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# Branched and linear fatty acid esters of hydroxy fatty acids (FAHFA) relevant to human health

Martin Riecan, Veronika Paluchova, Magno Lopes, Kristyna Brejchova, Ondrej Kuda *

*Institute of Physiology, Czech Academy of Sciences, 14220 Prague 4, Czech Republic*

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Fatty acid esters of hydroxy fatty acids (FAHFAs) represent a complex lipid class that contains both signaling mediators and structural components of lipid biofilms in humans. The majority of endogenous FAHFAs share a common chemical architecture, characterized by an estolide bond that links the hydroxy fatty acid (HFA) backbone and the fatty acid (FA). Two structurally and functionally distinct FAHFA superfamilies are recognized based on the position of the estolide bond: omega-FAHFAs and in-chain branched FAHFAs. The existing variety of possible HFAs and FAs combined with the position of the estolide bond generates a vast quantity of unique structures identified in FAHFA families.

In this review, we discuss the anti-diabetic and anti-inflammatory effects of branched FAHFAs and the role of omega-FAHFA-derived lipids as surfactants in the tear film lipid layer and dry eye disease. To emphasize potential pharmacological targets, we recapitulate the biosynthesis of the HFA backbone within the superfamilies together with the degradation pathways and the FAHFA regioisomer distribution in human and mouse adipose tissue. We propose a theoretical involvement of cytochrome P450 enzymes in the generation and degradation of saturated HFA backbones and present an overview of small-molecule inhibitors used in FAHFA research.

The FAHFA lipid class is huge and largely unexplored. Besides the unknown biological effects of individual FAHFAs, also the enigmatic enzymatic machinery behind their synthesis could provide new therapeutic approaches for inflammatory metabolic or eye diseases. Therefore, understanding the mechanisms of (FA)HFA synthesis at the molecular level should be the next step in FAHFA research.

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*Abbreviations:* CYP, cytochrome P450; HFA, hydroxy fatty acid; HSA, hydroxystearic acid; FA, fatty acid; TAG, triacylglycerol.

* Corresponding author at: Laboratory of Metabolism of Bioactive Lipids, Institute of Physiology of the Czech Academy of Sciences, Videnska 1083, 14220 Prague, Czech Republic.
*E-mail address:* ondrej.kuda@fgu.cas.cz (O. Kuda).

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## 1. Introduction

FAHFAs belong to a family of estolides that are defined as oligomers of a hydroxy fatty acid and a fatty acid. Hence, FAHFA is a monoestolide in which the carboxylic acid group of a fatty acid (FA) is esterified to the hydroxyl group of a hydroxy fatty acid (HFA). Depending on the length and saturation of their acyl chains and the position of the branching carbon, they are grouped into various super−/sub-families (Brejchova et al., 2020; Yore et al., 2014). For instance, 5-PAHSA common abbreviation expands to 16:0-(5-O-18:0), where "5" defines the ester position relative to carboxylic acid, 16:0 stands for palmitic acid (PA), and (5-O-18:0) stands for hydroxy stearic acid (HSA) (Ma et al., 2015; Yore et al., 2014).

We can divide FAHFAs related to human health into two main superfamilies based on the estolide bond position. These superfamilies include 1) in-chain "branched" FAHFAs, involved in the regulation of metabolism and immune reactions, and 2) "linear" (ω-hydroxylated) FAHFAs serving mainly as biosurfactants and skin barrier matrix (Fig. 1). The first superfamily contains several hundred members (Brejchova et al., 2020; Zhu et al., 2020) and the latter even more species (Butovich, 2017; Khanal et al., 2021; Vavrusova et al., 2020).

The biochemical difference between the families is also the origin of HFA. Only little is known about the branched saturated HFA synthesis that happens mainly in adipose tissue (Yore et al., 2014). More is known about unsaturated HFA synthesis that is driven mainly by enzymatic reactions (Hajeyah et al., 2020), while enzymes oxidizing the ω-carbon in sebaceous and meibomian glands are known (see below). Free FAHFAs can also be incorporated into complex lipids. Branched FAHFAs, mainly saturated, are stored in triacylglycerol (TAG) estolides (Brejchova et al., 2021; Brezinova et al., 2020; Paluchova, Oseeva, et al., 2020; Tan et al., 2019) and the ω-hydroxylated FAHFAs were found in the form of cholesteryl esters (Butovich, 2017; Butovich et al., 2009; Kaluzikova et al., 2017).

In this review, we discuss the endogenous and exogenous levels and potential sources of FAHFAs in humans. Next, we briefly summarize FAHFA biological effects related to human diseases for the most studied species. To transfer the therapeutic potential of FAHFAs into a drug, we need to identify the potential pharmacologic targets within the metabolic pathways. We review metabolic pathways of FAHFA synthesis and degradation, speculate on the missing parts, and summarize the small molecule inhibitors used in the literature. Strategies for total organic synthesis of FAHFA and optimal detection methods have been reviewed elsewhere (Balas et al., 2018; Brejchova et al., 2020; Hancock et al., 2018; Kokotou, 2020).

## 2. Sources of FAHFAs

FAHFAs are synthesized endogenously in specialized tissues (e.g. adipose tissue or meibomian glands) and the branched FAHFAs also

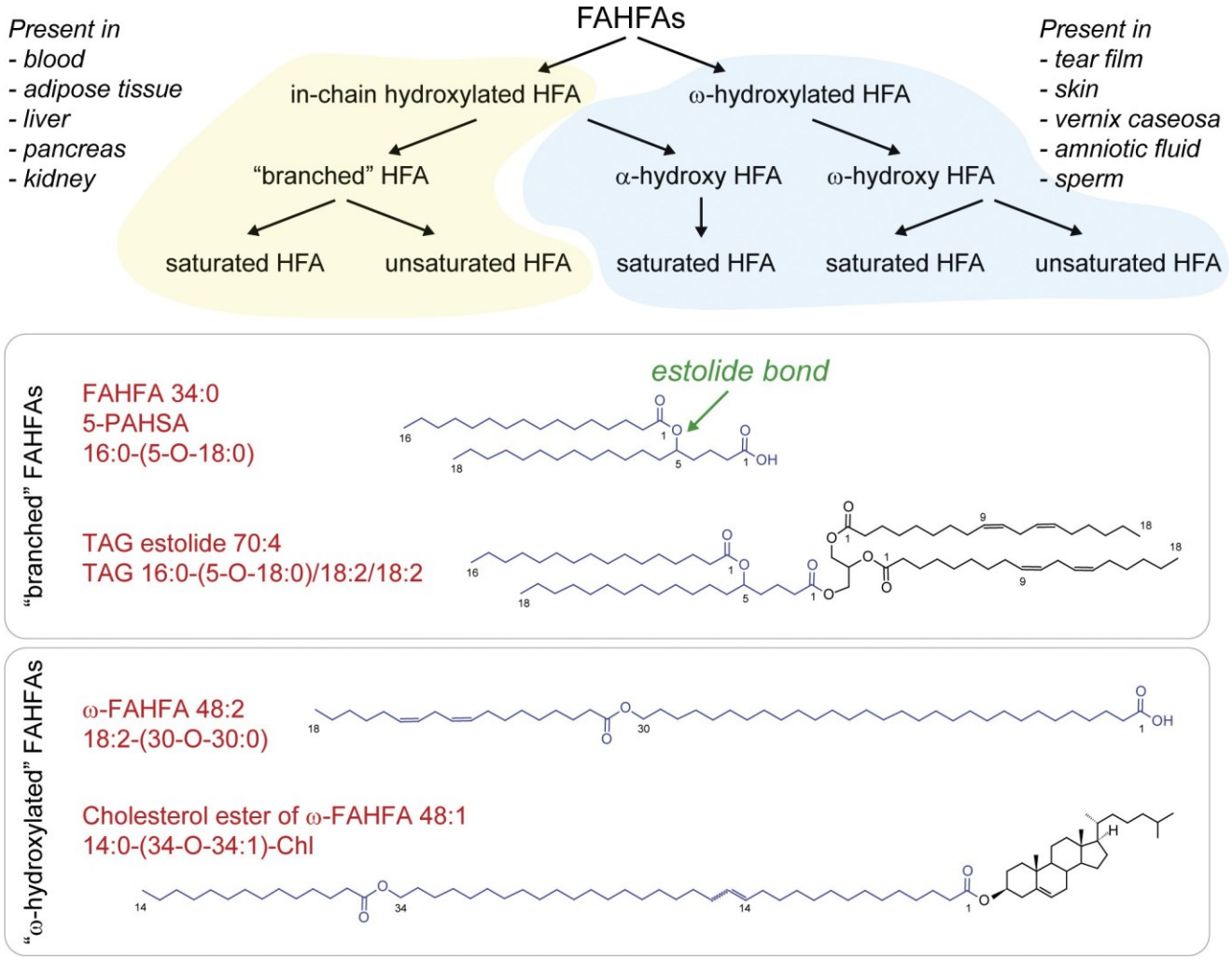


Fig. 1. Hierarchical scheme of FAHFA kingdom based on HFA structure and metabolic origin. The yellow cloud is related to metabolic regulations and the blue cloud is related to biosurfactants. Examples of branched (upper panel) and ω-hydroxylated (lower panel) FAHFA-related lipids. Structures of 5-PAHSA, a triacylglycerol estolide containing 5-PAHSA, ω-FAHFA, and cholesteryl ester of ω-FAHFA.

Table 1
Quantification of FAHFAs in human samples.

| Matrix | Main Analyte | Approx. Range | Other Detected FAHFAs | Ref. |
|---|---|---|---|---|
| adipose tissue | 9-PAHSA | 10–60 pmol/g | 5-PAHSA, 10-PAHSA, 12/13-PAHSA | Yore et al. (2014), Hammarstedt et al. (2018) |
| adipose tissue | 9-PAHSA | 10–50 pmol/g | 5-PAHSA, 10-PAHSA, 11-PAHSA, 12/13-PAHSA | Brezinova et al., 2020 |
| adipose tissue | 9-PAHSA | 10–50 pmol/g | all branched FAHFAs | Brejchova et al. (2020) |
| adipose tissue | TAG EST total | 10–60 pmol/mg | TAG EST 72:4, TAG EST 70:5, TAG EST 68:1 | Brezinova et al., 2020 |
| serum | 9-PAHSA | 2.5–11.5 nM | 5-PAHSA, 10-PAHSA, 12/13-PAHSA | Yore et al. (2014), Hammarstedt et al. (2018) |
| serum | 9-PAHSA | 4.1–4.6 nM | 5-PAHSA, 9-PAHSA, 12–13-PAHSA, 13-DHAHLA, 9-DHAHLA, 14-DHAHDHA | Kuda et al. (2016) |
| serum | 9-PAHSA | 0.3–0.8 nM | 5-PAHSA, 12-PAHSA, 13-PAHSA, 9-SAHSA, 12-SAHSA, 13-SAHSA | Zhu et al. (2017) |
| serum | 9-PAHSA | 2.5–20 nM | 5-PAHSA, 10-PAHSA, 11-PAHSA, 12/13-PAHSA | Brezinova et al. (2020) |
| serum | 9-OAHSA | 100–1000 nM | 9-POHSA | Dongoran et al. (2020) |
| serum | 9-PAHSA | 1–3 nM | 10-PAHSA, 12/13-PAHSA, 9-OAHSA, 9-SAHSA, 9-PAHPA | Kellerer et al. (2021) |
| plasma | PAHSAs | 15–20 nM | shotgun, no separation of regioisomers | Hu et al., 2020 |
| milk | 5-PAHSA | 0.2–1.6 nM | 5-PAHSA, 10-PAHSA, 11-PAHSA, 12/13-PAHSA | Brezinova et al. (2018) |
| meibum | OAHFAs | 2.9–3.3% total lipids | all ω-FAHFAs | Lam et al. (2011), Brown et al. (2013) |
| tears | OAHFAs | 3.6–5.2% total lipids | all ω-FAHFAs | Brown et al. (2013) |
| vernix caseosa | OAHFAs | ~0.04% total lipids | all ω-FAHFAs | Vavrusova et al. (2020) |

circulate in the blood (Table 1). Therefore, branched FAHFAs can be detected in organs that do not produce them independently (e.g. lungs, thymus, heart) and which are exposed to FAHFAs in the blood (Yore et al., 2014; Zhu et al., 2017). Besides endogenous synthesis, nutrition is another important source of FAHFAs as was recently shown in a cohort of vegans and omnivores (Kellerer et al., 2021). FAHFAs have been found in different plants (e.g. rice, olive oil, oat) (Kolar et al., 2019; Kuda, 2018; Syed, Lee, Peroni, et al., 2018; Zhu et al., 2018) or animal products (e.g. eggs, meat) (Ma et al., 2015; Pham et al., 2019). It is known that at least 5-PAHSA regioisomer is not digested upon oral intake and reaches the circulation in mice (Brezinova et al., 2018; Syed, Lee, Moraes-Vieira, et al., 2018; Yore et al., 2014).

## 3. FAHFA biological effects

FAHFA lipid class is very diverse and theoretically, any combination of an FA and an HFA is conceivable. Therefore, there are probably bioactive species with positive or negative effects on target receptors as well as species that exert their effects as amphiphilic structural lipids. We reviewed branched FAHFA biological effects in cell and animal models recently (Brejchova et al., 2020) and here we focus on the FAHFA effect related to human health and FAHFA therapeutic potential.

