99.9% $^{2}H_2O$ to track de novo lipogenesis in WAT. Moreover, mice were provided with drinking water with 10% $^{2}H_2O$ to stabilize the level of isotopic tracer in their body. We collected plasma and tissues of interest, such as epididymal white adipose tissue (eWAT), and quickly froze them in liquid nitrogen until extraction. ATGL knock-out and corresponding wild-type (WT) mice were either fasted for 12 hours before dissection to compare fed and fasted states, or they were subjected to CE for different periods of time to compare the effect of acute and chronic CE.

ATGL and HSL knock-out mice were used to test the effect of these lipases on TAG estolide metabolism (**Publication II**). Mice were kept at 25°C and a 10-hour dark cycle with free access to water and diet. Mice were fasted for 12 to 14 hours, and WAT was used for extraction and further analysis.

To study the role of Prdx6 in the biosynthesis of FAHFAs, we used three mouse models of genetically modified Prdx6 (**Publication III**) and wild-type mice as a control. The team

of prof. Fisher created knock-out mice, which were bread in the animal facility at the Institute for Environmental Medicine, Perelman School of Medicine of the University of Pennsylvania. All the modified mice had the C57BL/6J background. The knock-out model (Prdx6 null) with no activity of the Prdx6 enzyme was produced by the inactivation of the Prdx6 gene in embryonic stem cells, which were then used for breeding homozygous Prdx6 null mice from its heterozygous predecessor. The only disadvantage of Prdx6 null mice was the reduced fertility compared to WT mice. For the production of knock-in models (C47S and D140A) was used so-called recombineering method when the mutation was inserted with the help of E. coli at the Gene Targeting Core and Laboratory and the Transgenic and Chimeric Mouse Facility of the University of Pennsylvania. To abolish peroxidase activity linked with the cysteine at position 47 in the amino acid sequence of peroxiredoxin 6, the cysteine was mutated to serine, hence the mouse model designation C47S. The last knock-in model was created accordingly. As the $PLA_2$ activity relies on a catalytic triad, mutations of all three amino acids were tested, and consequently, a mutation of the aspartate at the position 140 to alanine (D140A) was selected as the suitable one because, unlike the other two mutations, it is not essential for binding of the phospholipid to the enzyme. In this study (**Publication III**), we used both sexes (males and females), 10-13 weeks old. All mice were kept at standard conditions described above. At the time of the experiment, they were fasted for 24 h and then re-fed overnight with drinking water supplemented with 15% sucrose before sacrifice. The liver and scWAT were collected, flash frozen in liquid nitrogen, and stored in a -80°C freezer upon extraction.

### 5.3 Human Samples

Human breast milk samples (**Publication IV**) were collected at the Institute for Mother, and Child Care in Prague with signed informed consent of participants of the study and the approval of the Research Ethics Board of the Institute for Mother and Child Care. Apart from the mode of delivery (vaginal birth vs. caesarean section) and term of delivery (preterm vs. term delivery), lactating mothers were healthy and did not suffer from acute infection or any disease, including diabetes. All study participants fulfilled the following criteria: BMI 19-24, gestational age 38 - 41 weeks for the term delivery group and < 32 weeks for the preterm delivery group, and had sufficient breast milk. Infants born in term were 72 h and 28 days old at the time of sample collection. These two time points were the same in a group of preterm newborns, with one extra time point corresponding to the $36^{th}$ week of gestational age. More information about breast milk donors is provided in Table 1. Breast milk samples were frozen

at -20°C within 30 min after collection and transported to an -80°C freezer within three days at the latest.

| | Term delivery | | Preterm delivery |
|---|---|---|---|
| | Vaginal birth (VB) | Caesarean section (CS) | Preterm birth (PB) |
| Maternal characteristics | | | |
| Sample size (n) | 27 | 26 | 20 |
| Age at birth (years) | 32.4 ± 0.8 | 32.6 ± 0.8 | 31.0 ± 0.9 |
| BMI (kg/m$^2$) | 22.2 ± 0.5 | 23.0 ± 0.5 | 21.7 ± 0.6 |
| Gestational weight gain (kg) | 12.2 ± 1.0 | 14.4 ± 1.3 | 8.9 ± 0.9 |
| Gravidity | 2.4 ± 0.2 | 2.2 ± 0.2 | 1.5 ± 0.1 |
| Parity | 1.7 ± 0.1 | 1.7 ± 0.1 | 1.3 ± 0.1 |
| Delivery method | | | |
| Vaginal | 27 | 0 | 6 |
| Caesarean section | 0 | 26 | 14 |
| Milk sample | | | |
| 72 h | 27 | 26 | 20 |
| 28 days | 18 | 21 | 15 |
| 36$^{th}$ gestational week | – | – | 10 |
| Newborn characteristics | | | |
| Gestational age at delivery (wk + d) | 40 + 3 ± 1d | 39 + 2 ± 1d | 29 + 4 ± 1d |
| Sex (M/F) | 11 / 16 | 17 / 9 | 8 / 12 |
| Birth weight (g) | 3613 ± 67 | 3323 ± 74 | 1255 ± 65 |
| Birth height (cm) | 50.4 ± 0.3 | 50.0 ± 0.3 | – |

Table 1. Anthropometrics of breast milk donors and their infants

## 6 Extraction Protocols

In accordance with the study's design, various extraction protocols were used or modified. Generally, we are primarily interested in metabolomics and lipidomics. However, for certain lipid classes, such as FAHFAs and isoprostanes, special extraction protocols had to be established and optimized according to the matrix type to ensure the maximum yield.

### 6.1 Lipidomics and Metabolomics

Lipidomics and metabolomics sample preparation workflow was based on the method published by Matyash (Matyash et al., 2008) and further modified. The liquid-liquid extraction (LLE) uses methyl *tert*-butyl methyl ether (MTBE), methanol, and water. Since a complex spectrum of analytes is extracted with the extraction protocol of which some may be unstable, the whole procedure is performed on ice with ice-cold solvents of LC-MS purity grade. The amounts of solvents used for extraction and aliquots were taken following LC-MS analysis and adjusted according to the used matrix. The main advantage of extracting polar and non-polar metabolites at the same time is that it saves the amount of samples needed for extraction, allowing more analyses to be performed.

### 6.1.1 Plasma

For extraction of polar and non-polar metabolites, 25 µL of plasma was used in a 1.5 mL tube, followed by the addition of 165 µL of methanol (MeOH), 600 µL of MTBE, and 165 µL of 10% MeOH/90% $H_2O$ with appropriate internal standards. Between each addition of a solvent, a tube was vortexed for 10 s and centrifuged at 16 000 × g for 10 min at 4°C to properly separate the polar and organic phases. Then aliquots for lipidomic (the upper phase) and metabolomic (the bottom phase) profiling were pipetted in separate plastic tubes, evaporated using vacuum concentrator SpeedVac (Savant SPD130LX, Thermo Scientific, Massachusetts, USA), combined with refrigerated vapor trap (Savant RVT450, Thermo Scientific, Massachusetts, USA). Then, resuspended in appropriate solvents, vortexed, centrifuged, and transferred into LC-MS vials.

### 6.1.2 Tissues

Tissues were cut into approximately 20 mg pieces and placed in a 2 mL tube with 275 µL of methanol and the same amount of 10% MeOH /90% $H_2O$. To provide a representative sample for analysis, all tissues had to be homogenized using a bead mill homogenizer MM400 (1.5 min, 30 Hz) (Retsch, Germany) followed by sonication. Then 1000 µL of MTBE was added and the homogenized mixture was vortexed for 20 s followed by centrifugation as described above. Aliquots for lipidomic and metabolomic analyses were transferred in a separate tube, and the protocol was followed as mentioned above. We used one more aliquot for TAGs and TAG EST analysis for adipose tissues due to the high fat content to avoid suppression of the signal for other non-polar analytes.

### 6.1.3 Cells

The extraction protocol for lipidomic and metabolomic profiling of cell samples was the same for non-adipose tissues. However, since the cells had to be flash-frozen to quench the metabolism at the specific time point, cells were lysed by adding methanol and a mixture of methanol and water directly to the well with cells and then frozen in liquid nitrogen. Before extraction, wells were scraped off with a cell lifter and transferred in a 2 mL tube. An appropriate amount of MTBE was added, and the whole mixture was sonicated until homogeneous. The protocol was then followed as described above.

### 6.1.4 Human Breast Milk

Firstly, samples were thawed in a water bath warmed up to 37°C for 5 min followed by 3 min sonication to ensure homogeneity of samples. Homogeneous breast milk (300 µL) was then pipetted in a 5 mL tube as fast as possible to prevent degradation of analytes followed

by addition of 660 µL of methanol, 2400 µL of MTBE, and 500 µL of 10% MeOH/90% $H_2O$. Samples were sonicated for 10 s in between the solvent addition and for 20 s after the addition of the mixture of methanol/water followed by brief sonication and centrifugation of the whole mixture. Similarly to the protocols described above, the appropriate amount of aliquots was transferred in a new tube, evaporated, cleaned up if needed, resuspended, centrifuged, and moved to an LC-MS vial.

### 6.2 FAHFAs

Two extraction protocols might be used for FAHFA extraction – either the MTBE protocol for lipidomic and metabolomic analysis mentioned above or a special protocol using citrate buffer and ethyl acetate based on the method published by Yore et al. in 2014 (Yore et al., 2014). In both cases, FAHFAs are present in the upper organic phase. Since FAHFAs are not the most abundant lipid species, the extract must be cleaned up and concentrated prior to the quantification. For this purpose, we are using solid phase extraction (SPE). Similarly to previous MTBE extraction, also the extraction of FAHFAs is performed on ice with pre-chilled solvents to ensure the maximum yield.

#### 6.2.1 Plasma

For extraction of FAHFAs from plasma, 150 µL of plasma and 350 µL of citrate buffer (pH = 3.6) were needed as acidic pH helps to protonate acidic lipids, which consequently facilitates their transition to the upper organic phase. Then 1 mL of ethyl acetate with internal standard was added to a 2 mL tube, and the whole mixture was vortexed for 10 s, followed by 20 min rotating at 4°C and centrifugation (12 000 × g, 10 min, 4°C). After spinning, the whole organic phase (1 mL) was transferred to a glass tube and dried using vacuum concentrator SpeedVac (Savant SPD130LX, Thermo Scientific, Massachusetts, USA) combined with refrigerated vapor trap (Savant RVT450, Thermo Scientific, Massachusetts). Evaporated samples were resuspended in 5% ethyl acetate in hexane (300 µL), vortexed, and loaded to the sorbent of pre-conditioned HyperSep SPE columns (500 mg/10mL, 40-60 µm, 70 Å, Thermo Fisher). FAHFAs were eluted with ethyl acetate, concentrated, resuspended in methanol, and analyzed using LC-MS straight away to minimalize loss due to degradation.

