**Univerzita Karlova**

**1. lékařská fakulta**

Autoreferát disertační práce

UNIVERZITA KARLOVA
1. lékařská fakulta

# Role of antioxidant defense in the synthesis of antidiabetic lipokines

Role antioxidační obrany v syntéze antidiabetických lipokinů

**Ing. Bc. Veronika Domanská**

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Předseda oborové rady: prof. MUDr. Zdeněk Kleibl, Ph.D.

Školicí pracoviště: Fyziologický ústav AV ČR, v.v.i.

Školitel: RNDr. Ondřej Kuda, Ph.D.

Disertační práce bude nejméně pět pracovních dnů před konáním obhajoby zveřejněna k nahlížení veřejnosti v tištěné podobě na Oddělení pro vědeckou činnost a zahraniční styky Děkanátu 1. lékařské fakulty.

**CONTENTS**

**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, which 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

**ABBREVIATIONS**

| | |
|---|---|
| ATGL | Adipose triglyceride lipase |
| C47S | Knock-in mouse model with mutating cysteine at position 47 to serine |
| CE | Cold exposure |
| CGI-58 | Comparative gene identification 58 |
| CS | Caesarean section |
| D140A | Knock-in mouse model with mutating aspartate at position 140 to alanine |
| DAG | Diacylglycerol |
| DNL | De novo lipogenesis |
| eWAT | Epididymal WAT |
| FA | Fatty acid |
| FAHFAs | Fatty acid esters of hydroxy fatty acids |
| FAHLAs | HLA-containing FAHFAs |
| GPX4 | Glutathione peroxidase 4 |
| HFA | Hydroxy fatty acid |
| HLA | Hydroxy linoleic acid |
| HpFA | Hydroperoxy fatty acid |
| HSL | Hormone-sensitive lipase |
| KO | Knock-out mouse |
| 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 |
| LOESS | Locally estimated scatterplot smoothing |
| MAG | Monoacylglycerol |
| OAHPA | Oleic acid ester of hydroxy palmitic acid |

| | |
|---|---|
| PAHSA | Palmitic acid ester of hydroxy stearic acid |
| PB | Preterm birth |
| PC | phosphatidylcholine |
| $PLA_2$ | Phospholipase A2 activity |
| Prdx6 | Peroxiredoxin 6 |
| Prdx6 null | Mouse model with deficient Prdx6 |
| QC | Quality control |
| RSD | Relative standard deviation |
| TAG | Triacylglycerol |
| TAG EST | TAG estolide |
| TN | Thermoneutrality |
| VB | Vaginal birth |
| WAT | White adipose tissue |
| WT | Wild-type mouse |

# 1 INTRODUCTION

Lipokines are a group of adipose tissue-derived bioactive lipids. Their bioactive activities are based on the connection to glucose and lipid metabolism. Many distinct lipid species belong to lipokines, however this dissertation thesis focuses on free fatty acid esters of hydroxy fatty acids (FAHFAs). These recently discovered compounds consist of a fatty acid (FA) and a hydroxy fatty acid (HFA) joined via an ester bond. A number of possible FAHFA structures resulting from combinations of FAs and HFAs and different regioisomers defined by branching position, makes this novel group of lipids quite extensive. Moreover, different FAHFA structure possess different biological activities with antidiabetic and anti-inflammatory being the most studied.

Since FAHFAs have been recently discovered, their metabolism particularly biosynthesis and degradation has not been fully elucidated yet. However, there are some lipolytic enzymes known to control metabolism of FAHFAs and their hydrolysis such as adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) among others. Regarding FAHFA biosynthesis, the synthesis of HFAs is of interest as FA synthesis is already well described. The dissertation thesis deals with the metabolism of FAHFAs and TAG estolides, the compounds from which FAHFAs are liberated upon demand. Among others, it focuses on the biosynthesis of FAHFA, particularly on the involvement of antioxidant enzyme Peroxiredoxin 6 in this process. To fulfill the objectives of the thesis, in vitro and in vivo experiments were carried out and samples measured via LC-MS.

# 2 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 influences 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.