### *3.1. Anti-diabetic effects*

Experiments in rodents show that saturated FAHFAs have the potential to improve glucose tolerance without major negative side effects. Low PAHSA levels were reported in pathophysiological metabolic situations (obesity, diabetes) in humans (Hammarstedt et al., 2018; Kellerer et al., 2021; Yore et al., 2014). Although no clinical study testing FAHFA supplementation was performed, therapeutic strategies to increase PAHSA levels in humans could potentially help with insulitis and glucose intolerance. Nutritional therapy could be a suitable approach to manipulate FAHFA levels in humans because the composition of food lipids affects the systemic FAHFA levels. A group of young omnivores had higher serum levels of total PAHSAs than vegans and one week of overfeeding with whipped cream significantly increased total FAHFA levels in circulation (Kellerer et al., 2021).

Diabetes mellitus is a metabolic disorder of carbohydrate metabolism characterized by high blood glucose levels (hyperglycemia) and usually resulting from insufficient production of the hormone insulin due to autoimmune destruction of insulin-producing β cells (type 1 diabetes, T1D) or an ineffective response of cells to insulin (type 2 diabetes, T2D). Despite major advances in glucose monitoring and insulin delivery techniques, many patients with diabetes develop long-term complications. The PAHSA family has been found to be positively associated with insulin sensitivity in mice and humans (Brezinova et al., 2020; Yore et al., 2014; Zhou et al., 2019). Daily oral 5- and 9-PAHSA administration to non-obese diabetic mice delayed the onset of T1D and markedly reduced its incidence by attenuating immune responses and exerting direct protective effects on β cell survival and function. PAHSAs also prevented impairment of glucose-stimulated insulin secretion, increases islet β cell proliferation and viability, and improved glucose tolerance in these mice (Syed et al., 2019). Oral administration of 5- or 9-PAHSA regioisomer in mice fed chow or high-fat diet improved glucose tolerance and augmented insulin and glucagon-like peptide 1 secretion in vivo without affecting adiposity (Syed, Lee, Moraes-Vieira, et al., 2018; Vijayakumar et al., 2017; Wang et al., 2018b; Yore et al., 2014). Across both murine and human islets of Langerhans, chronic exposure to 5-PAHSA demonstrated augmented glucose-stimulated insulin secretion (Bandak et al., 2018).

The 5- and 9-PAHSA suppressed lipolysis and affected hepatic glucose production through modulation of free FA levels in circulation, which was shown by hyperinsulinemic-euglycemic clamps in mice. Also, the 9-PAHSA inhibited isoproterenol-induced lipolysis in murine perigonadal adipose tissue explants (Zhou et al., 2019). On the other hand, 5-PAHSA was found to increase lipolysis and stimulate de novo lipogenesis in adipocytes (Paluchova, Oseeva, et al., 2020). 9-PAHSA also increased viability and decreased steatosis of HepG2 hepatocytes (Schultz Moreira et al., 2020). While PAHSA effects in adipose tissue are explored, much less is known about the effects on other organs involved in lipid and carbohydrate metabolism. For instance, the liver contains a PAHSA concentration profile different from adipose tissue (Yore et al., 2014) and hepatocyte lipid droplets (LDs) could serve as an independent source of FAHFAs.

Besides PAHSAs, also other FAHFAs have been reported to be effective in increasing insulin sensitivity. The long-term high intake of palmitic or oleic acid esters of hydroxypalmitic acids (9-PAHPA or 9-OAHPA, respectively) increased basal metabolism and insulin sensitivity in healthy mice (Benlebna, Balas, Bonafos, Pessemesse, Fouret, et al., 2020; Benlebna, Balas, Bonafos, Pessemesse, Vigor, et al., 2020). The oleic acid esters of hydroxystearic acids (OAHSAs) mirror the concentration profiles of PAHSAs and could serve as the alternative markers of PAHSA effects (Kolar et al., 2018).

### 3.2. Anti-inflammatory effects

Several members of the FAHFA family have generated increasing interest due to their anti-inflammatory effect on both the innate and adaptive branches of the immune system (Kolar et al., 2019; Kuda et al., 2016; Syed et al., 2019; Yore et al., 2014). Especially PAHSAs could be safe oral therapeutics that prevent ulcerative colitis, a chronic inflammatory disease affecting the colon (Lee et al., 2016). PAHSAs in a murine model of colitis attenuated colonic T cell activation and pro-inflammatory cytokine and chemokine expression (Lee et al., 2016). 5-PAHSA could also help with the maturation of intestinal epithelium during lactation in humans (Brezinova et al., 2018).

Chronic PAHSA treatment reduced the total number of $CD11c^+$ (pro-inflammatory) macrophages and macrophages expressing IL-1β and TNF-α in white adipose tissue of chow- and high fat diet-fed mice (Syed, Lee, Moraes-Vieira, et al., 2018; Yore et al., 2014). In vitro studies confirmed 9-PAHSA-, 9-POHSA-, and 9-OAHSA-mediated downregulation of lipopolysaccharide (LPS)-stimulated macrophage activation and cytokine production (Dongoran et al., 2020; Kolar et al., 2019; Kuda et al., 2016; Wang, Chang, et al., 2019). Mechanism of the anti-inflammatory effects seems to be associated with free fatty acid receptor 4 (GPR 120) activation and inhibiting the lipopolysaccharide activated NF-kB pathway (Lee et al., 2016; Syed, Lee, Moraes-Vieira, et al., 2018; Wang et al., 2018a), however, studies on the liver and adipose tissue crosstalk suggested that multiple G protein-coupled receptors (families) could be involved (Zhou et al., 2019). Moreover, 5-PAHSA binds Sialic acid-binding immunoglobulin-like lectins (Siglecs) Siglec5 and Siglec14, which belong to the family of cell-surface immune receptors on macrophages, monocytes, and neutrophils (Suematsu et al., 2019). Activation of this pathway suppresses interleukin-8 production in human monocytic cells and may contribute to the anti-inflammatory effects of PAHSAs (Suematsu et al., 2019).

Other interesting species are polyunsaturated FAHFAs based on hydroxylinoleic acid, which exerted anti-inflammatory and pro-resolving effects. 13-DHAHLA reduced macrophage activation by LPS, reduced pro-inflammatory cytokines, and enhanced the phagocytosis of zymosan particles in murine macrophages and human peripheral blood mononuclear cells (Kuda et al., 2016). Both 13(*S*)- and 13(*R*)-DHAHLA inhibited $PGE_2$- and antigen-induced chemotaxis and the degranulation of mast cells (Paluchova, Vik, et al., 2020). 13-LAHLA is highly abundant in oat oil and suppressed the lipopolysaccharide-stimulated secretion of cytokines and the expression of proinflammatory genes in RAW 264.7 macrophages (Kolar et al., 2019). Again, nutritional therapy can be used to increase levels of these anti-inflammatory lipids in humans. Fish oil supplementation increased levels of anti-inflammatory ω-3 FAHFAs, mainly 13-DHAHLA, in diabetic patients (Kuda et al., 2016). Intake of oat oil liposomes rich in 13-LAHLA prolonged postprandial lipid digestion, reduced total energy intake, and modified satiety and appetite in humans (Ohlsson et al., 2014), and topical application of colloidal oatmeal improved skin dryness, scaling, roughness, and itching in humans (Reynertson et al., 2015). Structurally related polyunsaturated FAHFAs were explored for their antioxidant effects including the suppression of lipid (per)oxidation (Gowda et al., 2021; Gowda, Fuda, et al., 2020).

However, not all FAHFAs are positively associated with health. Murine model of inflammatory renal disease (lupus nephritis) associated with systemic lupus erythematosus revealed significant elevation of the total FAHFA levels in the kidney reflecting the progression of the disease (Hu et al., 2021). Some FAHFAs could also serve as markers of colorectal cancer (Liu et al., 2020; Rodriguez et al., 2019). Detailed characterization of FAHFA species associated with pathophysiology is crucial for the correct interpretation of the result.

### 3.3. Biosurfactants

The ω-FAHFAs have specific properties related to surface tension. Their structure allows them to interact with both polar and non-polar phases and create specific lipid films (Schuett & Millar, 2013). Meibomian glands in the eyelid secrete a unique mixture of lipids to create a tear film that prevents water evaporation and reduces surface tension, reviewed in (Butovich, 2017). Meibum contains (O)-acylated ω-hydroxy fatty acids, known as OAHFAs among ophthalmic researchers, or ω-FAHFAs with extremely long FAs (C30-C36 HFA and C16-C18 FA) (Butovich et al., 2009; Khanal et al., 2021). Tears have their own composition of ω-FAHFAs (Khanal et al., 2021). The ω-FAHFAs are diminished in the tear film of dry eye patients (Lam et al., 2011; Lam et al., 2014; Miyamoto et al., 2020), and a metabolic dysbalance in meibogenesis results in high triglycerides-low waxes syndrome (Butovich & Suzuki, 2020). T2D could influence the composition of meibum lipids and contribute to meibomian gland degeneration and dry eye disease (Q. Yang et al., 2021). The ω-FAHFAs can be incorporated into cholesteryl esters (Chl-OAHFA) or even more complex structures like type 1 wax diesters 16:1-(34-O-34:1)-16:1 (Miyamoto et al., 2020). All these structures create an amphiphilic lipid layer that influences the stability of the tear film (Butovich, 2017) and may contribute to the anti-inflammatory lipid mix in bovine pink eye syndrome (Wood, Donohue, et al., 2018).

Physiologically related to meibum is sebum, a product of sebaceous glands, but its lipid composition is different (Butovich, 2017; Butovich et al., 2016; Butovich et al., 2019; Hinde et al., 2013). Keratinocytes synthesize ω-FAHFA and ω-O-acylceramides as lipid components essential for skin barrier (Hirabayashi et al., 2017). Acylceramides are critical for epidermal barrier function and the lack of epidermal ω-O-acylceramides is incompatible with survival in a desiccating environment (Vasireddy et al., 2007).

Vernix caseosa is a complex biofilm covering the human fetus and its sebum-related lipidome is only partially explored. It is a unique and extremely complex mixture containing hundreds of HFA-based species including cholesteryl esters of ω-FAHFAs, (O-acyl) α- and ω-hydroxy fatty acid and wax diesters (Kaluzikova et al., 2017; Nicolaides et al., 1970). Interestingly, ω-FAHFA contains ultra-long and unsaturated acyls, while α-FAHFAs comprise shorter saturated chains (Vavrusova et al., 2020). The ω-FAHFAs were identified also in amniotic fluid (Wood, Ball, et al., 2018) and are thought to participate in the delivery process as a lubricant or to help with vernix caseosa emulsification during development (Nishijima et al., 2019).

The therapeutic potential of ω-FAHFAs is apparent. Artificial tears based on OAHFA 18:1/34:1 have been preliminarily tested as a therapy for dry eye disease (Gan et al., 2017) and detailed analysis of vernix caseosa composition could uncover potential uses in cosmetic dermatology or wound healing.

## 4. Potential pharmacologic targets

The biology of FAHFAs is complex and we still do not know which FAHFAs are beneficial and which could be detrimental. It is clear that the family of PAHSAs and PAHPAs, and the polyunsaturated FAHFAs exert positive effects and that their levels are lower in pathophysiological states (Benlebna, Balas, Bonafos, Pessemesse, Fouret, et al., 2020; Kuda et al., 2016; Yore et al., 2014). Characterization of metabolic pathways, key enzymes, and regulation of FAHFA metabolism is critical for the development of therapeutic strategies and the identification of potential pharmacologic targets. Here we review the literature related to FAHFA biosynthesis and degradation including several speculations on the missing metabolites within the pathways.

## 5. FAHFA biosynthesis

### 5.1. Pathway intersection

The synthesis of FAHFAs can be divided into three steps: 1) formation of an HFA; 2) synthesis of an FA, and 3) HFA esterification with an FA.

First, the regioisomer distribution of hydroxyl positions on the acyl chain among the FAHFA kingdom suggests that a) branched HFA and ω-HFA are synthesized by different pathways, and b) branched saturated HFA are formed by different processes than branched unsaturated HFAs, reviewed in (Brejchova et al., 2020).

- Regarding the "linear" ω-HFA, Butovich and colleagues described the process of biosynthesis of meibum and ω-FAHFAs, meibogenesis (Butovich, 2017; Butovich et al., 2016). The synthesis of ω-FAHFA, ultra long FA, and related ceramides by CYP4F39/CYP4F22 was described by Hirabayashi and colleagues (Hirabayashi et al., 2017; Ohno et al., 2015). Very long chain and ultra long chain FAs are synthesized by an enzymatic cascade of elongases, desaturases, and acyltransferases (Butovich et al., 2016; Hirabayashi et al., 2017; Ohno et al., 2015; Sanders et al., 2008; Wilkerson et al., 2021). Based on their origin and function, α-FAHFAs are related to ω-FAHFAs (Vavrusova et al., 2020).
- The estolide bond positions in unsaturated branched FAHFAs largely follow known patterns of enzymatic and nonenzymatic acyl chain oxidation (Brejchova et al., 2020). A typical representative is hydroxy linoleic acid with two primary regioisomers: 9-HLA and 13-HLA, products of 15-lipoxygenase (Kolar et al., 2019; Kuda et al., 2016; Paluchova, Vik, et al., 2020; Vik et al., 2019; Wang, Kolar, et al., 2019). Cyclooxygenases, lipoxygenases, and oxidases of the cytochrome P450 family produce numerous polyunsaturated FA metabolites (reviewed in (Christie & Harwood, 2020; Hajeyah et al., 2020; Hardwick, 2008)).
- The exact source of branched saturated HFA is still unknown and several theoretical pathways and enzymes could be potentially involved in this process (summarized below).

Second, de novo lipogenesis pathway, providing the FA in FAHFA, is closely related to the synthesis of branched FAHFAs (Paluchova, Oseeva, et al., 2020; Yore et al., 2014), but the fatty acyl composition also mirrors diet (Paluchova, Vik, et al., 2020; Pham et al., 2019). Therefore, the FA might come from multiple sources.