#### 6.2.2 Cells and Tissues

For both cells and tissues, 500 µL of citrate buffer was used together with 50 mg in the case of tissues. For cells, citrate buffer was poured into a well of washed cells free of any media and frozen in liquid nitrogen. Then the cells were scraped off similarly to the MTBE protocol

stated above, and the whole volume was transferred to a 2 mL tube. The following steps were identical to the extraction of FAHFAs from plasma.

### 6.2.3 Human Breast Milk

To avoid repeating freeze-thaw cycles and minimalize the amount of sample required for extraction, we decided to use the part of the organic phase from lipidomic and metabolomics extraction to extract FAHFAs. The aliquot (2 mL) was evaporated and then handled the same way as described above.

## 6.3 Isoprotanes

The published protocol (Dupuy et al., 2016) for the extraction of isoprostanes was adapted for the liver tissue. The liver (200 mg) was mixed with 50 µL of an antioxidant (BHT), 1 mL of methanol, and 5 mM EGTS in water in a ratio 2:1 and internal standards. Then the mixture was homogenized using a Fast Prep homogenizer (MP Biomedical) for 30 s at 6.5 m/s, the homogenate was transferred in a tube, and the rest was reextracted with methanol (830 µL) and phosphate buffer (800 µL). Chloroform (3 mL) was added, the tube was vortexed for 30 s, and centrifuged (2 000 × g, 5 min, 4°C). The bottom organic phase was transferred in a Pyrex tube and evaporated using a nitrogen gas flow. Subsequently, extracted lipids were hydrolyzed with 1M KOH in methanol (950 µL) while rotating. When cooled to room temperature, 2 mL of formic acid (40 mM) were added, and the sample was transferred to pre-washed SPE columns (OASIS MAX 200 mg, 3 mL). IsoPs were eluted with a mixture of hexane/ethanol/ethyl acetate (70:29.4:0.6, v/v/v), dried with nitrogen gas, resuspended in water/acetonitrile (87:17, v/v), centrifuged through a filter at room temperature (10 000 × g, 1 min) and transferred into LC-MS vial.

# 7 LC-MS Analysis

All extracted analytes were measured with liquid chromatography coupled to mass spectrometry (LC-MS) or liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), which also provides information about the structure necessary for proper identification. Three different LC-MS systems were used in the conducted experiments.

## 7.1 Lipidomics and Metabolomics

The LC-MS used for the analysis of lipids and polar metabolites consisted of a Vanquish UHPLC System (Thermo Fisher Scientific, Bremen, Germany) and a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). LIMeX (Lipids, Metabolites, and eXposome) workflow was used for the untargeted analysis of complex lipids and polar metabolites (Lopes et al., 2021). Four different platforms were used for lipidomic analysis:

lipidomics of high-abundant TAG in positive ion mode, lipidomics of low-abundant TAG EST in positive ion mode, and lipidomics of minor polar lipids in both positive and negative ion mode. Lipidomic platforms were measured using an Acquity UPLC BEH C18 column (2.1×50 mm; 1.7 µm; Waters, The Netherlands). Polar metabolites were measured in negative ion mode with an Acquity UPLC HSS T3 column (2.1×50 mm; 1.8 µm; Waters, The Netherlands) and in positive ion mode with an Acquity UPLC BEH HILIC column (2.1×50 mm; 1.7 µm; Waters, The Netherlands). If necessary, MS/MS methods with inclusion lists were applied to annotate specific analytes.

### 7.2 FAHFAs

Target FAHFA analysis was performed by UltiMate 3000 RSLC UHPLC System coupled to a QTRAP 5500/SelecION mass spectrometer (SCIEX, Darmstadt, Germany) with Triart C18 ExRS column (2.0×250 mm; 3 µm; YMC, Japan). FAHFAs were measured in negative ion mode as before (Kuda et al., 2016).

### 7.3 Isoprostanes

As in the case of FAHFAs, also Isoprostanes were measured with targeted analysis using an Eksiment microLC 200 Plus System coupled to a QTRAP 5500 (Sciex, Darmstadt, Germany) with a Zorbax Eclipse Plus C-18 column (2.1×150 mm; 1.8 µm; Agilent, Germany).

## 8 Data Processing and Statistical Analysis

LC-MS and LC-MS/MS data from the LIMeX workflow were processed in MS-DIAL software ver. 4.70 using the tracking features if necessary. Metabolites were annotated using in-house retention time and m/z library and MS/MS libraries available from public sources (Massbank, Mona, LipidBlast). For the identification of estolides, an in silico library of theoretical MAG, DAG, and TAG estolides was calculated using Python scripts. Raw data were filtered through blank samples with relative standard deviation (RSD) < 30% and then normalized using the LOESS approach using QC pool samples for each matrix repeatedly injected every 10 samples. For experiments where analytes were isotopically labeled with $^{13}C$ or $^{2}H$ natural abundance of carbon and hydrogen atoms and tracer purity were adjusted with IsoCor 2.0.5, if needed. Data from target analyses of FAHFAs and IsoPs were processed with MultiQuant software from SCIEX. Data were further processed with MetaboAnalyst or GraphPad Prism (current version), where various statistical tests were applied (such as ANOVA, Student t-test, etc.) with the value $p < 0.05$ considered significant. All the graphs in the thesis are displayed as means ± SEM.

# RESULTS

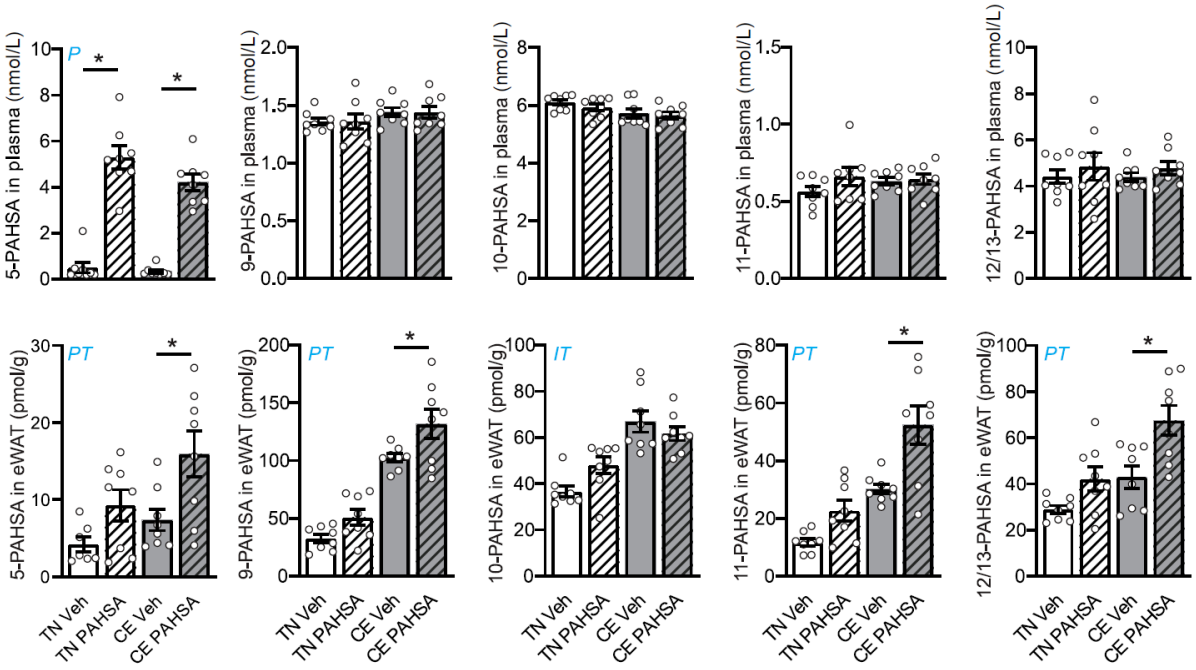
In the following chapters, there are clearly summarized the most important results from the enclosed publications.

## 9 Metabolism of 5-PAHSA

To study the metabolism of FAHFAs (**Publication I**), we focused on the specific member, palmitic acid ester of hydroxy stearic acid with a branching position at the 5$^{th}$ carbon atom called 5-PAHSA. The effect of 5-PAHSA on glucose and lipid metabolism and their link was explored using cold exposure and isotopic tracers ($^{13}$C and $^{2}$H) and compared to the effect of insulin.

### 9.1 The Effect of Cold Exposure on PAHSA Levels

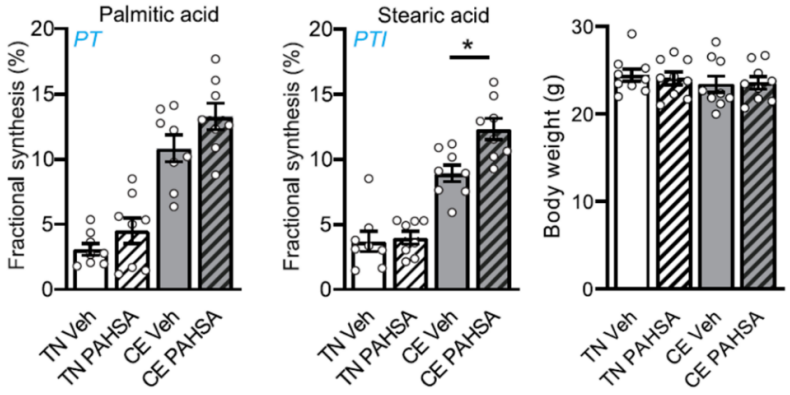
After seven days in a cold environment (CE, 6°C) or thermoneutrality conditions (TN, 30°C), the total body weight of mice was unchanged. However, the weight of WAT depots and the size of adipocytes were reduced. Simultaneously, levels of certain PAHSAs (regioisomers 5- and 9-) in WAT were increased (data not shown). Hence, we focused on 5-PAHSA and subjected mice kept in cold or thermoneutrality to gavage of this particular PAHSA regioisomer for three days. Administration of 5-PAHSA rose levels of 5-PAHSA in TN and CE but did not affect other regioisomers in plasma (Graph 1). In contrast, the effects of gavage were more pronounced in WAT, where almost all PAHSAs were elevated, especially in the group kept in CE.