# 3 MATERIAL AND METHODS

## 3.1 Samples

We used different cell lines, mouse models, and human samples to investigate FAHFA metabolism, synthesis, and their levels in various matrices. For in vitro experiments, we used two different cell lines – 3T3-L1 adipocytes and transfected HEK293T cells. Animal studies were carried out with C57BL/6J or C57BL/6N mice and genetically modified models derived from them (ATGL KO, HSL KO, and Prdx6 altered mouse models). Mice were kept in a stable environment and their use was approved by the local ethical committee. We also used human samples, specifically human breast milk

provided by the institute for Mother and Child Care in Prague with signed informed consent of participants of the study.

### 3.2 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.

### 3.3 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.

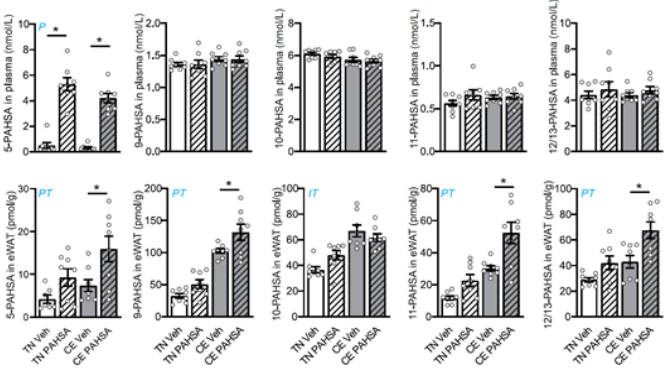
### 3.4 Data Processing and Statistical Analysis

LC-MS and LC-MS/MS data from the LIMeX workflow were processed in MS-DIAL software ver. 4.70. 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 was calculated using Python scripts. Raw data were filtered through blank samples with relative standard deviation (RSD) < 30% and then normalized for each matrix. 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.

# 4 RESULTS

## 4.1 Metabolism of 5-PAHSA (Publication I)

After seven days in a cold environment (CE, 6°C) or thermoneutrality conditions (TN, 30°C), we observed that administration of 5-PAHSA rose levels of 5-PAHSA in TN and CE but did not affect other regioisomers in plasma (Graph 1). 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

To investigate the role of 5-PAHSA in lipid metabolism, we used isotopic labeling ($^{2}H_2O$, $^{13}C_6$ glucose, 4-$^{2}H$ glucose and $^{13}C_5$ glutamine). The fractional rates of palmitic and stearic acid were elevated in a cold environment, but only stearic acid was significantly affected by 5-PAHSA treatment. 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. Short-chain TAGs such as TAG 48:0 were the most enriched with $^{2}H$ and its lipolytic product (DAG 32:0) was increased in the CE group gavaged with 5-PAHSA, which stimulated acylglycerol remodeling.

Administration of 5-PAHSA 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. 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 (Fig. 1).

There was no deuterium enrichment within FAHFA structure suggesting that FAHFAs might be stored intracellularly in TAG estolides and released upon demand. LC-MS analysis of epididymal adipose tissue (eWAT) of ATGL knock-out (KO) mice showed minimum levels of TAG EST and PAHSAs compared to wild-type (WT) mice meaning that the release of FAHFAs from TAG EST structure is controlled by ATGL.

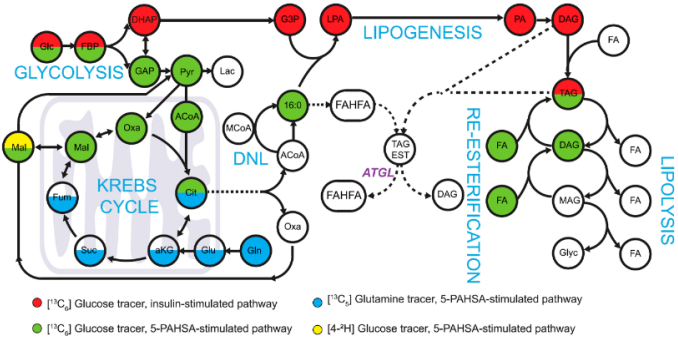
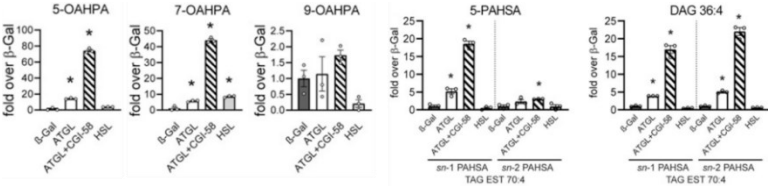