Third, the lipid acyltransferases involved in the final step of FAHFA biosynthesis remain to be characterized. The acyl-CoA wax alcohol acyltransferases (AWAT) 1 and 2 from human acyl-CoA:diacylglycerol acyltransferase (DGAT) 2 gene superfamily are responsible for sebum wax ester formation (Turkish et al., 2005). Unfortunately, only fatty alcohols and acyl-CoA as substrates were tested. Overexpression of DGAT1/2, monoacylglycerol acyltransferase (MGAT1/2), and Sterol O-acyltransferase (ACAT) in HEK293T cells did not lead to acyltransferase activity and higher FAHFA levels (Kahn et al., 2015).

We will focus on the branched FAHFA in the following paragraphs.

### 5.2. Hydroxy fatty acyl level

The origin of branched saturated HFA is puzzling. Non-enzymatic acyl chain oxidation by various active forms of oxygen is the most probable source of branched saturated HFA (Spickett, 2020). Adipocytes with compromised antioxidant defense and glutathione depletion produced higher levels of several PAHSA regioisomers (Kuda et al., 2018). However, FAHFA kingdom is much more diverse (Zhu et al., 2020) and more controlled synthesis of bioactive substances should be expected, for instance, a family of cytochrome P450 could be involved in the hydroxylation of FAs.

Cytochrome P450s (CYPs) are a superfamily of enzymes, which are responsible for the metabolism of numerous endogenous and exogenous compounds. The fourth family of CYP enzymes (CYP4) is involved in the metabolism of fatty acids, especially in the synthesis of bioactive fatty acid derivatives like eicosanoids (Edson & Rettie, 2013; Hardwick, 2008; Jarrar & Lee, 2019). The human-specific CYP4 gene family consists of six subfamilies CYP4A, B, F, V, X, and Z (Edson & Rettie, 2013). Their role in the metabolism of fatty acids, substrate specificity, and protein expression levels in different tissues was recently reviewed by Jarrar and Lee (Jarrar & Lee, 2019). However, data on substrate specificity are incomplete and the current knowledge is based on experiments focused on specific hypotheses rather than open substrate screening (Table 2).

UniProt database contains four human proteins that catalyze the oxidation of the terminal carbon (ω-oxidation) of saturated and unsaturated fatty acids (Database Uniprot, version as of 7/23/2021): CYP4A11, CYP4V2, CYP4F3, and CYP2E1.

CYP4A11 oxidizes lauric acid (C12:0) at both the ω- and ω-1 positions to form 12- and 11-hydroxylaurate, respectively (Adas et al., 1999; Dhar et al., 2008; Powell et al., 1996). Saturated fatty acid substrate specificity was explored for CYP4A11 and CYP2E1 in human liver microsomes. The rate of metabolite formation decreased when increasing the alkyl chain length of saturated fatty acids (from C12:0 to C16:0), while no hydroxylated metabolite was detected with stearic acid (Adas et al., 1999). CYP4F3 and CYP4F2 oxidize very long-chain fatty acids at the ω-position (Sanders et al., 2008). CYP4F22 ω-hydroxylase is required for ultra long-chain acylceramide production (Ohno et al., 2015) and mouse ortholog CYP4F39 synthesizes hydroxy lipids in a tear film (Miyamoto et al., 2020).

CYP4V2 is a selective ω-hydroxylase of saturated, medium-chain fatty acids with relatively high catalytic efficiency toward myristic acid (C14:0). The catalytic efficiency decreases in the following order: myristate > laurate > palmitate (Nakano et al., 2009). Overexpression of CYP4V2 in HepG2 cells altered polyunsaturated fatty acid (PUFA) homeostasis (Nakano et al., 2012) and CYP4V2 mutations led to decreased hydroxyl-PUFAs in retinal pigmented epithelial cells (Zhang et al., 2020). CYP4V2 gene is linked to Bietti's crystalline dystrophy, which is characterized by disrupted lipid homeostasis and excessive accumulation of PUFAs in retinal epithelia (Zhang et al., 2020). CYP37A1 (ortholog of human cytochrome P450 CYP4V2 in the nematode *Caenorhabditis elegans*) and its redox partner P450 oxidoreductase were linked to the conversion of multilocular lipid droplets to unilocular ones in the environment- and temperature-dependent way (Li et al., 2017). A role of CYP4V2 variants in lipid remodeling in patients with

Table 2
Specificity of hydroxylation position for fatty acids and CYPs.

| | CYP2E1 | CYP4A11 | CYP4F2 | CYP4F22 | CYP4V2 | CYP4Z1 | CYP4Z1 |
|---|---|---|---|---|---|---|---|
| Acyl chain | Adas et al. (1999) | Adas et al. (1999) | Sanders et al. (2008) | Ohno et al. (2015) | Nakano et al. (2009) | Zöllner et al. (2009) | McDonald et al. (2017) |
| C8:0 | | | | | ω | | |
| C12:0 | ω-1 | ω, ω-1 | | | ω | ω-2, ω-3, ω-4, ω-5 | ω-1, ω-2, ω-3, ω-4, ω-5 |
| C14:0 | ω-1 | ω, ω-1 | | | ω | ω-2, ω-3, ω-4, ω-5 | |
| C16:0 | ω-1 | ω | | | ω | | |
| C18:0 | ω-1 | | | | | | |
| C18:1 | ω, ω-1 | ω, ω-1 | | | | | |
| C18:2 | ω, ω-1 | ω, ω-1 | | | | | |
| C22:0 | | | ω | | | | |
| C26:0 | | | ω | | | | |
| C26:0–36:0 | | | | ω | | | |

hepatic steatosis was recently observed (J.P. Hardwick, personal communication).

CYP4Z1 is an 'orphan' CYP fatty acid hydroxylase that is associated with breast cancer (Yang et al., 2017). Zöllner and colleagues explored biotransformation of a saturated fatty acid library (C8:0 − C20:0), oleic, and linoleic acid in a yeast model (*Schizosaccharomyces pombe*) expressing human CYP4Z1 (Zöllner et al., 2009). They found that CYP4Z1 hydroxylated lauric and myristic acid 'in-chain', which in both cases led to the formation monohydroxylated products at positions ω-2, ω-3, ω-4, and ω-5, respectively (Zöllner et al., 2009). The other substrates were not metabolized. Interestingly, co-expression of human redox partner cytochrome P450 reductase resulted in higher yields of hydroxylated products, similar to the situation in *Caenorhabditis elegans* and CYP4V2 (Li et al., 2017; Zöllner et al., 2009). The following study in the human breast cancer cell line overexpressing CYP4Z1 identified two additional substrates as arachidonic acid (C20:4) and eicosadienoic acid (C20:2) (Yu et al., 2012).

The exceptional ability of CYP4Z1 to oxidize lauric acid (C12:0) was independently confirmed when CYP4Z1 yeast microsomes were incubated with lauric acid in the presence of NADPH (McDonald et al., 2017). Mass spectrometry-based assays enabled discrimination of the regioselectivity of hydroxylation reaction and the 7-OH, 8-OH, 9-OH, 10-OH, and 11-OH monohydroxylated products were formed in a ratio of ~1:20:3:8:4 (McDonald et al., 2017) (Table 2).

These lines of evidence show that our current knowledge about the catalytic properties and the substrate spectrum of human CYPs is still very limited. It is possible that some CYPs can in-chain hydroxylate saturated FAs under specific conditions. Therefore, more screening experiments on CYPs are needed to explore their potential in FAHFA biosynthesis. The enzyme of interest is the one that prefers 9th carbon from the carboxylic end group (Fig. 2). Hydroxystearic and hydroxypalmitic acids have the highest number of regioisomers among saturated HFAs (Fig. 2A, adapted from Zhu et al. (Zhu et al., 2020)). The 9-FAHFA regioisomers usually dominate LC–MS profiles of white adipose tissue and biological effects are known (Fig. 2B) (Benlebna, Balas, Bonafos, Pessemesse, Fouret, et al., 2020; Kuda et al., 2016; Yore et al., 2014). Based on the structural motif, the carboxylic function might be important for substrate position and position 9 from the carboxylic end group should be located within the action radius of the active site (Fig. 2C), similar to the modeled lauric acid binding site in CYP4Z1 (Yan et al., 2017). It is possible that a new role (the missing hydroxylation activity) will be found for the known enzyme (s), similar to the discovery of FAHFA hydrolases (Brejchova et al., 2021; Parsons et al., 2016). The androgen-dependent TFPI regulating protein (ADTRP) was originally identified as a protein involved in the anticoagulant protection of the endothelium (Lupu et al., 2011) and later as a FAHFA hydrolase (Parsons et al., 2016). Adipose triglyceride lipase (ATGL) is a well-known lipase (Zimmermann et al., 2004) that can also remodel FAHFAs in TAG estolides in the presence of a specific co-activator (Brejchova et al., 2021). Controlled post-translational modification, expression of a specific co-factor, substrate structure overlap, or close interaction of an enzymatic cascade with non-enzymatic reaction could yield the missing intermediates and HFA products.

Interestingly, an innovative synthetic strategy was used to generate various HFA regioisomers (Zhang, Eser, & Guo, 2021). Two fatty acid hydratases, FA-HY1 and FA-HY2, from *Lactobacillus acidophilus* metabolize fatty acyl chain substrates and hydrate various double bond positions. Regioselectivity of the reaction can be modified by targeted mutations of the enzymes and enantiomeric hydroxy fatty acids can be produced in large quantities. This reaction was coupled to a yeast lipase A from *Candida antarctica* and formed a bi-enzymatic cascade pathway yielding optically pure FAHFAs (Zhang, Hu, et al., 2021). A subfamily of short-chain FAHFAs (acetic acid or propanoic acid esterified long-chain HFA) is present in the gut (Gowda, Liang, et al., 2020; Hou et al., 2021). The intestinal microbiota has many bacterial enzymes that can catalyze the production of unusual HFAs and also other lower organisms create a pool of HFAs, reviewed by Hammerer and colleagues (Hammerer et al., 2017).

### 5.3. Phospholipid level

The FAs can be oxidized in a free form or as acyl chains of complex lipids as a part of epilipidome (Spickett, 2020). Metabolic pathways in plants synthesizing HFA to be stored in TAG oils use phospholipid intermediates for HFA trafficking (reviewed in (Brejchova et al., 2020; Lee et al., 2015)). Therefore, it is plausible that also mammalian pathway could be based on phospholipid scaffold (Fig. 3).

Genome-wide linkage analysis in recombinant inbred rat strains highlighted nuclear factor erythroid 2–related factor 2 (Nrf2) pathway and a phospholipid-remodeling enzyme Peroxiredoxin 6 (Prdx6) has been liked to PAHSA formation in adipose tissue (Kuda et al., 2018). The loss of Prdx6 resulted in lower PAHSA levels (Kuda et al., 2018). Prdx6 has peroxidase, phospholipase A2, and lysophosphatidylcholine acyltransferase activities, and can produce a phospholipid with a hydroxy fatty acid or even cleave this hydroxy fatty acid (Fisher, 2017). Prdx6 is widely expressed in all mouse organs with the highest levels in the lungs (Manevich & Fisher, 2005). Its major role is to repair peroxidized phospholipids in cell membranes in the lungs (Li et al., 2015). Remarkably, Prdx6 plays several distinct roles in antioxidant defense and it is closely connected with diabetic complications. Prdx6 null mice develop a phenotype similar to early-stage T2D (reduced glucose-dependent insulin secretion and increased insulin resistance) (Pacifici et al., 2014). Impaired insulin signaling negatively affected the communication between the liver and adipose tissue in Prdx6 null mice fed a high-fat diet (Arriga et al., 2019). The involvement of Prdx6 in insulin sensitivity and diabetes is supported also by several other publications. Intravenous application of recombinant PRDX6 reduced the inflammatory response and ameliorated the immune status of mice with streptozotocin-induced diabetes (Novoselova et al., 2021). Increased expression of PRDX6 inhibited high glucose-triggered oxidative stress and ferroptosis in a model of diabetic nephropathy (Zhang, Eser, & Guo, 2021). These reports confirm that peroxiredoxins and redox balance could be connected to metabolic pathways of de novo lipogenesis and FAHFA synthesis.

Although several phospholipids containing 9-HSA were identified in human colorectal cancer samples and their metabolism was related to FAHFA synthesis (Rodriguez et al., 2019), a phospholipid containing FAHFA chain was not reported so far. We can speculate that the esterification of HFA with an FA happens either in the form of free (H)FA or via an unknown complex lipid intermediate. The composition of FA in FAHFAs mimics dietary sources and production of de novo lipogenesis pathway via endoplasmic reticulum (ER) under carbohydrate-responsive element-binding protein (ChREBP) supervision (Brezinova et al., 2020; Kuda et al., 2016; Paluchova, Vik, et al., 2020; Yore et al., 2014). The availability of the HFA backbone and substrate specificity of the unknown acyltransferase(s) define the levels and combinations of FAHFAs (Nelson et al., 2017; Yore et al., 2014). Dietary experiments with linoleic acid and docosahexaenoic acid in mice showed that the highest levels of the expected product 22:6(13-O-18:2) (aka 13-DHAHLA) were achieved when the FA substrates were provided in a form of triacylglycerol-based oil (Paluchova, Vik, et al., 2020). This suggests that triacylglycerol composition is closely related to FAHFA synthesis.