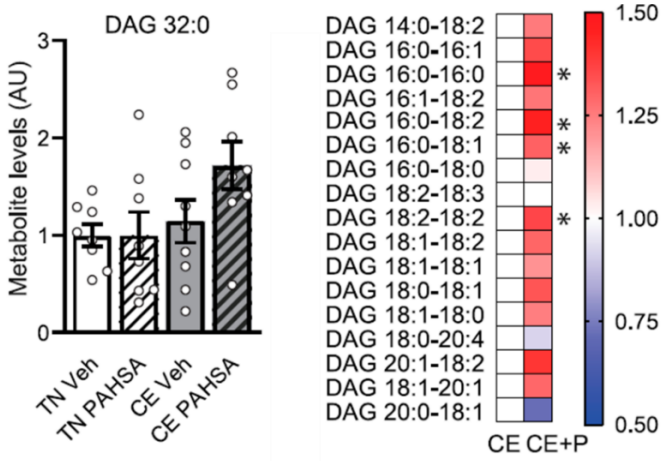

Graph 1. Effect of cold exposure on PAHSA levels in plasma and WAT

## 9.2 The Effect of 5-PAHSA on Glucose and Lipid Metabolism

To investigate the role of 5-PAHSA in lipid metabolism, we injected mice with heavy water ($^2H_2O$) two days prior to sacrification and substituted 10% of their drinking water with heavy water to maintain the stable level of isotopic tracer in their body. The fractional rate of palmitic and stearic acid were elevated in a cold environment, but only in the case of stearic acid, the effect of 5-PAHSA treatment was significant (Graph 2). Together with the fact that the body weight was not affected, it showed that DNL stimulated by 5-PAHSA did not lead to TAG production but rather to the energy-demanding TAG/FA cycle. Thanks to the LC-MS analysis, we were able to compare enrichment with $^2H$ in various lipid species, and we found out that short-chain TAGs such as TAG 48:0 (16:0_16:0_16:0) were the most labeled ones. Although we did not observe any change in the level of this short-chain TAG between the groups (not shown), its lipolytic product (DAG 32:0) was increased in the CE group gavaged with 5-PAHSA. Following analysis of several DAGs revealed that 5-PAHSA stimulated acylglycerol remodeling (Graph 3).




Graph 2. Fractional synthesis of palmitic and stearic acid in WAT




Graph 3. Effect of 5-PAHSA on DAGs levels

To investigate the influence of 5-PAHSA on glucose uptake in adipocytes, we used a $^{13}C$ tracer, namely $^{13}C_6$ glucose. Upregulated metabolites are highlighted in green, and those that were decreased by the effect of 5-PAHSA are shown in red (Fig. 7). Although 5-PAHSA administration stimulates carbon flow from glucose at the lower part of pentose phosphate pathway and glycolysis but more importantly to the Krebs cycle resulting to a higher level of citrate, which can be used as a precursor molecule for DNL.

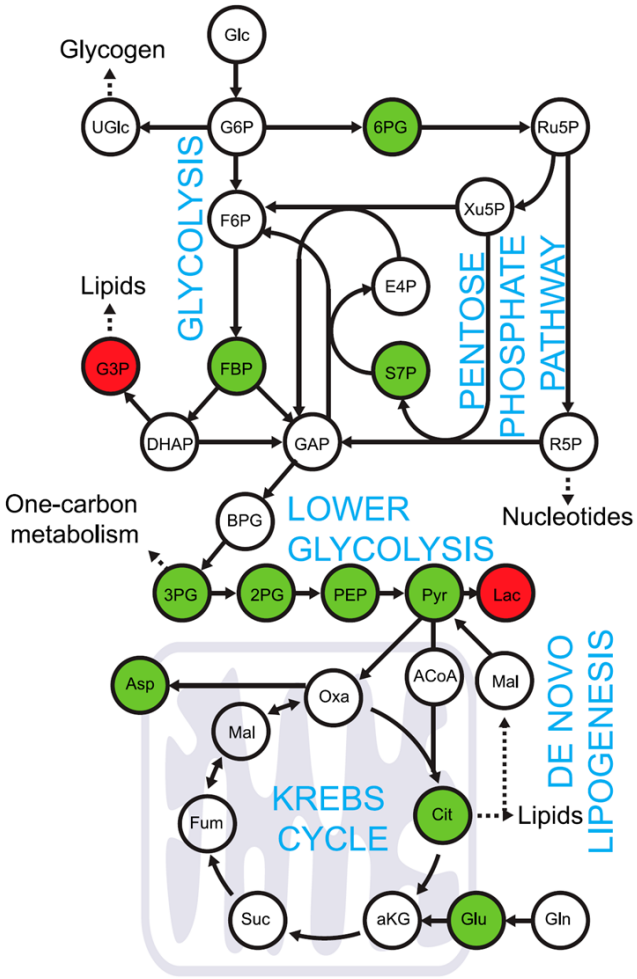



Figure 7. Glucose uptake in adipocytes treated with 5-PAHSA

The experiment was repeated, and 5-PAHSA stimulated pathways were also tracked with other isotopic tracers (4-$^{2}H$ glucose and $^{13}C_5$ glutamine) because carbons for DNL could be provided from various sources, including glutamine. The $^{13}C_6$ glucose was used to label the carbon atom flow in the pathway also stimulated with insulin, the most important antidiabetic substance, to compare the effect of insulin with the effect of 5-PAHSA. Stimulated pathways are colored according to the used tracer or treatment (5-PAHSA vs. insulin) (Fig. 8). Under ideal conditions, without the limitation of carbon atom sources, glutamine showed to be the primary source of carbons for the Krebs cycle. Carbons enter the cycle in the form of alpha-

ketoglutarate, which is then metabolized to succinate or, interestingly, backward the Krebs cycle to citrate in the reductive carboxylation process. Generally, insulin stimulates glucose uptake and potentiates carbon flux to TAG accumulation through glycerol-3-phosphate, while 5-PAHSA directs the carbon flow towards the Krebs cycle, DNL, and it also stimulates the remodeling of TAGs.

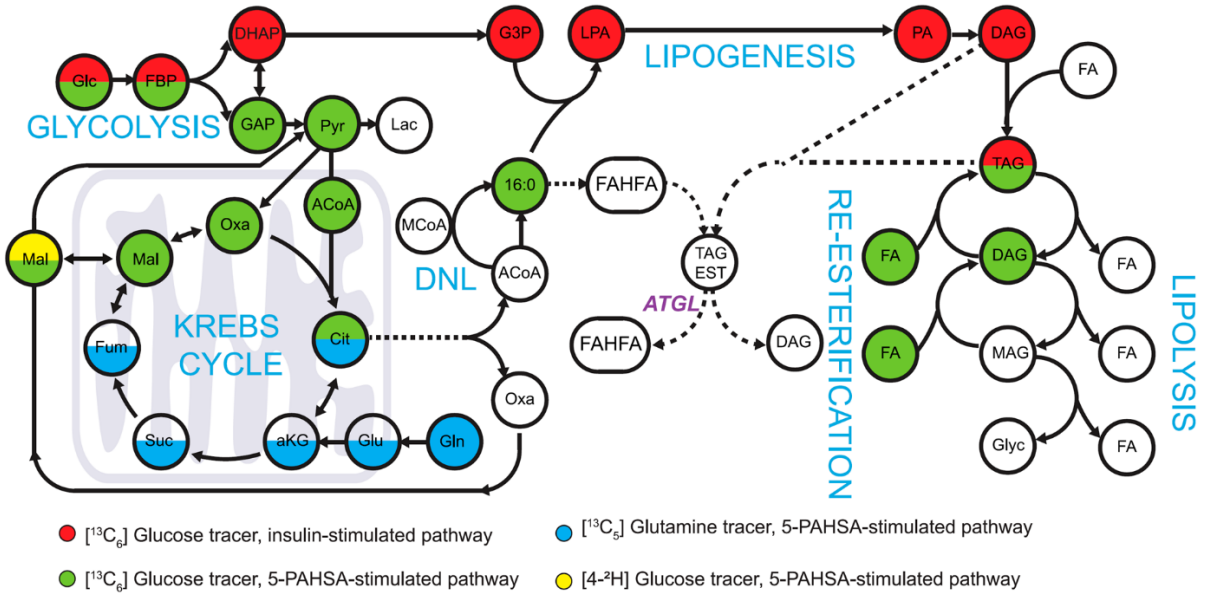
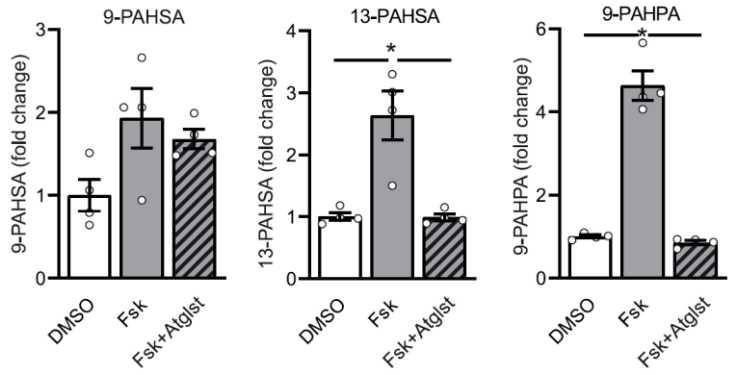


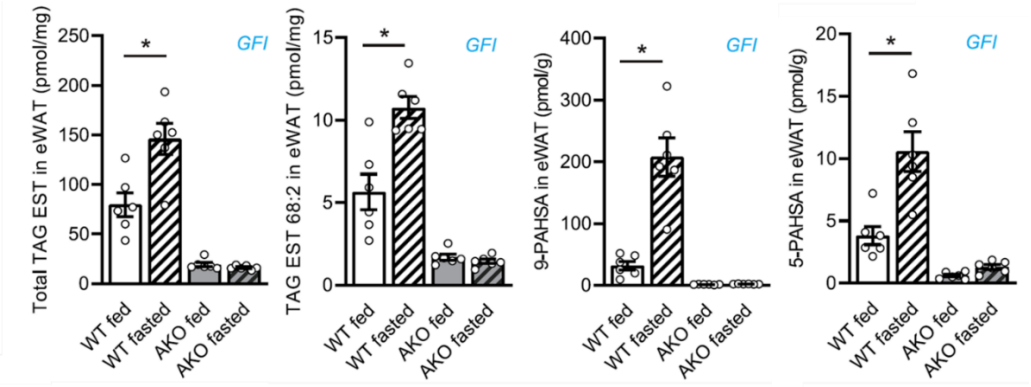

Figure 8. Summary of metabolic pathways affected with 5-PAHSA and their comparison to insulin-stimulated pathway

### 9.3 Intracellular FAHFA Reservoir

The experiment with heavy water was designed to isotopically label products of DNL and possibly other substances that use DNL products as precursors for their biosynthesis, such as FAHFAs. The fact that no deuterium enrichment was observed within the FAHFA structure suggested that FAHFAs might be stored intracellularly and released upon demand. We focused on TAG-containing FAHFAs or so-called TAG estolides. For this purpose, 3T3-L1 adipocytes were incubated with forskolin, which stimulates lipolysis, or with Atglistatin, an inhibitor of ATGL. While forskolin elevated levels of FAHFA, the inhibition of ATGL decreased them (Graph 4). Then, we tested eWAT from WT and AKO (ATGL deficient) mice in a fed and fasted state for the presence of TAG estolides (Graph 5). LC-MS analysis revealed that higher levels of TAG EST in fasted WT mice and selected PAHSAs followed the same trend. On the contrary, mice lacking ATGL showed minimum levels of TAG EST and PAHSAs compared to WT mice showing that the release of FAHFAs from TAG EST structure is controlled by ATGL.