Figure 1. Metabolic pathways affected with 5-PAHSA and insulin

### 4.2 Metabolism of TAG Estolides (Publication II)

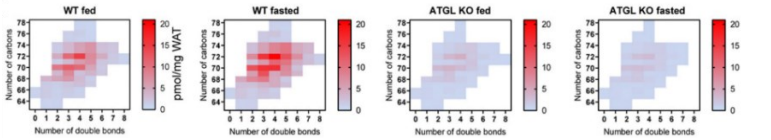
ATGL alone, or in combination with the activator CGI-58, was shown to be the key enzyme for the hydrolysis of the glycerol ester bond, thus releasing the free FAHFA from the TAG EST structure (Graph 2).

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.



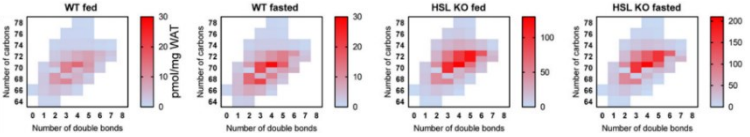

Graph 2. FAHFAs released from TAG estolides by ATGL or HSL

Transacylase activity of ATGL translocated 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. At last, we analyzed eWAT ATGL KO mice to verify if the ATGL plays a crucial role in TAG estolide metabolism also in vivo model. 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 3).




Graph 3. Levels of TAG EST in eWAT of ATGL KO mice

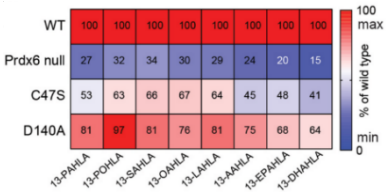
Apart from the hydrolysis of the glycerol ester bond, we also assessed the hydrolysis of the estolide bond, which is the ester bond within the FAHFA structure. Such hydrolysis increases the level of a particular FA and HSL turned out to be was an effective hydrolytic enzyme for all FAHFAs bound within TAG EST, with exceptional hydrolytic activity for 16-OAHPA with a linear structure. We analyzed HSL KO mice, 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 4).




Graph 4. Levels of TAG EST in eWAT of HSL KO mice

### 4.3 Peroxiredoxin 6 in FAHFA Biosynthesis (Publication III)

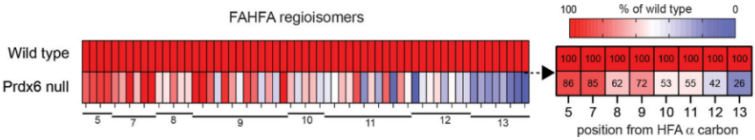
Although we did not observe any major effect of the genetic modifications of Prdx6 in neither of the mouse models, there was a significant influence on FAHFA levels, especially in Prdx6 deficient mice (Prdx6 null) and mice lacking the peroxidase activity (C47S). We focused on FAHFAs containing hydroxy oleic acid in their structure, namely on 13-hydroxy oleic acid (13-HLA) (Graph 5). 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 5. Levels of FAHFAs containing 13-HLA (FAHLAs) in scWAT

The analysis of selected HLA-containing TAGs revealed their downregulation in most Prdx6 mouse models, especially in Prdx6 null and C47S mice, suggesting Prdx6 participation in FAHFA synthetic pathway. We also tracked specific phosphatidylcholine (PC) and its corresponding peroxidized or hydroxylated forms in in vitro experiment to test the involvement of Prdx6 in synthesis of HFAs. We noticed similar profiles not only in oxidized PCs but also in related FAHLAs, however, 13-HLA was decreased only in the group with both inhibitors corresponding with the whole body knockout of Prdx6.

Comparison of all measured regioisomers of various FAHFAs in Prdx6 null mice with WT mice showed 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 (Graph 6). However, we did not observe such a trend in C47S or D140A mice.