### 5.4. Triacylglycerol level

Branched FAHFAs are stored in the form of TAG estolides, TAG-FAHFA, in adipose tissue of mice and humans (Brezinova et al., 2020; Paluchova, Oseeva, et al., 2020; Tan et al., 2019). Physiological stimuli like fasting/feeding or cold exposure trigger lipolysis and free FAHFAs are liberated from TAG estolides by lipases (Paluchova, Oseeva, et al., 2020; Tan et al., 2019). Two major metabolic lipases, adipose

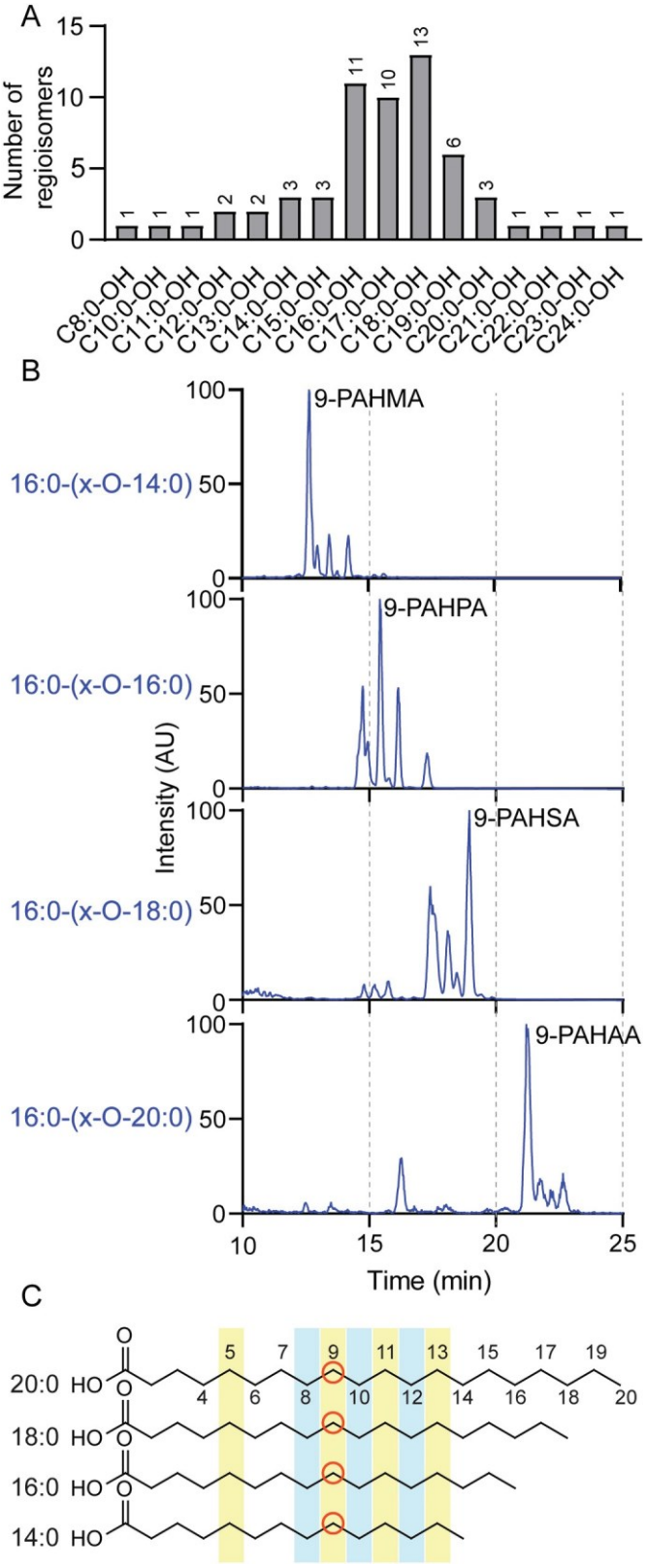


Fig. 2. Dominant regioisomers of saturated HFAs. A) Regioisomers of saturated HFAs detected in mouse visceral adipose tissue. Adapted from Zhu et al. (Zhu et al., 2020). B) LC–MS profiles of four saturated FAHFAs extracted from mouse adipose tissue. PAHMA, PAHPA, PAHSA, and PAHAA aligned MRM traces adapted from Brejchova et al. (Brejchova et al., 2020)). 9-FAHFA regioisomer dominates the composition. C) Alignment of saturated FA highlighting the 9th carbon from the carboxylic end group (red circle) and other known positions of PAHSA estolide bond.

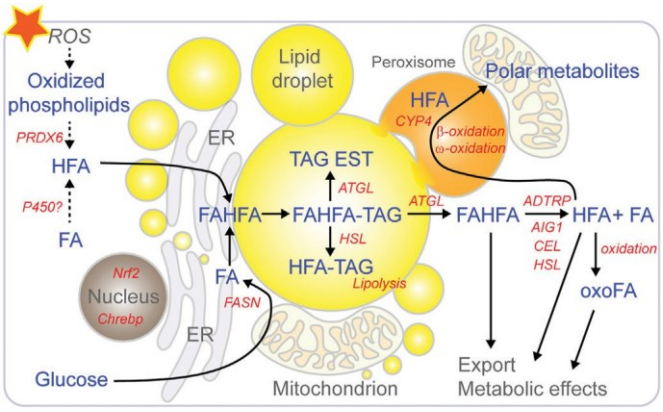


Fig. 3. Schematic representation of known potential PAHSA-related pathways. AIG1, androgen-induced gene 1; ADTRP, androgen-dependent TFPI regulating protein; ATGL, adipose triglyceride lipase; CEL, carboxyl ester lipase; ChREBP, carbohydrate-responsive element-binding protein; CYP4, cytochrome P450 family 4; ER, endoplasmic reticulum; FASN, fatty acid synthase; HSL, hormone-sensitive lipase; Nrf2, nuclear factor erythroid 2–related factor 2; oxoFA, carbonyl-containing FA; P450, cytochrome P450 family; PRDX6, peroxiredoxin 6; ROS, reactive oxygen species;

triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) have distinct roles in the catabolism of TAG estolides (Brejchova et al., 2021). ATGL alone or coactivated by comparative gene identification-58 efficiently liberated FAHFAs from TAG estolides with a preference for more compact structures where the estolide branching carbon atom is located near the glycerol ester bond. ATGL was also involved in remodeling and acyl-transferring reactions leading to the formation of new TAG estolides with alternative acyl compositions (migration of fatty acyls of FAHFA), or TAG double and triple monoestolides (two or three FAHFA acyl chains esterified to the glycerol backbone). HSL represented a potent estolide bond hydrolase for both TAG estolides and free FAHFAs (Brejchova et al., 2021). Combined actions of both enzymes create a pool of neutral lipid intermediates that could yield alternative FAHFA acyl combinations and control the release of specific FAHFAs.

The major part of FAHFA metabolism most probably happens on LDs. LDs are present in most eukaryotic cells and are dynamically synthesized and/or degraded as the body makes adjustments to achieve homeostasis (Olzmann & Carvalho, 2019). Recently, LDs have been recognized as independent organelles involved in lipid synthesis, remodeling, trafficking and also as important players of the cellular stress response (Jarc & Petan, 2020). LD lipolytic machinery provides a highly regulated, on-demand source of signaling lipids and a sink for oxidized lipids: lipids derived from their neutral lipid core, or the phospholipid monolayer, directly act as signaling mediators or are converted into ones (Bailey et al., 2015; Dichlberger et al., 2014; Jarc et al., 2018; Jarc & Petan, 2020). The FAHFAs could also modify local LD surface (Schuett & Millar, 2013). It is a perfect niche for FAHFAs.

## 6. FAHFA degradation

Several FAHFA hydrolases including androgen-induced gene 1 (AIG1) protein, androgen-dependent TFPI regulating protein (ADTRP), carboxyl ester lipase (CEL), ATGL, and HSL have already been identified as potential FAHFA hydrolases (Brejchova et al., 2021; Erikci Ertunc et al., 2020; Kolar et al., 2016; Parsons et al., 2016). Induced mutant mouse models proved that AIG1, ADTRP, and HSL are active FAHFA estolide bond hydrolases in vivo (Brejchova et al., 2021; Erikci Ertunc et al., 2020). Aig1 and Adtrp knockout and double knockout mice, respectively, were used to demonstrate that both proteins are responsible for regulating FAHFA hydrolysis in addition to regulation of TFPI-

dependent anticoagulant activity, preserving vascular integrity and normal cardiovascular function (Erikci Ertunc et al., 2020; Lupu et al., 2021). Interestingly, both enzymes contributed differentially to FAHFA hydrolysis rate in the liver, brown adipose tissue, and kidney and their membrane localization suggests a role in the regulation of circulating FAHFA levels (Erikci Ertunc et al., 2020; Lupu et al., 2021; Patel et al., 2018). AIG1 is highly expressed in the liver, adipose tissue, heart, and lung, and is involved in the control of endoplasmic reticulum $Ca^{2+}$ concentrations (Kuda et al., 2016; Nickel et al., 2016). CEL, known as bile salt-stimulated lipase, is associated with a monogenic form of diabetes due to mutations in the CEL gene and preferentially hydrolyzes FAHFAs over TAGs, cholesteryl esters, and phospholipids (Kolar et al., 2016). The pancreas secretes CEL to hydrolyze lipid species in the gut, but it is also secreted by the mammary gland during lactation into milk, where it could control FAHFA levels (Brezinova et al., 2018). In contrast to the previous hydrolases, ATGL and HSL are intracellular enzymes associated with the LDs. Both ATGL and HSL hydrolyzed free FAHFA in vitro (Brejchova et al., 2021).

Once hydrolyzed, free HFA can join the HFA pool and be used for FAHFA synthesis (Nelson et al., 2017). Alternatively, HFAs will be degraded. We can speculate on the degradation of HFA backbones based on the eicosanoid metabolism (Diczfalusy, 1994; Edson & Rettie, 2013; Gordon et al., 1990). In general, HFAs are most probably degraded via β- and ω- oxidation in peroxisomes (Fig. 4). Similar to eicosanoids, the 9-HSA backbone could be shortened by two cycles of β-oxidation to 5-OH-tetradecanoic acid (Diczfalusy, 1994; Gordon et al., 1990). Upon ω-oxidation of the methyl tail to carboxylic function, the chain-shortened product could be subsequently β-oxidized up to 5-OH-10-carboxy-decanoic acid, and eventually converted to a more polar metabolite or coupled to glutathione via the hydroxy group. Regioisomers of various HFA could hierarchically converge on a common substructure – a short-chain hydroxy (di)carboxylic acid. However, such metabolites are yet to be explored.

The alternative saturated HFA metabolic pathway could be oxidation to oxo-saturated fatty acids. Recently, several regioisomers of oxo-stearic acids were found in human plasma (Batsika et al., 2021). The

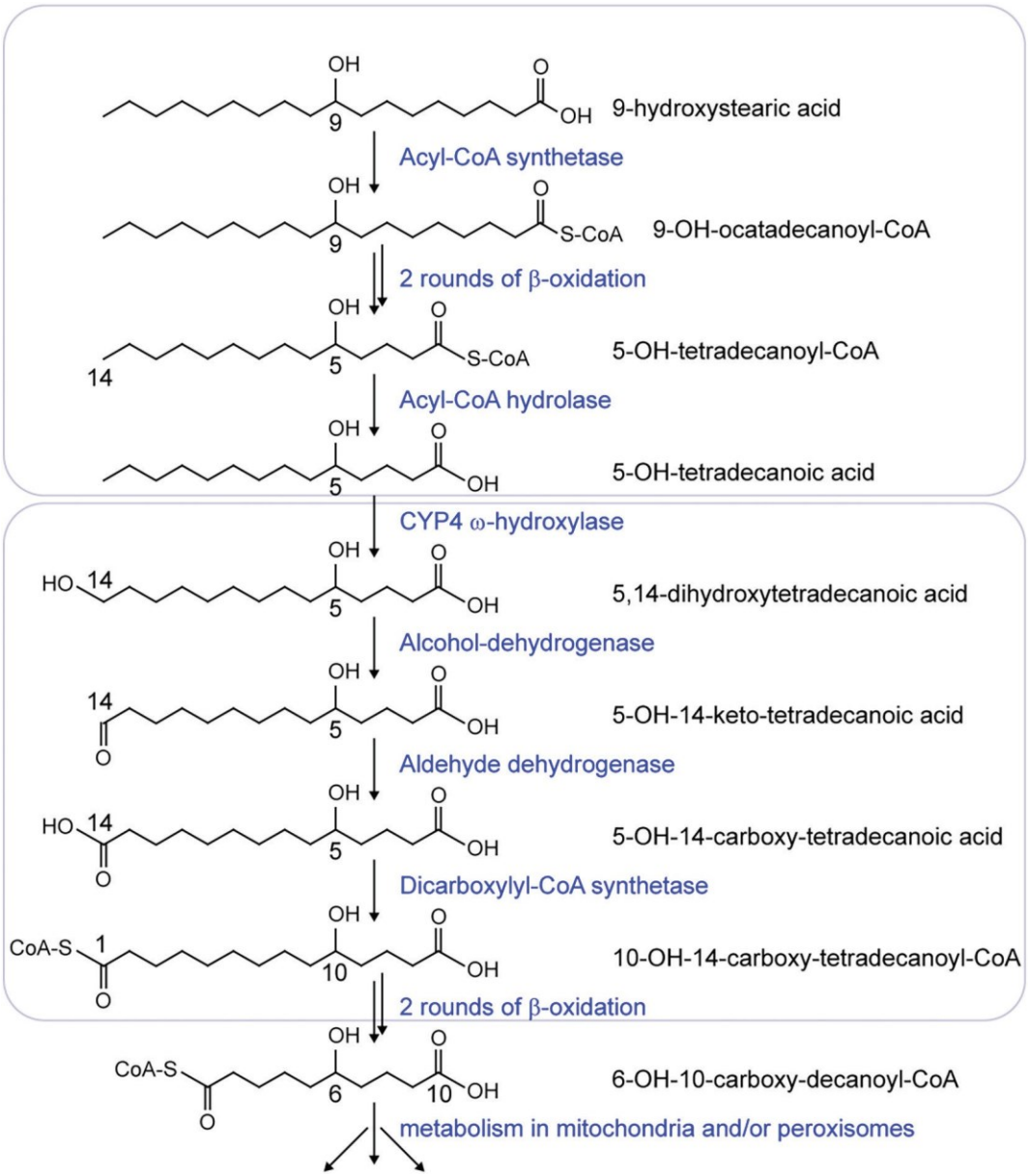


Fig. 4. Putative pathway of 9-hydroxystearic acid degradation. β-oxidation from the carboxylic end group (upper panel) followed by the ω- and β-oxidation from the methyl tail (lower panel).

concentration ranged from 4.9 to 19.5 ng/mL for 4-, 6-, 9-, and 10-oxostearic acids, respectively. It is currently unknown whether the oxostearic acids are produced by human metabolism or whether they come from the diet. Both saturated oxo-fatty acids and saturated hydroxyl fatty acids were found in cow milk (Batsika et al., 2021; Kokotou et al., 2020) and mouse visceral adipose tissue (Zhu et al., 2020) and mainly anti-inflammatory actions were reported for both species (Batsika et al., 2021; Calonghi et al., 2019; Kokotou et al., 2020; Parolin et al., 2012).