Graoh 4. FAHFA levels in 3T3-L1 adipocytes incubated with Forskolin and/or Atglistatin




Graph 5. TAG estolide and PAHSA levels in eWAT of WT and AKO mice

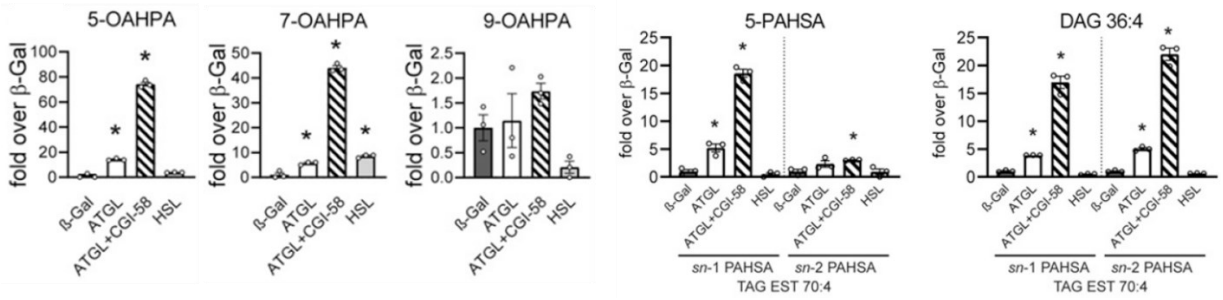
## 10 Metabolism of TAG estolides

As the new class of lipids called TAG estolides was identified and we confirmed their presence in our experiments (**Publication I**), we decided to focus more on their metabolism, particularly their catabolic enzymes (**Publication II**). In the previous study, we proved the relationship between TAG EST and ATGL, an important lipolytic enzyme. In this work, we focused not only on ATGL but also on HSL. For this purpose, HEK293T lysates were used as the source of enzymes of interest. Cell lysates were incubated with TAG estolide standards, which were used as substrates. Overexpressed β-galactosidase (β-Gal) served as a negative control, while the combination of ATGL and its activator CGI-58 showed an enhanced effect of ATGL.

### 10.1 The role of ATGL in FAHFA metabolism

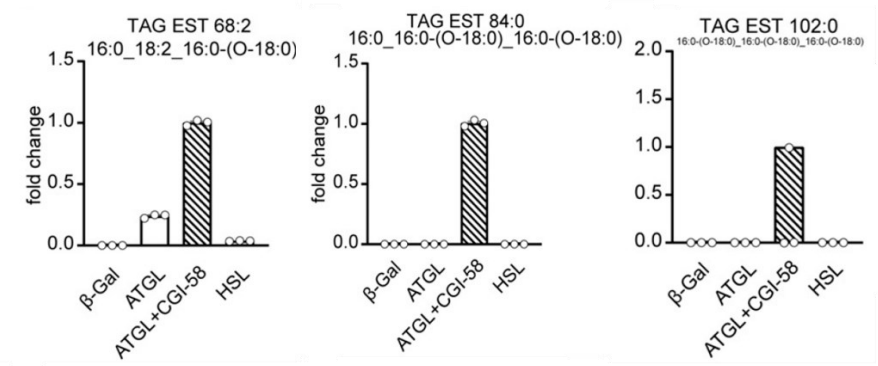
ATGL alone, or in combination with the activator CGI-58, was shown to be the key enzyme for the hydrolysis of the glycerol ester bond, thus releasing the free FAHFA from the TAG EST structure (Graph 6). As expected, the enzymatic activity of ATGL was amplified in the presence of CGI-58. ATGL activity was not affected by the type of FAHFA bound, but interestingly, the branching position significantly influenced ATGL hydrolytic properties. The closer the branching position was to the alpha-carbon atom, the stronger the catalytic effect of ATGL+CGI-58 was. Moreover, ATGL was able to cleave the 5-PAHSA bound at both

*sn-1,3* and *sn-2* positions, resulting in increased levels of DAG 36:4, the product of the hydrolysis reaction, equally in both stereospecific positions.



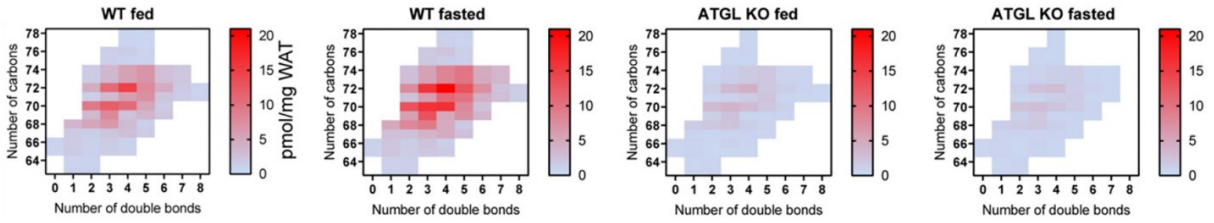

Graph 6. FAHFAs released from TAG estolides by ATGL or HSL

Apart from hydrolase activity, ATGL also possesses transacylase activity enabling the enzyme to translocate acyl from MAG and DAG to another molecule of DAG, which served as an acceptor, leading to the formation of a new TAG. If the activity of ATGL was enhanced with the activator, then more complex double or triple TAG estolides might be created (Graph 7).




Graph 7. TAG estolide remodeling

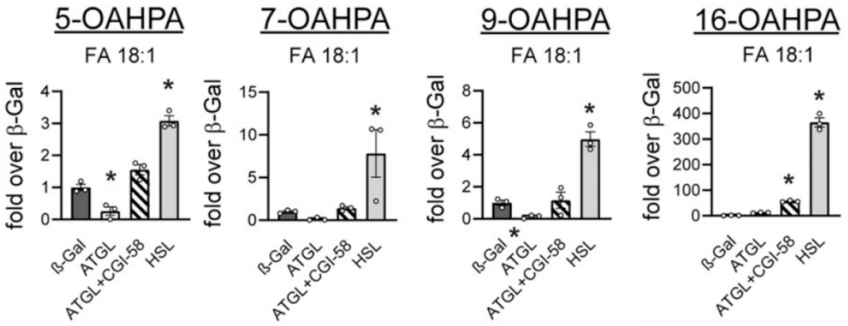
At last, we analyzed eWAT ATGL knock-out (KO) mice to verify if the ATGL plays a crucial role in TAG estolide metabolism also in vivo model. Similarly to what was shown in the previous publication (**Publication I**), the whole spectrum of TAG estolides was affected by deficiency of ATGL and decreased in adipose tissue of ATGL KO mice regardless of the fed or fasted state (Graph 8).




Graph 8. Levels of TAG EST in eWAT of ATGL KO mice

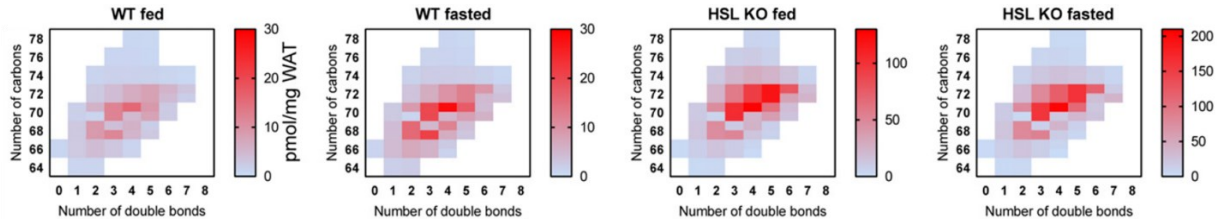
### 10.2 The role of HSL in FAHFA metabolism

Apart from the hydrolysis of the glycerol ester bond, we also assessed the hydrolysis of the estolide bond, which is the ester bond within the FAHFA structure. Such hydrolysis increases the level of a particular FA, for example, oleic acid in the case of OAHPAs. HSL was an effective hydrolytic enzyme for all FAHFAs bound within TAG EST, with exceptional hydrolytic activity for 16-OAHPA, which is a member ω-FAHFAs, that are linear molecules (Graph 9).




Graph 9. Products of OAHPA estolide bond hydrolysis

HSL KO mice were analyzed for the presence of TAG estolides in eWAT, likewise ATGL KO mice. While we identified fewer TAG EST species in ATGL KO mice, in HSL deficient mice, we observed the exact opposite in both fed and fasted groups when compared to WT mice meaning that HSL plays a crucial role in TAG EST catabolism (Graph 10).




Graph 10. Levels of TAG EST in eWAT of HSL KO mice

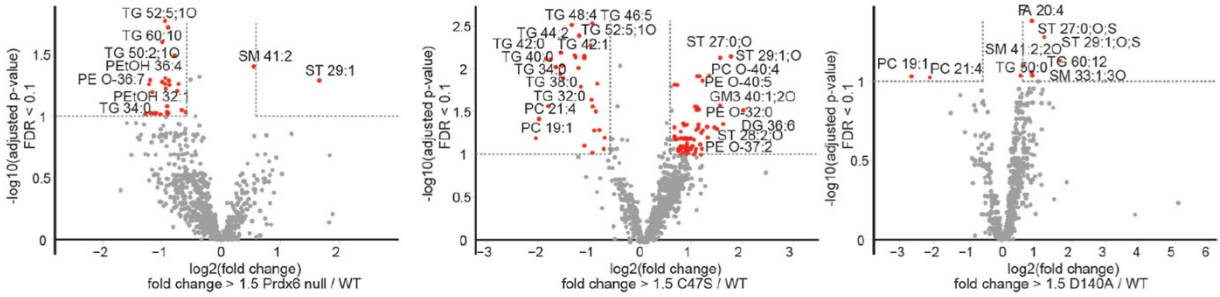
## 11 Peroxiredoxin 6 in FAHFA Biosynthesis

Lately, we have focused on the metabolism of FAHFAs; specifically, we tried to elucidate the role of the antioxidant enzyme peroxiredoxin 6 in this process (**Publication III**). To do so, we used mouse models of genetically altered Prdx6. Since we had two knock-in models for abolished peroxidase and phospholipase $A_2$ activities, we were able to test, which enzymatic activity of Prdx6 is involved in the production of hydroxy fatty acid that might serve as a precursor of FAHFAs.