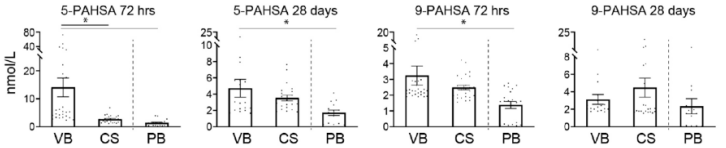



Graph 6. Representation of FAHFA regioisomers in scWAT

### 4.4 Bioactive Lipids in Human Breast Milk (Publication IV)

Following on from previous work (Brezinova et al., 2018), we focused on members of the PAHSA family, 5- and 9- PAHSA (Graph 7). 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 7. The effect of mode of delivery and lactation stage on PAHSA levels

We also looked at other FAHFAs, but 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. 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. Thus, we concluded that levels of TAG EST in the human breast are stable regardless of the mode of delivery and stage of lactation.

## 5 DISCUSSION

Cold exposure and fasting increased levels of FAHFAs, including 5-PAHSA in WAT (Kuda et al., 2018; Yore et al., 2014) (**Publication I**). 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). Moreover, 5-PAHSA directed carbon flux to the Krebs cycle, and synthesis of DNL precursors. In

contrast, the insulin-stimulated pathway led to excessive lipid storage. We managed to identify PAHSA-containing TAG estolides in our lipidomic data, which supported recently published work describing the discovery of this novel lipid group (Tan et al., 2019). The absence of ATGL in AKO mice caused a decline in FAHFA levels as well as in levels of TAG EST, 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).

ATGL mainly participated in releasing FAHFAs from the TAG estolide structure by cleaving the ester bond between glycerol and FAHFA (**Publication II**). As a substrate, ATGL preferred TAGs and FAs of a minimum chain length of eight carbons favoring unsaturated FAs (Eichmann et al., 2012). When ATGL was used in combination with CGI-58, 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). Complex double or triple TAG estolides were observed under experimental conditions, however, they were not detected double or triple estolides in human or mouse samples (Brezinova et al., 2020; Paluchova 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 et al., 2020). In contrast, HSL is far less substrate-specific as it catalyzes reactions with various substrates, including TAGs, DAGs, MAGs, and others (Kraemer & Shen, 2002). 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, which is in agreement with previous studies (Tan et al., 2019).

The study of peroxiredoxin 6 (**Publication III**), which was already linked with FAHFA biosynthesis (Kuda et al., 2018), showed only mild effects

of Prdx6 deletion in scWAT lipidome. 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). Significantly decreased levels of 13-FAHLAs in Prdx6 null and C47S mice proved the influence of Prdx6 peroxidase activity on FAHFA levels. Levels of 13-HLA-containing hydroxy TAGs, which belong among ATGL substrates, were similar as levels of 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). Levels of peroxidized PC, hydroxylated PC, and 13-HLA-containing FAHFAs showed that Prdx6 peroxidase activity is linked with FAHFA metabolism, but at the same time it suggested that Prdx6 is not the only enzyme from the peroxidase family, and glutathione peroxidase 4 (GPX4) could also have a role in the synthesis of FAHFA precursors (Kagan et al., 2017). Substrate specificity of Prdx6 was observed in Prdx6 deleted mice, however, not 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).

The analysis of human breast milk (**Publication IV**) was the first time TAG estolides were identified in this matrix, 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 (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. The core of milk fat globules can potentially store TAG estolides (Lee et al., 2018). The fact that ATGL 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).

## 6 CONCLUSION

We proved that cold exposure 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 5-PAHSA and insulin to find out that the administration of 5-PAHSA lipokine has certain advantages over insulin, although its effect was substantially weaker.

Our laboratory also confirmed the role of 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.

We also focused on antioxidant enzyme peroxiredoxin 6 and showed which of its enzymatic activity specifically participates in FAHFA biosynthesis. Moreover, we explained the substrate specificity of Prdx6 towards HpFAs with the hydroperoxyl group located further from the α-carbon atom.

Finally, analysis human breast milk revealed 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.

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## LIST OF PUBLICATIONS

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