## 7. Potential pharmacologic tools in FAHFA research

Nowadays, basic research in FAHFA metabolism focuses on the identification of new biological effects and the exploration of potential metabolic pathways. Mainly tools based on gene modifications (knock out mice, siRNA) and FAHFA administration (gavage, osmotic minipumps, supplemented diet) are used. However, several FAHFA-related proteins were targeted with small molecule inhibitors (Table 3). G-protein coupled receptors GPR120 and GPR40 are two membrane receptors involved in PAHSA signaling and their inhibition abrogates the beneficial effect of PAHSAs (Syed, Lee, Moraes-Vieira, et al., 2018; Yore et al., 2014). Modification of the chemical structure of FAHFAs (a truncation of both acyl chains) most probably affects the FAHFA-receptor interactions and modifies the anti-inflammatory effects (Wang, Chang, et al., 2019). Detailed knowledge of the structure–activity relationship could serve as a primer for the design of FAHFA analogs targeted to specific receptors. Enzymes involved in the main lipid-processing pathways (ATGL, HSL, MAGL, DGAT) play a role in FAHFA metabolism, but their inhibition is more complicated for interpretation and does not represent a suitable drug target. The most promising strategy could be the inhibition of FAHFA-degrading enzymes. Several classes/generations of AIG1 and ADTRP inhibitors were developed up to the level of selective and in vivo–active compounds (Erikci Ertunc et al., 2020; Parsons et al., 2016). Downstream degradation of HFA, probably by CYPs in peroxisomes, could be also targeted to increase levels of free HFAs. HFAs can be recycled into new FAHFAs as was shown with 9-hydroxyheptadecanoic acid (Yore et al., 2014). Also, CEL inhibitor WWL92 was successfully tested in vitro and in mice (Kahn et al., 2015; Kolar et al., 2016). The potential targets in the synthetic pathway are unknown, but a family of CYP4 inhibitors could be possibly used to investigate the role of CYPs in HFA backbone generation.

## 8. Conclusion and future perspectives

FAHFA kingdom is very diverse and only selected highly abundant molecules were characterized in detail. We can estimate that the number of FAHFAs in humans is close to 1000 species, while their role, presence, and composition change during the lifespan (Brejchova et al.,

Table 3
Potential pharmacological tools in FAHFA research.

| Target | Inhibitor | Concentration | Model | Effect | Ref. |
|---|---|---|---|---|---|
| GPR120 | AH7614 | 100 μM | Paneth and dendritic cells | inhibition of GPR120 signaling in Paneth cells and dendritic cells | Lee et al. (2016) |
| GPR40 | GW1100 | 10 μM | HEK293T, human islets | inhibition of 9-PAHSA effects in human islets | Syed, Lee, Moraes-Vieira, et al. (2018) |
| GPR40 | DC260126 | 5 mg/kg | C57Bl/6 J mice | attenuation of the beneficial effects of PAHSAs on insulin sensitivity and glucose tolerance | Syed, Lee, Moraes-Vieira, et al. (2018) |
| GPR40 | GW1100 | 10 μM | MIN6 cells | no effect on beta cell proliferation | Syed et al. (2019) |
| unknown | modified FAHFAs | 25 μM | RAW 264.7 macrophages | alteration of anti-inflammatory actions by modification of chemical structure of FAHFAs | Wang, Chang, et al. (2019) |
| DGAT1 | PF 04620110 | 5 μM | 3T3-L1 adipocytes | partial inhibition of TAG-FAHFA synthesis | Tan et al. (2019) |
| DGAT2 | PF 06424439 | 5 μM | 3T3-L1 adipocytes | partial inhibition of TAG-FAHFA synthesis | Tan et al. (2019) |
| ATGL | Atglistatin | 10 μM | 3T3-L1 adipocytes | modification of free FAHFA levels during lipolysis | Tan et al. (2019) |
| HSL-MAGL | CAY10499 | 0.1 μM | 3T3-L1 adipocytes | modification of free FAHFA levels during lipolysis | Tan et al. (2019) |
| ATGL | Atglistatin | 10 μM | 3T3-L1 adipocytes | modification of free FAHFA levels during lipolysis | Paluchova, Oseeva, et al. (2020) |
| AIG1 | ABD-110207 | 12 nM | HEK293T cells | inhibition of FAHFA hydrolysis | Erikci Ertunc et al. (2020) |
| ADTRP | ABD-110207 | 5.4 nM | HEK293T cells | inhibition of FAHFA hydrolysis | Erikci Ertunc et al. (2020) |
| AIG1/ADTRP | ABD-110207 | 5 mg/kg | C57Bl/6 J mice | inhibition of FAHFA hydrolysis | Erikci Ertunc et al. (2020) |
| AIG1 | KC01 | 0.21 ± 0.08 μM IC50 | HEK293T cells | inhibition of FAHFA hydrolysis | Parsons et al. (2016) |
| AIG1 | JJH260 | 0.57 ± 0.14 μM IC50 | HEK293T cells | inhibition of FAHFA hydrolysis | Parsons et al. (2016) |
| ADTRP | KC01 | 1.3 ± 0.4 μM IC50 | HEK293T cells | inhibition of FAHFA hydrolysis | Parsons et al. (2016) |
| ADTRP | JJH260 | 8.5 ± 3 μM IC50 | HEK293T cells | inhibition of FAHFA hydrolysis | Parsons et al. (2016) |
| CEL | WWL92 | 40 μM | HEK293T cells | inhibition of FAHFA hydrolysis | Kolar et al. (2016) |
| CEL | WWL92 | 20 μM | Liver and pancreas lysates | inhibition of FAHFA hydrolysis | Kahn et al. (2015) |
| CYP4A11 | Plumbagin | 0.9 μM | recombinant CYP4A11 | inhibition of 12-hydroxy lauric acid formation | Choi et al. (2018) |
| CYP4A11 | HET001 | 7.1 μM | recombinant CYP4A11 | inhibition of 12-hydroxy lauric acid formation | Choi et al. (2018) |
| CYP4A11 | HET0016 | 12 nM IC50 | human liver microsomes | inhibition of 12-hydroxy lauric acid formation | Yamaori et al. (2018) |
| CYP4A11 | Epalrestat | 1.82 uM IC50 | human liver microsomes | inhibition of 12-hydroxy lauric acid formation | Yamaori et al. (2018) |
| CYP4V2 | HET0016 | 38 nM IC50 | microsomal CYP4V2 | inhibition of 12-hydroxy lauric acid formation | Nakano et al. (2009) |
| CYP4A | 11-dodecynoic acid | 50 μM | microsomes | inhibition of lauric acid hydroxylation | Helvig et al. (1997) |
| CYP4Z1 | Ozagrel derivatives | 63 ± 19 nM | MCF-7 cells | inhibition of ω-hydroxylation | Machalz et al. (2021) |
| CYP4Z1 | HET0016 | 15 μM | yeast microsomes | inhibition of lauric acid hydroxylation | McDonald et al. (2017) |

2020; Brezinova et al., 2020; Khanal et al., 2021; Vavrusova et al., 2020; Zhu et al., 2020). Our understanding of FAHFA signaling potency and its integration with parallel regulations of metabolic pathways is very limited to date. Besides unknown biological effects, the biosynthesis of FAHFAs represents a future challenge and also an opportunity to find new therapeutic targets. New functions for old or orphan enzymes could be unraveled and specific inhibitors could be synthesized as was done for AIG1 and ADTRP.

Nutritional therapy and precision nutrition can be used to alter FAHFA levels. The collection of enzyme inhibitors is growing slowly and is closely linked to the identification of new molecular targets. Wider application of analytical methods focused on FAHFA estolide bond position and double bond position(s) will be needed (Hancock et al., 2018; Marshall et al., 2016) because the precise quantification of FAHFAs is a prerequisite for successful identification of the (patho) physiological condition. The role of FAHFA stereocenter will gain more attention when the research moves to the lipid–protein interaction level, structure-activity relationships, and potential drug targets (Brezinova et al., 2018; Nelson et al., 2017).

The anti-inflammatory and anti-diabetic effects of branched FAHFAs in metabolic research and immunology and the ω-FAHFA therapeutic potential of ω-FAHFAs in dermatology and ophthalmology represent the potential application of FAHFAs in human medicine.

## Declaration of Competing Interest

The authors declare no competing interests.

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# Tissue-specific sex-dependent difference in the metabolism of fatty acid esters of hydroxy fatty acids

Martin Riecan [a], Veronika Domanska [a], Cristina Lupu [b], Maulin Patel [b], Michaela Vondrackova [a], Martin Rossmeisl [c], Alan Saghatelian [d], Florea Lupu [b], Ondrej Kuda [a,*]

[a] Metabolism of Bioactive Lipids, Institute of Physiology of the Czech Academy of Sciences, Videnska 1083, 14200 Prague, Czechia
[b] Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation, Oklahoma City, O7 73104, USA
[c] Adipose Tissue Biology, Institute of Physiology of the Czech Academy of Sciences, Videnska 1083, 14200 Prague, Czechia
[d] Clayton Foundation Laboratories for Peptide Biology, Salk Institute for Biological Studies, La Jolla, CA, USA

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ABSTRACT

Fatty acid esters of hydroxy fatty acids (FAHFAs) are endogenous bioactive lipids known for their anti-inflammatory and anti-diabetic properties. Despite their therapeutic potential, little is known about the sex-specific variations in FAHFA metabolism. This study investigated the role of sex and Androgen Dependent TFPI Regulating Protein (ADTRP), a FAHFA hydrolase. Additionally, tissue-specific differences in FAHFA levels, focusing on the perigonadal white adipose tissue (pgWAT), subcutaneous white adipose tissue (scWAT), brown adipose tissue (BAT), plasma, and liver, were evaluated using metabolomics and lipidomics. We found that female mice exhibited higher FAHFA levels in pgWAT, scWAT, and BAT compared to males. FAHFA levels were inversely related to testosterone and *Adtrp* mRNA, which showed significantly lower expression in females compared with males in pgWAT and scWAT. However, no significant differences between the sexes were observed in plasma and liver FAHFA levels. *Adtrp* deletion had minimal impact on both sexes' metabolome and lipidome of pgWAT. However, we discovered higher endogenous levels of triacylglycerol estolides containing FAHFAs, a FAHFA metabolic reservoir, in the pgWAT of female mice. These findings suggest that sex-dependent differences in FAHFA levels occur primarily in specific WAT depots and may modulate local insulin sensitivity in adipocytes, and the role of ADTRP is limited to adipose depots. However, further investigations are warranted to fully comprehend the underlying mechanisms and implications of sex-dependent regulation of human FAHFA metabolism.

## 1. Introduction

Fatty acid esters of hydroxy fatty acids (FAHFAs) are a class of endogenous bioactive lipids belonging to the estolide family (LIPID MAPS [FA0709] subclass). They are defined as oligomers of a hydroxy fatty acid (HFA) and a fatty acid (FA) [1,2]. Specifically, 9-PAHSA stands for palmitic acid (PA) and hydroxy stearic acid (HSA), with the ester linkage at the "9" position relative to the carboxylic function [1]. Recently, FAHFAs have attracted interest because of their remarkable anti-inflammatory, anti-diabetic, and antioxidant effects [1–6].

A complete understanding of the molecular mechanisms regulating FAHFAs in organisms still needs to be achieved. However, several FAHFA hydrolytic enzymes have been identified: adipose triglyceride lipase (ATGL), carboxyl ester lipase (CEL), hormone-sensitive lipase (HSL), androgen-induced protein 1 gene (AIG1), and androgen-dependent tissue factor pathway inhibitor regulating protein (ADTRP) [7–11]. ATGL is a well-known lipase that can remodel FAHFAs stored in triacylglycerol estolides (TG EST) in the presence of a specific co-activator named comparative gene identification-58 (CGI-58) [11,12] and also drives FAHFA biosynthesis [13]. The putative pathway scheme (Fig. 1) [14] summarizes FAHFA-related metabolic reactions and enzymes that are dependent on sex, namely ADTRP, AIG1, HSL, ATGL, and diacylglycerol O-Acyltransferase 1 (DGAT1) [15–18].

The ADTRP was first identified as a protein involved in regulating tissue factor pathway inhibitor, responsible for the anticoagulant protection of the endothelium and preservation of normal cardiovascular

*Abbreviations:* FAHFA, Fatty acid esters of hydroxy fatty acids; WAT, white adipose tissue.

* Corresponding author.
*E-mail address:* ondrej.kuda@fgu.cas.cz (O. Kuda).

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function [19,20]. Later studies revealed a hydrolytic activity leading to the degradation of FAHFAs [8,9]. Androgens, namely testosterone, induce transcriptional activation of ADTRP through direct binding of the androgen receptor (AR) to the androgen response element (ARE) at the ADTRP promoter region [21].