### 11.1 Lipidomic analysis of Prdx6 mouse models

To assess the effect of genetic modifications on the whole lipidome, we proceeded untargeted lipidomic analysis and compared each mouse model with WT mice in volcano plots (Graph 11).

We did not observe any significant effect of the genetic modifications of Prdx6 in neither of the mouse models. Prdx6 null model with no activity of Prdx6 had lower levels of TAGs with oxidized oleic acid in their structure. The C47S mouse model with deleted peroxidase activity showed to have the most altered lipidome out of all models with several downregulated short-chain TAGs and upregulated lipid clusters consisting of phosphatidylethanolamines and sulfate conjugates of steroids.




Graph 11. Lipidomic analysis of scWAT in mouse models with genetically modified Prdx6

## 11.2 The role of Prdx6 in FAHFA biosynthesis

Since the lipidome was not fundamentally affected, we focused on FAHFAs in scWAT. We suggested a scheme of FAHFA biosynthesis based on the latest information available (Fig. 9). Membrane phospholipids readily oxidize during oxidative stress and therefore, are a source of peroxidized phospholipids, which are then reduced to the corresponding alcohols by Prdx6. TAG-OH is then formed from hydroxylated FA and further used in the biosynthesis of FAHFAs.

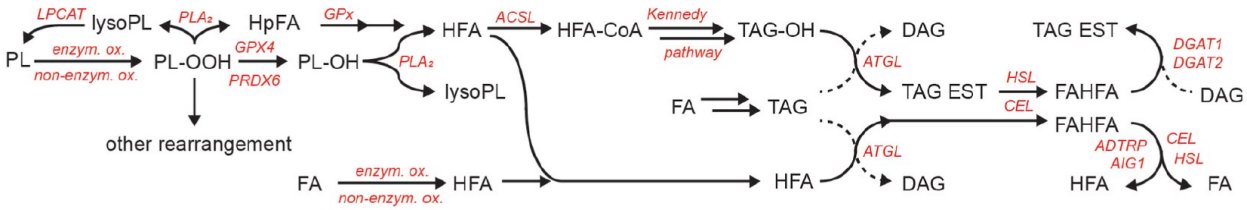
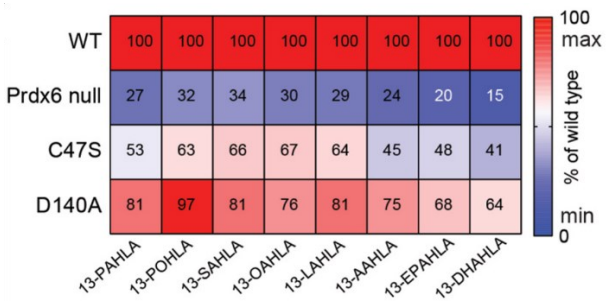


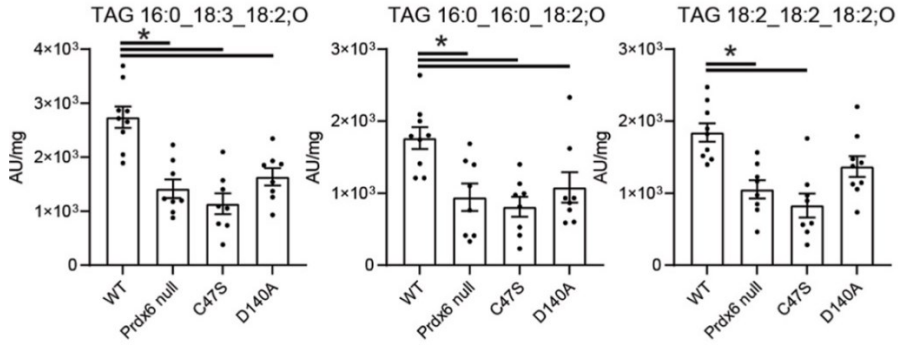

Figure 9. Suggested scheme of FAHFA biosynthesis

Based on information from Graph 15, we focused on FAHFAs containing hydroxy oleic acid in their structure, namely on 13-hydroxy oleic acid (13-HLA) (Graph 12). Levels of all measured FAHLAs in Prdx6 mouse models are displayed as a percentage of WT mice. Knock-out mice (Prdx6 null) had the most decreased levels of FAHLAs, as expected. C47S mice followed this trend, and the Prdx6 malfunction in D140A mice affected FAHLAs levels the least, meaning that the peroxidase activity of Prdx6 is essential for the biosynthesis of FAHFAs.



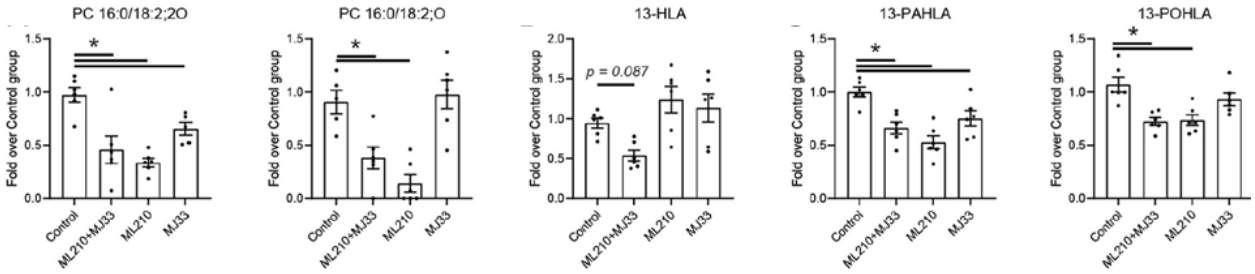

Graph 12. Levels of FAHFAs containing 13-HLA (FAHLAs) in scWAT

Following the scheme in Fig. 9, we also analyzed several TAG with hydroxylated oleic acid (18:2) bound to the glycerol backbone to test the involvement of the Prdx6 in FAHFA synthetic pathway (Graph 13). Selected HLA-containing TAGs we downregulated in most Prdx6 mouse models, especially in Prdx6 null and C47S mice.




Graph 13. Levels of TAG-containing HLA in scWAT

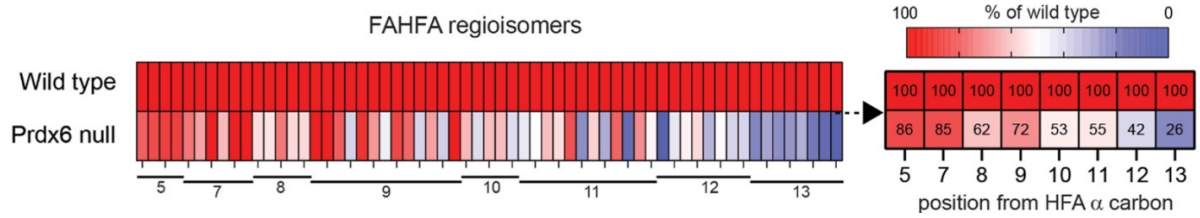
Then, we focused on the other part of suggested FAHFA metabolism biosynthesic pathway, namely on involvement of Prdx6 in production of HFAs, which could be further incorporated in oxidized TAGs and used as precursors for TAG EST formation. We incubated 3T3-L1 cell line with inhibitors of phospholipid peroxidase activity (ML210) (Kagan et al., 2017), of $PLA_2$ activity (MJ33) (Fisher et al., 1992) or their combination to imitate mouse models of Prdx6. We tracked the PC 16:0/18:2 and its corresponding peroxidized (PC 16:0/18:0;2O) or hydroxylated (PC 16:0/18:2;O) forms and noticed similar profiles not only in oxidized PCs but also in related FAHLAs as in scWAT of tested mouse models (Graph 14). However, 13-HLA was decreased only in the group with both inhibitors corresponding with the whole body knockout of Prdx6.




Graph 14. Analysis of oxidized PCs and corresponding HLA and FAHLAs

### 11.3 Substrate specificity of Prdx6

Next, we compared all measured regioisomers of various FAHFAs in Prdx6 null mice with WT mice to investigate the regioselectivity and substrate specificity of Prdx6 (Graph 15). We observed that the impact of Prdx6 deletion is more significant in FAHFAs with the position of the branching atom further from the α-carbon atom. This trend was more recognizable when we pooled the relative concentration of FAHFAs for each regioisomer measured. However, we did not observe such a trend in C47S or D140A mice. To confirm obtained data, we created a model of Prdx6 dimer with a bound molecule of oxidized phosphatidylcholine (Fig. 10), and we calculated the theoretical distance between the hydroperoxyl group of oxidized FA and thiol group of the Cys47 residue responsible for peroxidase activity for several hydroperoxy fatty acids. Graph 16 supports data shown in Graph 18 because the distance between the hydroperoxyl group and active site of Prdx6 decreases with increasing distance of the branching position from the α-carbon, meaning that these regioisomers (9- and higher) are easily accessible for Prdx6.




Graph 15. Representation of FAHFA regioisomers in scWAT

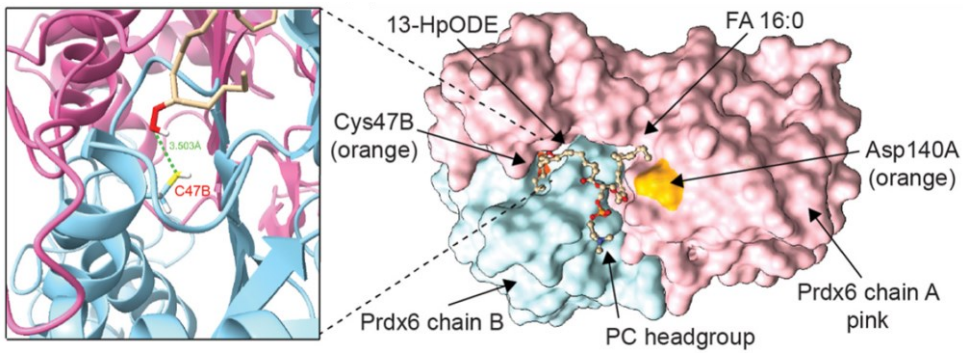
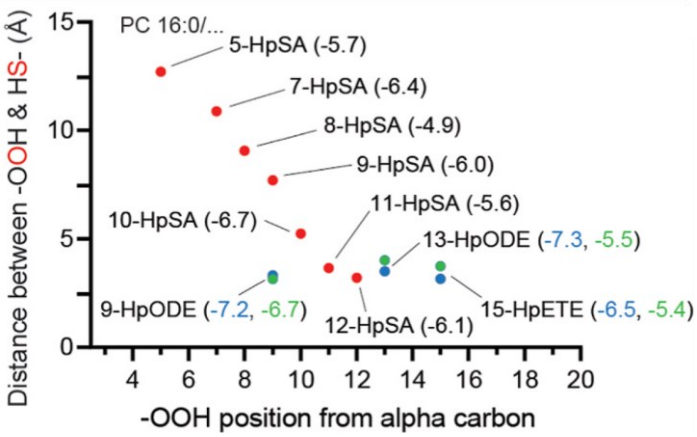



Figure 10. Model of Prdx6 dimer



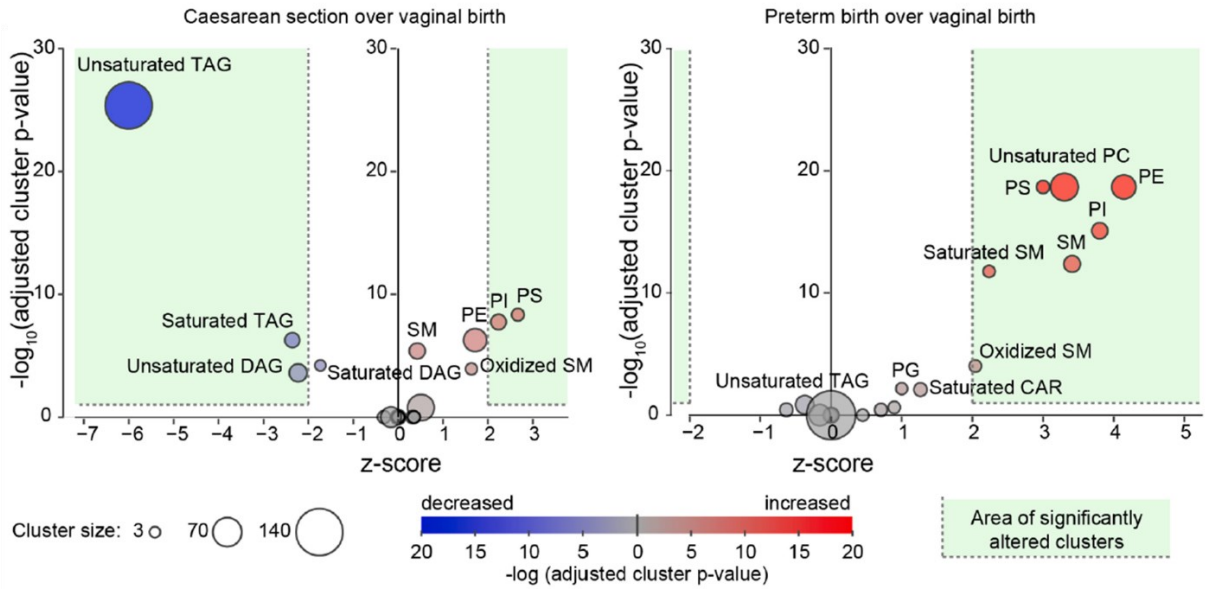

Graph 16. Model of a distance between the oxidized group and active site in Prdx6 for various HpFAs

## 12 Bioactive Lipids in Human Breast Milk

Our laboratory has already analyzed human breast milk samples from lean and obese mothers and reported lower levels of PAHSAs in the milk of obese mothers (Brezinova et al., 2018). In this study (**Publication IV**), we focused on how the influence of lactation stage, gestational age, and mode of delivery affect breast milk composition. We performed metabolomic and lipidomic untargeted profiling with target analysis of FAHFAs and TAG estolides.

### 12.1 Metabolomic and lipidomic profiling

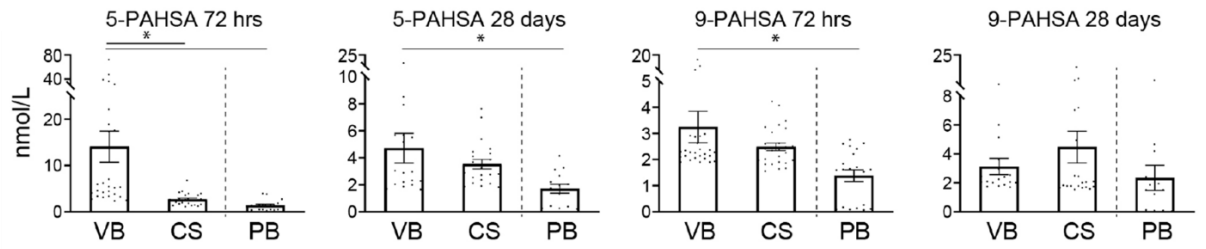
We analyzed breast milk donated 72 h after childbirth and compared three different modes of delivery (Caesarean section, preterm birth, and vaginal birth). Vaginal birth (VB) was considered a control group (Graph 17). LC-MS analysis revealed 148 polar analytes such as amino acids and 541 lipid species, including acylglycerols. Analytes were clustered according to the chemical similarity and colored blue if they were downregulated compared to vaginal birth or colored red if upregulated. While colostrum from the caesarean section (CS) group was characterized by lower levels of unsaturated TAGs, such lipid species remained unchanged in the preterm birth (PB) group. On the other hand, the rise of various glycerophospholipids was more pronounced in preterm birth colostrum than in the caesarean section group.




Graph 17. Difference in colostrum metabolome

### 12.2 FAHFA levels in human breast milk

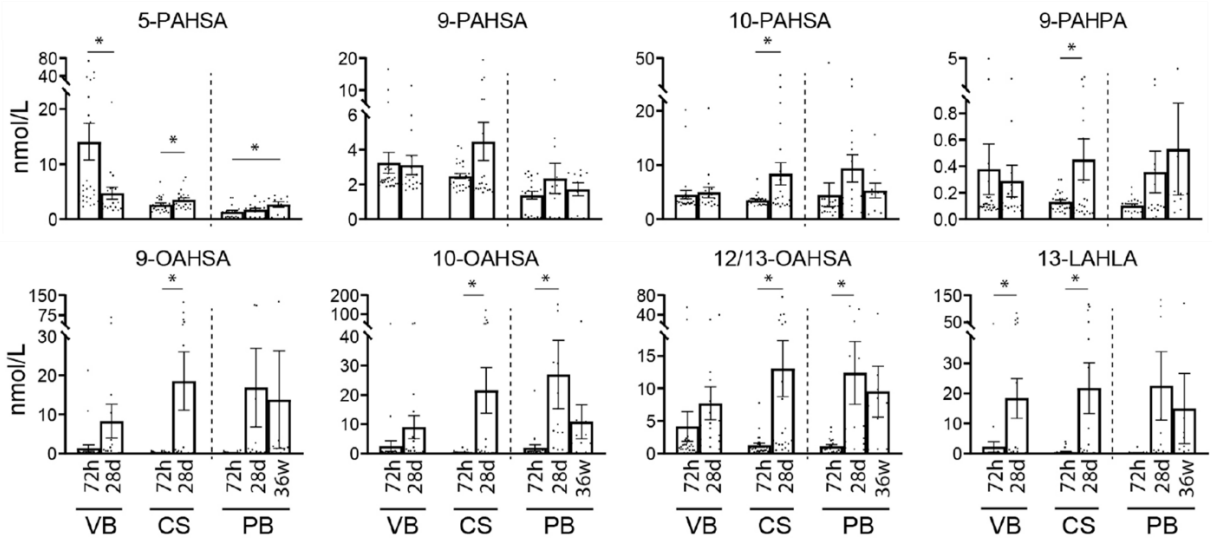
Following on from previous work (Brezinova et al., 2018), we focused on members of the PAHSA family, 5- and 9- PAHSA (Graph 18). The decrease of 5-PAHSA in colostrum (after 72 h) was significant in both groups of non-standard delivery (CS and PB). Over time the difference was compensated in the CS group, nevertheless, the shortage of 5-PAHSA remained significant even after 28 days. The level of 9-PAHSA was not affected to such an extent by the mode of delivery, and all differences were settled within 28 days.




Graph 18. The influence of mode of delivery and lactation stage on PAHSA levels

We also looked at other FAHFAs, and this time we included the time point of 36 weeks for the PB group. Since infants in this group were born before the 32nd week, we collected breast milk at the time corresponding to the 36th week of gestation, too, to see how the FAHFA levels would evolve over time. 5-PAHSA was the only member of the FAHFA family whose concentration was affected by the mode of delivery in all tested groups. Milk of mothers who have undergone vaginal birth was affected by the lactation stage the least as only 5-PAHSA, and 13-LAHLA have changed in mature milk. For all FAHFAs measured in CS milk,

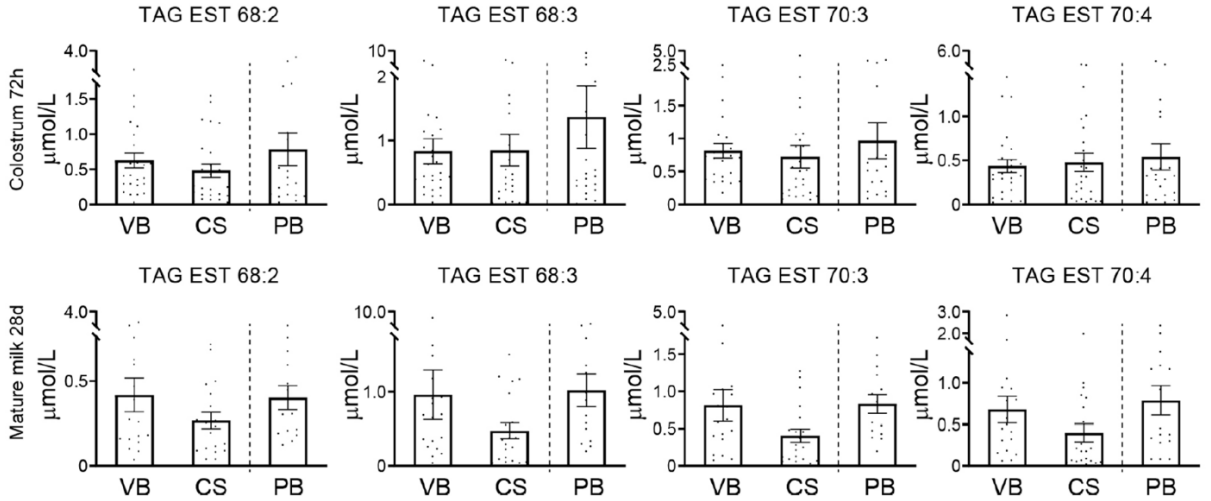
the concentration of which changed during lactation, we observed an increase in mature milk compared to colostrum (Graph 19).




Graph 19. The influence of mode of delivery, gestational age and lactation stage on FAHFA levels

### 12.3 TAG EST levels in human breast milk

In this study, we focused on TAG estolides, too, as they are involved in FAHFA metabolism. We managed to quantify various TAG EST species in human breast milk but did not observe any differences between tested delivery modes. Moreover, there was no significant change during the lactation period (Graph 20). Thus, we concluded that levels of TAG EST in the human breast are stable regardless of the mode of delivery and stage of lactation.