We hypothesized that since FAHFAs are degraded by enzymes whose activity is influenced by androgen levels, we should be able to find sex-linked differences in FAHFA levels. We aimed to perform lipidomic, metabolomic, and gene expression analyses in mice to define the sex-dependent metabolism of FAHFAs.

## 2. Materia1s and methods

### 2.1. Sample sources

Two different transgenic mouse models were used in this study. First, transgenic *Adtrp* knockout (KO) and wild-type (WT) mice (MODEL 1) were generated using CRISPR/Cas9-mediated indel mutations at the Salk Institute for Biological Studies in California, as previously published [9]. Male and female WT and KO mice were maintained in the animal facility of the Institute of Physiology of the Czech Academy of Sciences in a controlled environment with a 12-h light/dark cycle and unrestricted access to standard diet and drinking water. Perigonadal white adipose tissue (pgWAT) samples were collected between 7:00 and 9:00 am from fed mice (4 to 6 months old) into cryovials, rapidly frozen in liquid nitrogen, and stored at −80 °C until extraction.

The second mouse model (MODEL 2) was bred and maintained in animal core facilities of the Oklahoma Medical Research Foundation in Oklahoma City. Mice with the null allele for the *Adtrp* gene were generated as before [22], using the bacterial artificial chromosome gene targeting strategy. We studied female and male WT and KO mice fed a standard diet. Plasma, liver, pgWAT, subcutaneous WAT (scWAT), and interscapular brown adipose tissue (BAT) samples were collected from 8-month-old mice fed ad libitum and housed in a controlled environment under a 12-h light/dark cycle with unrestricted access to water. The mice fasted for 5 h (7:00 am) before being killed at noon. Samples were frozen in liquid nitrogen and stored at −80 °C until extraction.

### 2.2. Sample extraction for lipidomic and metabolomics profiling

Tissue samples (10–25 mg) were processed using bi-phase extraction with cold methanol, methyl *tert*-butyl ether (MTBE), and water as before [14,23]. The extracts were aliquoted for the lipidomics and metabolomics profiling platforms as described in detail previously [14,24].

### 2.3. Sample extraction for FAHFA analysis

Tissues (50–100 mg) and plasma (150 μL) samples were extracted as previously described [1,5,14,23]. Briefly, samples were homogenized using the bead mill in a mixture of citric acid buffer and ethyl acetate (ratio 1:2, *v*/v) containing 1 ng of [$^{13}C_4$]-9-PAHSA (Cayman Pharma, Neratovice, Czech Republic) as an internal standard for quantification. The organic phase was collected and dried using a Savant SpeedVac (ThermoFisher Scientific, Bremen, Germany). Extracts were resuspended in hexane and ethylacetate mixture (ratio 95:5, *v*/v) and purified by solid-phase extraction (SPE) using HyperSep SPE columns (500 mg/10 mL, 40–60 μm, 70 Å; Thermo Scientific). FAHFAs were eluted with ethyl acetate and dried [25]. The purified extracts were resuspended in 50 μL of methanol and immediately analyzed.

### 2.4. Lipidomic and metabolomic profiling

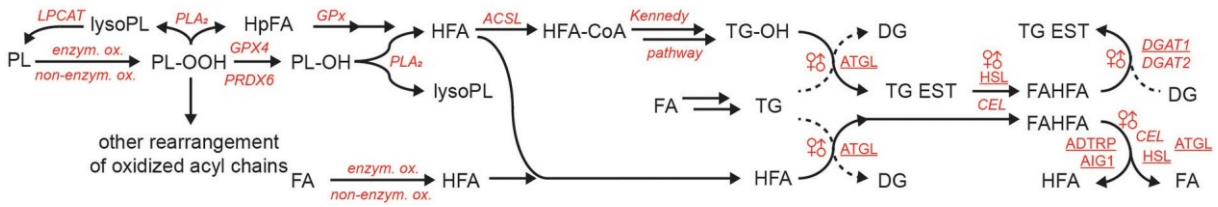
Lipidomic and metabolomic profiling of mouse tissues was conducted using liquid chromatography-mass spectrometry (LC-MS) systems consisting of a Vanquish ultra-high performance liquid-chromatography (UHPLC) System (Thermo Fisher Scientific, Bremen, Germany) coupled to a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) operated in the negative and positive ion mode, as described previously [23]. Internal standards such as 1-cyclohexyluriedo-3-dodecanoic acid and Val-Tyr-Val were used to control the platform's stability and possible differences in injection volumes [26]. Obtained untargeted lipidomic and metabolomic data were processed using MS-DIAL ver. 4.92 [27]. Raw data were filtered using blank samples and quality control (QC) pool samples with relative standard deviation (RSD) < 30 % and then normalized using the LOESS approach utilizing QC pool samples for each matrix regularly injected between 10 actual samples [26]. Lipids and metabolites were annotated using an in-house retention time–*m*/*z* library and MS/MS libraries (NIST20, LipidBlast, MassBank, MoNA). See Lipidomics Minimal Reporting Checklist (SI Material) for details.

### 2.5. FAHFA regioisomer analysis

Analysis of FAHFA regioisomers was conducted on an LC-MS system consisting of a UHPLC Ultimate 3000 RSLC (Thermo Scientific) coupled with a QTRAP 5500/SelexION, a hybrid triple-quadrupole, and a linear ion trap mass spectrometer equipped with an ion mobility cell (SCIEX, Massachusetts, USA). The measurement was based on a pre-calculated multiple reaction monitoring (MRM) method using one quantifier (FA) and two qualifier transitions (HFA, HFA-$H_2O$) for FAHFA [5,28,29]. The quantifier ion was used as a survey scan for information-dependent acquisition in a linear ion trap for enhanced-resolution MS/MS and second-generation (MS/MS/MS) product ion spectra [5]. This approach was used to identify FAHFA regioisomers based on the branching position of the backbone HFA using MS/MS/MS, as previously described [30]. The amount of FAHFAs was quantified by normalizing its peak height to the peak height of the internal standard [$^{13}C_4$]-9-PAHSA obtained from MultiQuant software (SCIEX) and the total tissue weight/plasma volume [31].

### 2.6. Testosterone analysis

Analysis of testosterone was conducted on the same LC-MS system as for FAHFAs. The measurement was based on a pre-calculated MRM




**/ig. 1.** Putative scheme of FAHFA synthesis adapted from [14]. Enzymes that are regulated by sex hormones are underlined. ACSL, acyl-CoA synthetase; ADTRP, Androgen-dependent TFPI-regulating protein; AIG1, Androgen-induced protein 1; ATGL, adipose triglyceride lipase; CEL, carboxyl ester lipase; DG, diacylglycerol; DGAT, diacylglycerol acyltransferase; FA, fatty acid; GPx, peroxidase activity; GPX4, glutathione peroxidase 4; HFA, hydroxy fatty acid, e.g., 13-HLA; HpFA, hydroperoxy FA; HSL, hormone-sensitive lipase; LPCAT, acyltransferase activity; lysoPL, lysophospholipid; PLA2, phospholipase A2 activity; PL, phospholipid, e.g. PC; PL-OH, hydroxylated PC; PL-OOH, peroxidated PL; TG, triacylglycerol; TG EST, triacylglycerol estolide (TG-FAHFA).

method using one quantifier and one qualifier transition, as described previously [24]. Androgens were separated on an Acquity UPLC BEH C18 column (50 mm × 2.1 mm i.d.; 1.7 µm particle size) coupled to an Acquity UPLC BEH C18 VanGuard pre-column (5 mm × 2.1 mm i.d.; 1.7 µm particle size) (Waters, Milford, MA, USA). The column was maintained at 45 °C at a 0.4 mL/min flow rate. The mobile phase consisted of (A) water and (B) acetonitrile. Analytes were separated under the following gradient: 0 min 15 % (B); 0–3 min from 15 % to 30 % (B); 3–5 min from 30 % to 100 % (B); 5–7 min 100 % (B); 7–7.20 min from 100 % to 15 % (B); 7.20–9 min 15 % (B). Following extraction, the sample of 100 µL of lipidomic fraction from MTBE extraction was evaporated and resuspended in 50 µL of the mixture of methanol and water (ratio 1:1, *v*/v) prior to analysis. A sample volume of 5 µL was injected.

### 2.7. Gene expression analysis

Total RNA isolated from mouse tissues (MODEL 2) using Trizol (Thermo Fisher Scientific) was purified and transcribed into cDNA as described [22]. qPCR was performed using predesigned primers (PrimeTime, Integrated DNA Technologies Inc., Coralville, IA, USA) for mouse *Aig1* and androgen receptor (*Ar*), and RT²SYBR Green master mix (Qiagen, Hilden, Germany) in a CFX96 Real Time System (Bio-Rad Labs, Inc., Hercules, CA, USA). *Adtrp* primers (forward: CCA AGA AAA TGG CCT GCA AGA G; and reverse: GAA GGA ATG AGG AGA AGC TAA GG) were designed to recognize exons 3–4, which are deleted in the MOUSE 2 model of *Adtrp*-KO [22].

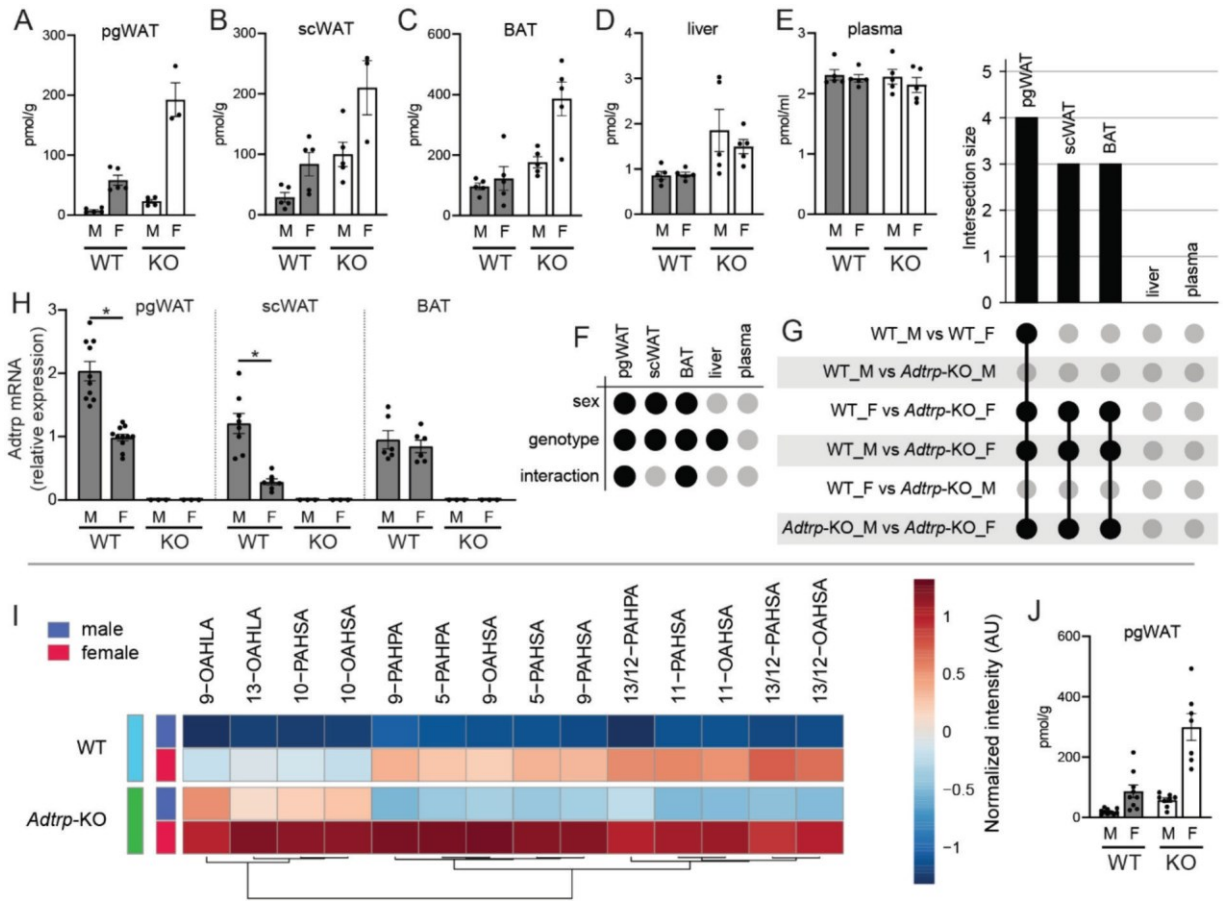
### 2.8. Data analysis and statistics

Data processing was performed using MetaboAnalyst 5.0 RRID: SCR_015539 (log10 data transformation and auto-scaling) [32]. GraphPad Prism 9 RRID:SCR_002798 was used to construct bar graphs and for statistical analyses. Plotly RRID:SCR_013991 was used to draw complex plots [33]. Student's *t*-test analysis and two-way ANOVA (Tukey's multiple comparisons test) were used to compare study groups, and $p < 0.05$ was considered significant. The UpSet plot was used to visualize two-way ANOVA results [34]. LORA was used to perform an over-representation analysis [35]. Single-cell portal was used to explore mouse WAT data study SCP1376 [36,37].

## 3. Resu1ts

### 3.1. FAHFA levels in the pgWAT are higher in female mice

To determine whether biological sex influences FAHFA levels, we used targeted LC-MS methods to measure the concentration of 9-PAHSA, the most studied FAHFA representative, in pgWAT, scWAT, BAT, liver, and plasma samples (Fig. 2). We found sex-dependent differences in




**/ig. 2.** Levels of 9-PAHSA levels in mouse organs. Data are means ± SE, $n = 7–10$. Grey bars, WT mice; white bars, *Adtrp*-KO mice; M, males; F, females. A-E: Levels of 9-PAHSA in pgWAT, scWAT, BAT, liver, and plasma from MODEL 2 mice [22]. Data are means ± SE, $n = 3–5$. F, Results of two-way ANOVA for panels A-E. Filled circles indicate a statistically significant effect of sex, genotype, or interaction, F-values 113.1, 52.02, 33.05 respectively, df = 1. Bonferroni's multiple comparison test, $p < 0.05$. Panel A statistics are similar to panel B. G: UpSet plot highlighting pairwise comparison by two-way ANOVA. Filled circles connected by a line indicate statistically significantly different results. The size of the intersection indicates the number of significant tests. H: Levels of *Adtrp* mRNA in pgWAT ($t = 6.838$, df = 19), scWAT ($t = 5.242$, df = 13) and BAT. Data are means ± SE, $n = 6–11$. * Statistically significant difference, $p < 0.05$, *t*-test. I: Levels of FAHFAs in pgWAT, MODEL 2. Data are means, $n = 3–5$, auto-scaled, and log10 transformed. t: Levels of 9-PAHSA in pgWAT from MODEL 1 mice [9].

pgWAT, scWAT, and BAT (see Fig. 2F & G for statistics), indicating that females generally had higher 9-PAHSA levels in the adipose tissues. PAHSA levels in the liver and plasma were not different between sexes.

The absence of ADTRP in male mice resulted in elevated levels of 9-PAHSA in pgWAT, consistent with previous data [9]. An even more pronounced effect of ADTRP absence was observed in female mice, which generally had higher levels of 9-PAHSA in pgWAT than males. Similar results were obtained in mice generated by a different gene targeting strategy (i.e., in Model 1; Figs. 2t, S1).

Next, we examined other ADTRP-expressing tissues related to lipid metabolism. Sex-related differences in 9-PAHSA levels were observed in scWAT and BAT, whereas genotype-related differences were observed in all three adipose depots and the liver (Fig. 2B-E). No difference was observed in plasma (Fig. 2E). Fig. 2H summarizes the results of the 2-way ANOVA (sex vs. genotype), and the UpSet plot highlights the results of the multiple comparison tests (Fig. 2G). Other FAHFAs in pgWAT were also regulated in a sex-dependent manner, similar to 9-PAHSA (Fig. 2I).

FAHFA levels appear to be inversely related to *Adtrp* mRNA levels, which showed significantly lower expression in both pgWAT and scWAT of WT females compared to males (Fig. 2H). This result is consistent with previous findings that defined ADTRP as a FAHFA hydrolase in WAT [8,9]. As expected, *Adtrp* mRNA levels were undetectable in *Adtrp*-KO mice.

Although the FAHFA levels remained unchanged in AIG-1 null mice [9], suggesting a predominant role for ADTRP in FAHFA hydrolysis in WAT and BAT, we observed sex-dependent differences and an increase of *Aig1* mRNA in *Adtrp*-KO males (Fig. 3A). To further explore a possible imbalance in steroid signaling related to the *Adtrp* absence, we measured testosterone levels and androgen receptor mRNA levels in pgWAT. The *Ar* mRNA was upregulated in *Adtrp*-KO animals, but only trends in tissue testosterone levels and plasma levels are observed (Figs. 3B, C, S2).

However, when we replotted the data for wild-type males only, we observed a relationship between the anatomical position of the fat depot (distance from the gonads) and the levels of 9-PAHSA, testosterone, and *Adtrp* mRNA (Fig. 3D) - the lower the levels of testosterone and Adtrp mRNA, the higher the concentration of PAHSA.

### 3.2. ADTRP deletion only slightly affected the metabolome and lipidome of pgWAT in both sexes

Next, we focused only on the pgWAT, where the most prominent differences between males and females were observed, and we examined the metabolome and lipidome profiles of both genotypes of MODEL 1 as the FAHFA metabolism is interlinked with phospholipid and acylglycerol metabolism (Fig. 4, Tables S1, S2). The effect of genotype on the metabolome was weak (Table S1), while the impact on the lipidome was mild (Table S2). Using loose statistical parameters (|fold change| > 1.2 and $p$-value < 0.05), we detected 41 downregulated and 1 upregulated lipid in male KO mice (Fig. 4A) and 8 downregulated lipids in female KO mice (Fig. 4B). The downregulated clusters consisted of glycerolipids [GL] and glycerophospholipids [GP], and the common lipids for both sexes were triacylglycerols and triacylglycerol estolides (Fig. 4C). Lipid overrepresentation analysis showed that the downregulated clusters were significantly enriched with glycerolipids composed of 7 acyl chains. The lipid molecule representing the most enriched structural features was TG 18:0_18:1_20:1 (Fig. 4D).

### 3.3. Endogenous levels of TG estolides are higher in the pgWAT of females

The esterified form of FAHFAs, TG estolides, were only slightly affected by ADTRP deficiency in pgWAT (Fig. 4), similar to what was previously reported in female mice [9]. However, when we examined only the effect of sex, we found higher levels of total TG estolides in females (Fig. 5A). Heatmaps of the individual TG estolide species showed clustering based on total lipid unsaturation, but the separation

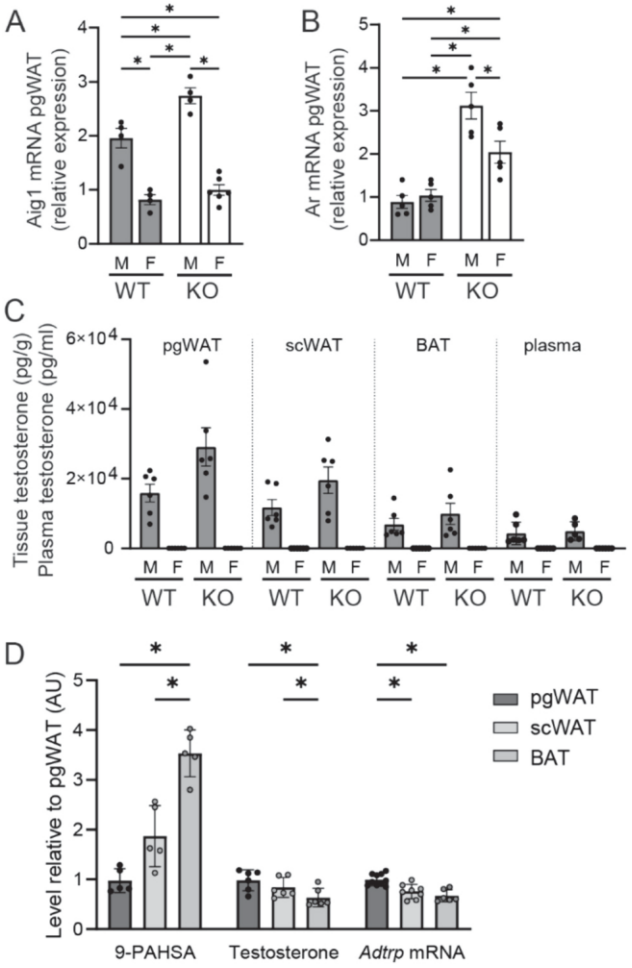


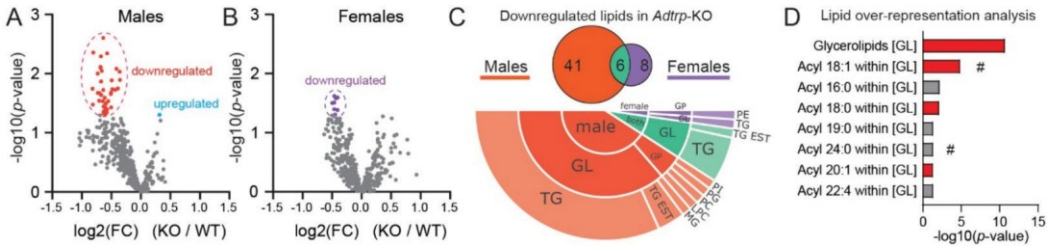

**Fig. 3.** Gene expression in adipose tissue depots, MODEL 2 mice [22]. A, B: *Aig1* (F-value 121.6, 13.52, 5.323 for sex, genotype, and interaction, df = 1) and *Ar* (F-value 4.264, 51.43, 7.459 for sex, genotype, and interaction, df = 1) mRNA expression in pgWAT. Data are means ± SE, $n$ = 4–6. * Statistically significant difference, $p$ < 0.05, two-way ANOVA, Tukey's multiple comparisons test. C: Testosterone levels in adipose depots and plasma. Data are means ± SE, $n$ = 5–6. D: Relative levels of 9-PAHSA, testosterone, and *Adtrp* mRNA in male adipose tissue depots, relativized to pgWAT and transformed by square root. Data are means ± SD. * Statistically significant difference, $p$ < 0.05. Two-way ANOVA, mixed effect analysis with Tukey's multiple comparisons (F-value 104.9, 31.67 for features and depots).

was not statistically significant (Figs. 5B and S3).

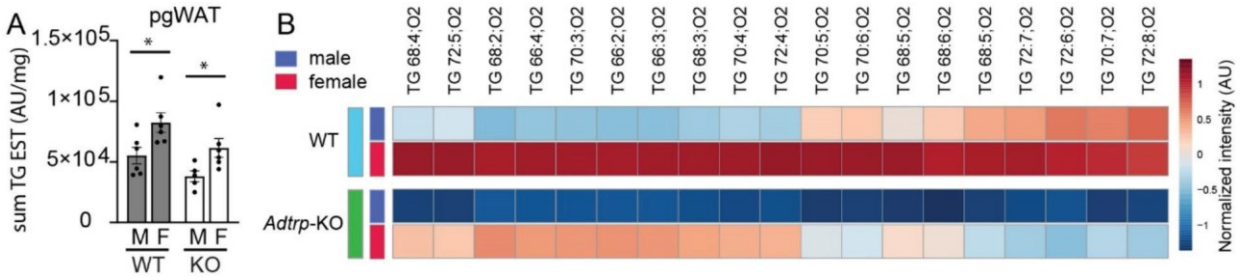
## 4. Discussion

Previous studies have shown that endogenous FAHFA levels in rodents and humans can be influenced by age [38], dietary composition [5,23,39], exercise training [31], and metabolic disorders such as obesity and diabetes [1,40]. The effect of sex on FAHFA and TG estolide levels in adipose tissue was a prominent finding in this study. We showed that females have higher levels of FAHFAs and TG estolides than males and that the systemic deletion of FAHFA hydrolase ADTRP affected only adipose tissue depots and not the systemic FAHFA levels. Notably, Nelson et al. published a paper showing the sex-based heterogeneity of FAHFAs in trained runners [41]. Circulating FAHFAs were increased in females in a manner modulated by specific adipose depot sizes, blood glucose, and lean body mass [41]. This suggests that sex-dependent FAHFA levels are controlled by another mechanism, probably unrelated to ADTRP actions in the adipose depots.

Defour et al. showed that manipulation of *Adtrp* in the liver did not




**/ig. 4.** Effect of *Adtrp*-KO on the lipidome. A: Volcano plot of pgWAT lipidome from male mice, MODEL 1. The red cluster marks 41 downregulated lipids, and the blue dot marks upregulated lipids (|fold change| > 1.2 and $p$-value < 0.05, $t$-test). B: Volcano plot for female mice. Purple cluster marks 8 downregulated lipids. C: Venn diagram of clusters from both sexes and color-coded lipid class composition of the clusters. Abbreviations follow the LIPID MAPS nomenclature: glycerolipids [GL], and glycerophospholipids [GP] classes. D: Lipid overrepresentation analysis of downregulated clusters; # marks features specific to lipids shared by both sexes. Red bars indicate building blocks representing the most enriched structural features.




**/ig. 5.** Effect of *Adtrp*-KO on TG estolides. A: The sum of TG estolide levels in pgWAT for both sexes and genotypes, MODEL 1. Data are mean ± SE, $n$ = 5–6. WT, wild type; KO, *Adtrp* knockout. * Statistically significant difference between genotypes at $p$ < 0.05, two-way ANOVA (F-value 12.50, 7.049, 0.07 for sex, genotype and interaction, df = 1). B: Heatmap representation of individual lipids. See Fig. S1 for a detailed version. Data are auto-scaled and log10 transformed, means, $n$ = 5–6.

affect systemic FAHFA levels and concluded that *Adtrp* was involved in PPAR-driven programming in adipose tissue [42] and the increased adipose tissue levels of FAHFA were not associated with systemic changes like improved glucose tolerance [9]. The contribution of the liver to systemic FAHFA metabolism is lower than that of adipose tissue [13,42]. The role of ADTRP in adipose tissue was further pinpointed to thermogenic activity and the secretion of S100 calcium-binding protein [43]. However, the ADTRP can be regulated by many signals, including androgens, oxidized LDLs, LDL receptors LDLR and CD36, LOX1, transcription factors GATA2, PPARα, and PPARγ (reviewed in [44]), and its FAHFA hydrolase activity might be adipose tissue-specific action.

The most significant effect of sex on tissue FAHFA and TG estolide levels was observed in pgWAT, probably due to its close contact with the gonads. In contrast, more distant adipose depots, such as scWAT and BAT, may be exposed to lower levels of androgens since the local androgen-dependent *Adtrp* expression appears lower.

Importantly, adipose tissue has been established as an important site for steroid storage and metabolism [45]. Locally produced or stored androgens could modulate the exposure of adipocytes to active androgens and potentially modulate levels of FAHFA hydrolases. The expression of steroid-converting enzymes differs in different adipose tissue depots, e.g., higher levels of 17β-hydroxysteroid dehydrogenase were found in omental than in subcutaneous adipose tissue [46]. Moreover, dihydrotestosterone stimulated lipolysis and upregulated Atgl and Hsl in male rat pgWAT [18], suggesting a potential steroid effect on the FAHFA biosynthesis, degradation, and FAHFA release from TG estolides [10,12].