Graph 20. Levels of TAG EST in breast milk and their evolution during lactation

# DISCUSSION

To fulfill the set goals of the dissertation thesis, we focused on various FAHFAs and related fields of study to elucidate the biosynthesis and metabolism of FAHFAs, their role in living organisms, and characterize multiple biological matrices, including human breast milk through FAHFA and TAG estolide content.

Firstly (**Publication I**), we used 5-PAHSA, a member of the FAHFA family with known biological activities, to investigate its connection to glucose and lipid metabolism through a combination of in vitro and in vivo experiments. The use of several isotopic tracers ($^{13}C_6$ glucose, $^{13}C_5$ glutamine, 4-$^{2}H$ glucose, and $^{2}H_2O$) was essential to reveal which metabolic pathways, in particular, were affected by 5-PAHSA lipokine or applied experimental conditions.

This study showed that cold exposure stimulated DNL together with energetically demanding TAG/FA cycling (Flachs et al., 2017). Moreover, cold exposure and fasting increased levels of FAHFAs, including 5-PAHSA in WAT (Kuda et al., 2018; Yore et al., 2014). CE enhanced the effects of 5-PAHSA as we observed elevated levels of this lipokine in eWAT, but the impact of 5-PAHSA on DNL was more substantial. Besides that, 5-PAHSA upregulated lipid remodeling that did not support lipid storage, thereby prevented weight gain (Syed et al., 2018). We also designed a $^{2}H$ enrichment experiment to track de novo lipogenesis, but palmitic acid, the primary product of de novo lipogenesis, was only minimally enriched with deuterium. Apart from a low level of deuterium enrichment, a wide range of TAGs with many different FAs bound to the glycerol backbone caused the dilution of the tracer leading to inconclusive results. PAHSAs were not labeled either in any of the tested groups, suggesting the presence of a reservoir that would release PAHSAs upon stimuli. The fact that we identified PAHSA-containing TAG estolides in our lipidomic data supported recently published work describing the discovery of this novel lipid group (Tan et al., 2019).

The metabolism of TAG estolides is controlled by lipolytic enzymes (ATGL or HSL), which can liberate FAHFAs from TAG EST structure during CE or fasting (Tan et al., 2019). Inhibition of ATGL using atglistatin on forskolin-treated cells or the absence of ATGL in AKO mice caused a decline in FAHFA levels. Even though the effect was more pronounced in adipocytes, levels of TAG EST remained unchanged due to the short incubation time. Nevertheless, in AKO mice, we observed decreased levels of TAG EST similarly to FAHFA levels, indicating that not only hydrolytic but also transacylation activity of ATGL participate in the metabolism of TAG estolides (Jenkins et al., 2004; Lake et al., 2005).

We used 3T3-L1 adipocytes and isotopic tracers in media with limited carbon sources to explore, unlike Krycer et al. (Krycer et al., 2017), to study the effect of 5-PAHSA solely and compared it to insulin. We observed that 5-PAHSA stimulated lipogenesis without excessive accumulation of TAGs, which is in accordance with previous data (Hammarstedt et al., 2018). 5-PAHSA also triggered TAG remodeling with selective re-esterification of monounsaturated FAs, directed carbon flux to the Krebs cycle, and synthesis of DNL precursors. In contrast, the insulin-stimulated pathway led to excessive lipid storage. However, it is worth mentioning that the biological effects of 5-PAHSA were smaller than insulin, which remains the most effective substance used for diabetes treatment. Potentially, 5-PAHSA could be used in combination with insulin for therapeutic reasons. We also used $^{13}C$ glutamine as a primary carbon source in 5-PAHSA preincubated adipocytes, which enhanced the effect of 5-PAHSA on DNL and limited TAG accumulation even more compared to the usage of $^{13}C$ glucose as a primary carbon source.

Next (**Publication II**), we focused more on the novel group of TAG estolides to reveal more about their metabolism and the enzymes that participate in it. It was already shown that ATGL and HSL, which are predominantly expressed in adipose tissues, are engaged in TAG EST metabolism (Tan et al., 2019).

Other tissues, such as the liver, muscle, and heart, contain lower amounts of these lipases (Zimmermann et al., 2004). In spite of it, their deficiency is linked with higher mortality and several health disorders showing the necessity of lipolytic enzymes in non-adipose tissues (Albert et al., 2014; Schweiger et al., 2009). Although ATGL and HSL play an essential role in TAG EST catabolism, each has different substrate specificity.

ATGL mainly participated in releasing FAHFAs from the TAG estolide structure by cleaving the ester bond between glycerol and FAHFA. However, its ability to cleave the estolide ester bond within FAHFAs was relatively weak. As a substrate, ATGL preferred TAGs and FAs of a minimum chain length of eight carbons favoring unsaturated FAs (Eichmann et al., 2012). Besides that, ATGL also preferred TAG estolides with more compact FAHFAs bound with the branching carbon atom located closer to the glycerol backbone, i.e., preferring 5-PAHSA-containing TAG estolide to 10-PAHSA-containing TAG estolide. When ATGL was used in combination with CGI-58, which amplified the activity of ATGL, we observed that ATGL hydrolyzed ester bonds in TAGs at both stereospecific positions (*sn-1* and *sn-2),* unlike most other lipases (Eichmann et al., 2012; Rogalska et al., 1993). The same trend was observed in the reaction with 5-PAHSA-containing TAG estolide, which led to the liberation of 5-PAHSA and the formation of the corresponding DAG regardless of the *sn-* position.

We showed previously that in addition to hydrolysis, TAG estolides could be remodeled by transacylase activity, which both ATGL and HSL accept and transfer acyl groups to acylglycerols (Jenkins et al., 2004; Paluchova, Oseeva, et al., 2020; Zhang et al., 2019). We assume that CGI-58-activated ATGL was responsible for TAG EST production via the remodeling process when it utilized DAG estolides as both acyl acceptors and donors. Complex double or triple TAG estolides, which contain two or three molecules of FAHFAs attached to the glycerol backbone, could be generated through this mechanism. Though this was observed only under experimental conditions in PAHSA-containing substrates, the presence of double or triple estolides in human or mouse samples was not detected (Brezinova et al., 2020; Paluchova, Oseeva, et al., 2020; Tan et al., 2019). Further experiments with ATGL KO mice supported the importance of this enzyme in the metabolism of TAG estolides, as mice deficient in ATGL had significantly decreased levels of TAG EST and free FAHFAs (Paluchova, Oseeva, et al., 2020). The fact that PAHSA levels increased in white adipose tissue during fasting when expression of ATGL is stimulated pointed out the dependent relationship in PAHSA production and its possible physiological relevance.

ATGL, also called PNPLA2 – patatin-like phospholipase domain containing 2, is not the only enzyme from the PNPLA family with transacylation activity (Kienesberger et al., 2009), namely PNPLA1 and PNPLA3. While PNPLA1 is responsible for forming estolide bonds in ω-*O*-acylceramides and its function is connected exclusively with the skin barrier (Kien et al., 2018), PNPLA3 is highly expressed in adipose tissue and the liver where it affects TAG metabolism similarly to ATGL (Y. Wang et al., 2019; Yang et al., 2019). However, their effect on FAHFA or TAG estolide metabolism has not yet been elucidated.

In contrast to ATGL, HSL is far less substrate-specific as it catalyzes reactions with various substrates, including TAGs, DAGs, MAGs, cholesteryl esters, and others (Kraemer & Shen, 2002). Concerning that, we observed different efficiency of ATGL and HSL to hydrolyze ester bonds within the TAG EST structure. Although HSL was capable of cleaving TAG estolides to form FAHFAs and DAGs, its ability to hydrolyze the FAHFA-glycerol ester bond was poor compared to its strong hydrolytic activity on ester bond within FAHFAs. These findings were in agreement with previous studies on 3T3-L1 cells (Tan et al., 2019) as well as the data obtained from HSL KO mice where deficiency of HSL caused a rise in free FAHFAs. There are various other hydrolases excluding HSL that could potentially cleave ester bonds in FAHFA molecules, such as ADTRP and AIG1 (Erikci Ertunc et al., 2020; Parsons et al., 2016) or carboxylesterase 3 (CES3), which might also be able to release FAHFAs from TAG estolides (Kolar et al., 2016; Soni et al., 2004) together with other TAG hydrolases including PNPLA3

(Yang et al., 2019) and DDHD2 (Araki et al., 2016). However, only ATGL, HSL, ADTRP, and AIG1 have been demonstrably linked with the metabolism of FAHFA or TAG EST.

Within the study of FAHFA metabolism, we also focused on FAHFA biosynthesis, especially on the role of antioxidant defense in forming HFA that could be potentially used as a precursor to FAHFAs (**Publication III**). From all antioxidant enzymes, we selected peroxiredoxin 6 as it was already linked with FAHFA biosynthesis (Kuda et al., 2018) to elucidate what enzymatic activity participates in this process. Hence, we used mouse models with genetically altered Prdx6, either entirely deleted or partially active.

A comparison of genetically modified mouse models showed only mild effects of Prdx6 deletion in scWAT lipidome. In more detail, we observed a drop in specific oxidized lipids with oxidized linoleic acid FA 18:2;O (HLA) bound to the glycerol backbone in Prdx6 null mice, although we expected that complete inactivation of Prdx6 will increase oxidized lipids. An antioxidant system ensuring lipid protection against oxidation could be stimulated in the absence of Prdx6, leaving just the Prdx6-specific pathway to be dysregulated, which is in agreement with an unchanged level of oxidative stress. Similarly, knock-in D140A mice did not significantly differ from WT mice. The lipidome of scWAT was most affected in C47S knock-in mice lacking peroxidase activity. The effect of oxidative stress amplified by the Prdx6 mutation could probably be more noticeable in tissues that are more likely exposed to oxidative stress conditions, such as the lungs (Lien et al., 2012).

We based our further research on the lipidomic data from the Prdx6 null model and focused on FAHFAs with bound HLA, particularly those containing the most abundant 13-HLA. Mice with deleted Prdx6 had significantly decreased levels of 13-FAHLAs and so had C47S mice, although to a lesser extent. This proved the influence of Prdx6 peroxidase activity on FAHFA levels and showed that the effect of $PLA_2$ activity was negligible, by contrast. Subsequently, we followed the suggested FAHFA biosynthesis scheme and looked at 13-HLA-containing hydroxy TAGs, which belong among ATGL substrates, and we found the same trend as in FAHLAs (Brejchova et al., 2021; Patel et al., 2022). However, analysis of specific lipids involved in FAHFA metabolism is complicated as the remodeling process generates many different structure combinations (Brejchova et al., 2021).

To explore the involvement of Prdx6 in the process of FAHFA biosynthesis, we analyzed oxidized phosphatidyl cholines which are known to be the primary substrates of Prdx6 in 3T3-L1 adipocytes, which were incubated in the presence of Prdx6 inhibitor to mimic genetically modified mouse models. Levels of peroxidized PC, hydroxylated PC, and 13-HLA-containing FAHFAs were comparable to those obtained from mouse models showing that Prdx6

peroxidase activity is linked with FAHFA metabolism. Nevertheless, the results also suggested that Prdx6 is not the only enzyme from the peroxidase family, and glutathione peroxidase 4 (GPX4), which ML210 primarily inhibits, could also have a role in the synthesis of FAHFA precursors (Kagan et al., 2017). Inhibition of peroxidase activity caused, against expectations, an increase in peroxidized lipids. Still, it is worth mentioning that we are currently unable to analyze the broad spectrum of oxidized lipids to inspect all possible combinations (A. Criscuolo, 2021). Furthermore, it was published that levels of (per)oxidized phospholipids in human plasma may be affected differently under oxidative stress depending on which fatty acid is bound within their structure (A. Criscuolo, 2021).

When analyzing the set of all measured FAHFAs, we have noticed an exciting trend in the abundance of FAHFA regioisomers. Mice with inactivated Prdx6 had significantly decreased levels of FAHFA with branching positions far from the α-carbon atom. The further the branching position, the more pronounced the negative effect of Prdx6 deletion was, suggesting a strong preference for HpFAs with hydroperoxyl group further form the $9^{th}$ carbon atom, which the protein model of Prdx6 supported. However, such a trend was not observed in other mouse models, including C47S, where $PLA_2$ activity could cover the missing peroxidase activity and provide peroxidized acyl chains for other pathways to repair oxidative damage (Lien et al., 2012).

Theoretically, with increasing levels of oxidative stress, the antioxidant system in adipose tissue might be stimulated and compensate for the abolished function of Prdx6. Oxidized lipids can be either degraded (D. Chen et al., 2021) or transformed into the lipid species we did not analyze. This results in a decrease of oxidized lipids (Jarc & Petan, 2020), including hydroxy TAGs within lipid droplets that can be eventually used in TAG EST synthesis, which is directly linked to FAHFA metabolism (Tan et al., 2019). Affecting the link between glycemic and lipogenic metabolism, together with the decrease of FAHFAs caused by Prdx6 modifications, could result in liver disease and diabetes (Arriga et al., 2019).

Thanks to the collaboration with the Institute for the Care of Mother and Child in Prague, which provided us with human breast milk to proceed lipidomic and metabolomic analysis, including measurement of TAG estolides and FAHFAs (**Publication IV**), which levels have already been shown to be negatively affected in obesity (Brezinova et al., 2018). In this study, we compared the influence of delivery mode (caesarean section and vaginal birth) and term of delivery (preterm and term) on milk composition and level of lipokines at different stages of lactation.

Differences we observed in metabolic profiles of milk from tested delivery modes were expected as they can negatively affect breastfeeding, especially delay the initiation (Hobbs et

al., 2016), which might affects colostrum primarily, the first milk body produces. Our data showed downregulated unsaturated TAGs in the CS group and upregulated phospholipids and sphingomyelins in both groups in accordance with already published work (Pérez-Gálvez et al., 2020). Such differences could affect the milk fat globule size and their digestibility (Garcia et al., 2014).

This was the first time TAG estolides were identified in human breast milk, and so far, the mechanism of TAG EST and FAHFA production is not fully understood. Although they could be produced in breast white adipose tissue that is fully de-differentiated during lactation (Q. A. Wang et al., 2018), the mammary gland is capable of producing mainly shorter- to medium-chain FAs (6-14 carbon atoms) via de novo lipogenesis (Libertini & Smith, 1978). Since FAHFA levels in the body are linked either with de novo lipogenesis or food intake (Brejchova et al., 2020; Yore et al., 2014), longer-chain FAs (mostly 16-18 carbon atoms) are produced endogenously in the liver or adipose tissue and then probably transported to the mammary gland where estolide bond might be formed. We measured similar levels of TAGs containing linoleic acid (LA) in colostrum, but LA-containing 13-LAHLA was identified in mature milk only. The discrepancy can be caused by the stereospecific composition of TAGs unique to breast milk with a proportion of saturated fatty acids located at the *sn-2* position (Innis, 2011; Miles & Calder, 2017). The core of milk fat globules can potentially store TAG estolides (Lee et al., 2018). The fact that ATGL, the enzyme with the function to cleave FAHFAs but also remodel acyl chains on TAGs, is highly expressed in the goat mammary suggests that the mammary gland might be a place where FAHFAs and TAG estolides are metabolized with the help of appropriate hydrolases such as ATGL (Li et al., 2015).

# CONCLUSION

The dissertation thesis focused on novel groups of lipokines called FAHFAs, which are known to possess antidiabetic and anti-inflammatory properties. The main goal of the thesis was to study the metabolism of FAHFAs and other compounds that can be involved in their biosynthesis, such as TAG estolides and oxidized lipids.

We proved that cold exposure noticeably affects white adipose tissue by enhancing de novo lipogenesis and energy-consuming TAG/FA cycling. A cold environment affects the whole metabolome of white adipose tissue and upregulates FAHFAs. Besides that, we tracked metabolic pathways stimulated with a member of the FAHFA family (5-PAHSA) and insulin to find out that the administration of 5-PAHSA has certain advantages over insulin, although its effect was substantially weaker.

Our laboratory also confirmed the role of recently discovered lipids, TAG estolides, in FAHFA metabolism. We further focused on studying TAG estolides, especially their catabolism, during which FAHFAs are released. The investigation showed that ATGL and HSL are both lipases capable of hydrolyzing and remodeling TAG estolides, although with different substrate specificity.

In the next part of the research, we followed up on the recent work of our team that showed the dependence of FAHFA levels on the antioxidant enzyme peroxiredoxin 6. In this project, we tried to elucidate that peroxidase activity is responsible for this fact. Moreover, we also explained the substrate specificity of Prdx6 towards HpFAs with the hydroperoxyl group located further from the α-carbon atom.

Finally, we proceeded with metabolic and lipidomic analysis, including targeted analysis of FAHFAs and TAG estolides in human breast milk to find out that timing and mode of delivery negatively affect milk composition, including FAHFAs with the most differences observed in colostrum. However, the stage of lactation influences levels of certain FAHFAs too.

In conclusion, the objectives of the theses have been fulfilled, giving more information about FAHFA lipokines and related TAG estolides. This research elucidated essential parts of FAHFA metabolism and provided the basis for further investigation of these bioactive compounds, which could potentially have pharmacological uses in the future.

# LIST OF PUBLICATIONS

## 13 Publications Related to the Dissertation

### Publication I

**Paluchova V**, Oseeva M, Brezinova M, Cajka T, Bardova K, Adamcova K, Zacek P, Brejchova K, Balas L, Chodounska H, Kudova E, Schreiber R, Zechner R, Durand T, Rossmeisl M, Abumrad NA, Kopecky J, Kuda O. Lipokine 5-PAHSA Is Regulated by Adipose Triglyceride Lipase and Primes Adipocytes for De Novo Lipogenesis in Mice. *Diabetes*. 2020 Mar;69(3):300-312. doi: 10.2337/db19-0494. Epub 2019 Dec 5. PMID: 31806624; PMCID: PMC7118252. **IF (2020) = 9.461**

**Author contribution**: animal studies and cell culture experiments, methodology, LC-MS analysis, data curation, investigation, writing – review & editing

### Publication II

Brejchova K, Radner FPW, Balas L, **Paluchova V**, Cajka T, Chodounska H, Kudova E, Schratter M, Schreiber R, Durand T, Zechner R, Kuda O. Distinct roles of adipose triglyceride lipase and hormone-sensitive lipase in the catabolism of triacylglycerol estolides. *Proc Natl Acad Sci U S A*. 2021 Jan 12;118(2):e2020999118. doi: 10.1073/pnas.2020999118. PMID: 33372146; PMCID: PMC7812821. **IF (2021) = 12.779**

**Author contribution**: methodology, data curation, formal analysis, investigation

### Publication III

**Paluchova V**, Cajka T, Durand T, Vigor C, Dodia C, Chatterjee S, Fisher AB, Kuda O. The role of peroxiredoxin 6 in biosynthesis of FAHFAs. *Free Radic Biol Med*. 2022 Nov 17:S0891-5849(22)00978-9. doi: 10.1016/j.freeradbiomed.2022.11.015. Epub ahead of print. PMID: 36403738. **IF (2022) = 8.101**

**Author contribution**: animal studies and cell culture experiments, methodology, LC-MS analysis, data curation, writing – original draft, writing – review & editing

**Publication IV**

Brejchova K, **Paluchova V**, Brezinova M, Cajka T, Balas L, Durand T, Krizova M, Stranak Z, Kuda O. Triacylglycerols containing branched palmitic acid ester of hydroxystearic acid (PAHSA) are present in the breast milk and hydrolyzed by carboxyl ester lipase. *Food Chem*. 2022 Sep 15;388:132983. doi: 10.1016/j.foodchem.2022.132983. Epub 2022 Apr 15. PMID: 35486985. **IF (2022) = 9.231**

**Author contribution**: methodology, LC-MS analysis, data curation, writing – review & editing

The above-mentioned publications, which are related to the topic of the dissertation thesis, are the result of the cooperation of several scientists. The author of the dissertation participated in these publications and her contribution is stated above. The co-authors are aware of it and agree with this statement.

I hereby confirm that the contribution of Ing. Bc. Veronika Domanská to the submitted publications corresponds to the fact.

In Prague .....

RNDr. Ondřej Kuda, Ph.D.

## 14 Other Publications

Riecan M, **Paluchova V**, Lopes M, Brejchova K, Kuda O. Branched and linear fatty acid esters of hydroxy fatty acids (FAHFA) relevant to human health. *Pharmacol Ther*. 2022 Mar;231:107972. doi: 10.1016/j.pharmthera.2021.107972. Epub 2021 Aug 25. PMID: 34453998. **IF (2022) = 13.400**

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# APPENDICES

**Appendix I**

Publication I - Lipokine 5-PAHSA is regulated by adipose triglyceride lipase and primes adipocytes for de novo lipogenesis in mice.

**Appendix II**

Publication II - Distinct roles of adipose triglyceride lipase and hormone-sensitive lipase in the catabolism of triacylglycerol estolides.

**Appendix III**

Publication III - The role of peroxiredoxin 6 in biosynthesis of FAHFAs.

**Appendix IV**

Publication IV - Triacylglycerols containing branched palmitic acid ester of hydroxystearic acid (PAHSA) are present in the breast milk and hydrolyzed by carboxyl ester lipase.