The complete absence of *Adtrp* had only a minor effect on the pgWAT metabolome and lipidome. From the global perspective, no differences between WT and *Adtrp*-KO were detected, as reported previously [9]. Only the targeted approach (targeted FAHFA analysis and single metabolite analysis without the FDR correction) showed that the FAHFAs and TG estolides were altered by *Adtrp* deletion. Interestingly, the sex differences in PAHSAs persisted and were even greater upon *Adtrp* deletion, which suggests that other enzymes involved in FAHFA metabolism contribute to sex differences in FAHFA levels. Given the differences in TG estolides, the FAHFA cycling via Atgl, Hsl, and Dgat1 might be affected by sex hormones [15–18].

Emont et al. published a single-cell atlas of mouse WAT, including data for both sexes [36]. Visualization of FAHFA-related genes showed that *Adtrp* (and the other acylglycerol metabolism-related genes) is expressed only in adipocytes and not in other cell types within WAT (Fig. 6A). However, the other FAHFA hydrolase *Aig1* is expressed mainly in epithelial and endothelial cells. The same panel clustered on sex showed that the overall expression of *Adtrp* and *Aig1* is comparable among the sexes and that females have higher expression of acylglycerol metabolism-related genes (Fig. 6B). Clustering on WAT depot location showed different distributions of gene expression in inguinal WAT (subcutaneous), periovarian, and perigonadal (male) WAT (Fig. 6C) [36]. Analysis of the cellular composition of WAT suggests that sex differences in FAHFA levels could be influenced by many players within the local environment of WAT, favoring synthesis and disfavoring degradation in females.

## 5. Conc1usion

FAHFA levels are sex-dependent, and higher levels in females are most pronounced in adipose tissue depots, particularly pgWAT, which is closest to gonads. The role of ADTRP, which is connected to many complex regulations [44], in FAHFA metabolism is limited to adipose depots in mice. Future studies using human cohorts and different fat depots will be needed to explore sex-specific FAHFA metabolism in the WAT cellular microenvironment and its impact on systemic metabolism.

## /unding

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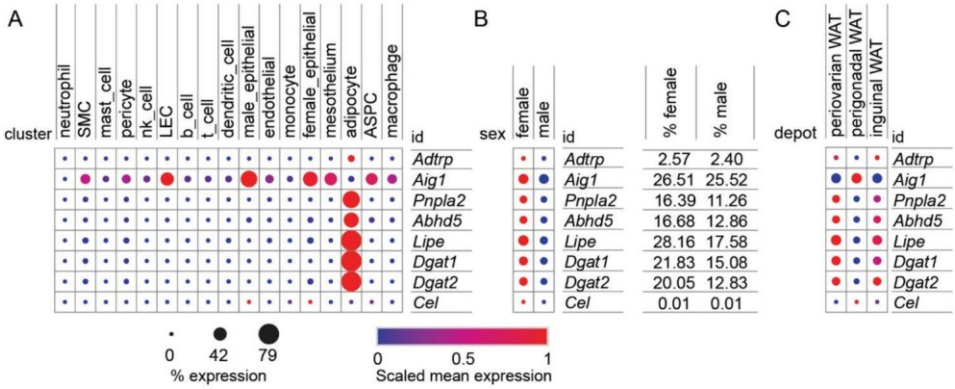


**/ig. 6.** Visualization of FAHFA-related genes in WAT using single-cell mouse WAT atlas [36]. A: Dot plot of selected mouse genes within the cluster of cell types. Scaling is relative to each gene's expression across all cells in a given annotation selection. *Abhd5*, Alpha/beta Hydrolase Domain Containing 5, CGI-58 Atgl co-factor; *Dgat1*, Diacylglycerol O-Acyltransferase 1; *Dgat2*, Diacylglycerol O-Acyltransferase 2; *Lipe*, Hormone-Sensitive Lipase; *Pnpla2*, Patatin Like Phospholipase Domain Containing 2, Atgl; ASPC, adipose stem and progenitor cells; LEC, lymphatic endothelial cells; SMC, vascular smooth muscle cells; B: The same data clustered according to sex. The percentage of gene expression is provided to highlight the difference between males and females. C: The same data is clustered according to the WAT depot location.

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## Ethics approva1 and consent to participate

All animal procedures were approved by the Institutional Animal Care and Use Committees of the Oklahoma Medical Research Foundation by the Institutional Animal Care and Use Committee (IACUC) and the Committee for Animal Protection of the Ministry of Agriculture of the Czech Republic (Approval Number: 81/2016) for the care and use of laboratory animals.

## CRediT authorship contribution statement

Conceptualization: F.L. and O.K.; Data curation: V.D. and M.Ro.; Formal analysis: M.R., V.D., M.P., M.V. and O.K.; Funding acquisition: M.Ro., F.L. and O.K.; Investigation: M.R., C.L., M.P. and O.K.; Methodology: M.R., V.D., M.P., M.V. and O.K.; Project administration: M.Ro., A. S., F.L. and O.K.; Resources: C.L., M.Ro., A.S., F.L. and O.K.; Software: M. V. and O.K.; Supervision: O.K.; Validation: M.Ro. and F.L.; Visualization: M.R., M.V. and O.K.; Writing – original draft: M.R. and O.K.; Writing - review & editing: M.R., V.D., C.L., M.P., M.V., M.Ro., A.S., F.L. and O.K.;

## Dec1aration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data avai1abi1ity

Supplementary material

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## Appendix A. Supp1ementary data

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# Analysis of fatty acid esters of hydroxy fatty acids in edible mushrooms

Felipe Martínez-Ramírez, Martin Riecan, Tomas Cajka, Ondrej Kuda *

*Institute of Physiology, Czech Academy of Sciences, 14220, Prague 4, Czech Republic*

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ABSTRACT

Edible mushrooms are consumed worldwide. The overall amount of lipids in mushrooms is small and consists of complex lipids, such as triacylglycerols, phospholipids, and sterols. Nutritional studies have outlined the lipid compositions of mushrooms but have not analyzed their fatty acid esters of hydroxy fatty acid (FAHFA) profiles. FAHFAs are a class of anti-inflammatory and antidiabetic lipids frequently found in human food. This study aimed to characterize the FAHFA profiles of various edible mushrooms.

Using a liquid chromatography-mass spectrometry-based lipidomics approach, we identified 34 FAHFAs in seven members of the fungal order Agaricales, also known as gilled mushrooms. Linoleic acid (LA)-containing FAHFAs were the most abundant among all the species. Shiitake (*Lentinula edodes*) and enokitake (*Flammulina velutipes*) mushrooms showed the most divergent FAHFA profiles, being rich in short-chain FAHFAs and very long-chain FAHFAs, respectively. The brown (*Hypsizygus tessulatus*) and white (*Hypsizygus ulmarius*) varieties of shimeji have closely related FAHFA compositions and quantities of FAHFA diversity can be used for chemotaxonomic classification of the mushroom family, similar to mitochondrial (mt)DNA information. Adding mushrooms to the diet provides several micronutrients, including FAHFAs, with minimal impact on the overall calories from fat.

## 1. Introduction

Edible mushrooms are consumed worldwide as part of the human diet (Bolaniran et al., 2021; Gunc Ergonul, Akata, Kalyoncu, & Ergonul, 2013). The nutritional value of edible mushrooms is well accepted; however, their use is limited compared to that of other foods (Kalac, 2009; Sande et al., 2019). As dry matter, mushrooms are well known as a source of proteins and structural polysaccharides with low lipid content (Hanus, Shkrob, & Dembitsky, 2008; Kalac, 2009; Pereira, Barros, Martins, & Ferreira, 2012). Lipids are included in the fat content of edible mushrooms as fatty acids; mono-, di-, and triacylglycerols; sterols; sterol esters; and phospholipids. Each of the fungal species has a different fatty acid composition, which is a crucial part of the organelles that make up 30–70% of fungi (Feeney et al., 2014; Raman et al., 2020). In addition to these compounds, mushrooms are an important source of vitamins, particularly vitamins B1 and B3, with lower amounts of vitamins B2 and B12 (Feeney et al., 2014; Krishnamoorthi, Srinivash, Mahalingam, & Malaikozhundan, 2022). Mushrooms have therefore been added to the human diet as an important source of nutrients (Bolaniran et al., 2021; Gunc Ergonul et al., 2013). In this context, mushrooms are being sought as new sources of nourishment because of concerns such as high caloric value, allergies, and high cholesterol content when consuming other foods (Karine, Paul, Andre, & Russell, 2006; Sande et al., 2019).

Essential fatty acids (for example, linoleic acid [LA]) constitute a significant portion of the lipid content in mushrooms (Sande et al., 2019). Another type of fatty acid (FA) found in fungi is the fatty acid esters of hydroxy fatty acids (FAHFAs) (Celis Ramirez et al., 2020; Watanabe et al., 2022). Initially discovered in mammal adipose tissue (Yore et al., 2014), FAHFAs have been detected in foods commonly consumed by humans, such as meat, seafood, rice, and fungi (Liberati-Cizmek et al., 2019; Pham et al., 2019; Yore et al., 2014; Zhu, Yan, Zhang, Xiao, & Feng, 2018). FAHFAs are a novel group of endogenous bioactive lipids with beneficial effects on human health (Brejchova et al., 2020) and are preserved from fungi to mammals (Celis Ramirez et al., 2020). Recently, FAHFAs have attracted interest because of their outstanding antioxidant, antidiabetic, and anti-inflammatory effects (Aryal et al., 2021; Brejchova et al., 2020; Kolar et al., 2019; Kuda et al., 2016; Gowda et al., 2021; Yore et al., 2014).

FAHFAs exist at low concentrations in plants and animals and possess high structural diversity across species (Brejchova et al., 2020;

* Corresponding author. Metabolism of Bioactive Lipids, Institute of Physiology of the Czech Academy of Sciences, Videnska 1083, 14220, Prague 4, Czech Republic.

*E-mail address:* ondrej.kuda@fgu.cas.cz (O. Kuda).

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**Abbreviations**

| | |
|---|---|
| FA | fatty acid |
| FAHFA | fatty acid esters of hydroxy fatty acid |
| HFA | hydroxy fatty acid |
| LC-MS | liquid chromatography-mass spectrometry |
| MTBE | methyl *tert*-butyl ether |
| LA | linoleic acid |
| OA | oleic acid |
| PA | palmitic acid |
| AAHDDA | arachidic acid ester of hydroxy docosadienoic acid |
| DHAHDHA | docosahexaenoic acid ester of hydroxy docosahexaenoic acid |
| LAHARA | linoleic acid ester of hydroxy arachidonic acid |
| LAHLA | linoleic acid ester of hydroxy linoleic acid |
| LAHOA | linoleic acid ester of hydroxy oleic acid |
| LAHPA | linoleic acid ester of hydroxy palmitic acid |
| LAHSA | linoleic acid ester of hydroxy stearic acid |
| OAHOA | oleic acid ester of hydroxy oleic acid |
| OAHSA | oleic acid ester of hydroxy stearic acid |
| PAHLA | palmitic acid ester of hydroxy linoleic acid |
| PAHOA | palmitic acid ester of hydroxy oleic acid |
| PAHPA | palmitic acid ester of hydroxy palmitic acid |
| PAHSA | palmitic acid ester of hydroxy stearic acid |
| SAHLA | stearic acid ester of hydroxy linoleic acid |
| SAHPA | stearic acid ester of hydroxy palmitic acid |
| SAHSA | stearic acid ester of hydroxy stearic acid |
| UHPLC | ultra-high performance liquid-chromatography |

Celis Ramirez et al., 2020; Watanabe et al., 2022; Zhu, Yan, Ni, & Feng, 2020). Because FAHFAs exhibit a wide isomeric variety in mammals, it is expected that FAHFA regioisomers in the fungal realm also vary (Brejchova et al., 2020). Interest in this lipid class is increasing in human consumption (Kellerer et al., 2021), and data for physiological, chemotaxonomic, and intragenic differentiation would be useful (Karine et al., 2006).

In this study, we characterized the FAHFA profiles of different species of commercially available edible mushrooms. As a result, we are enhancing our knowledge of FAHFAs with regard to the characterization of FAHFAs in edible mushrooms and their chemotaxonomic relevance.

## 2. Experimenta1 section

### 2.1. Mushroom samples

Seven commercially available mushrooms were obtained from a local supermarket in Prague, Czech Republic. The mushrooms were identified and labeled as edible macrofungi belonging to the division Basidiomycota and class Agaricomycetes. Mushrooms were transferred to the laboratory for proper cleaning and storage to avoid contamination. All samples were kept in sealed bags in the dark and stored at −80 °C before the extraction process. Their characterization is provided in Table S1.

### 2.2. Sample extraction

Mushroom caps (60 mg, in triplicate) were weighed in 2 mL polypropylene sample tubes and stored at −80 °C until analysis. First, lipids were isolated using bi-phase extraction with methanol, methyl *tert*-butyl ether (MTBE), and water (Matyash, Liebisch, Kurzchalia, Shevchenko, & Schwudke, 2008). Frozen samples were homogenized using a bead mill (30 Hz, 1.5 min) with 275 μL methanol containing 3.7 ng [$^{13}C_4$]-9-PAHSA (Cayman Pharma, Neratovice, Czech Republic) as an internal standard for quantification. Then, 1 mL of MTBE was added and the mixture was shaken, followed by the addition of 275 μL of methanol/water mixture (1:9, v/v). After shaking (30 s), the tubes were centrifuged for 10 min at 16,000 g (4 °C). The organic layer (1 mL) was then transferred to a new tube, followed by solvent evaporation. Finally, the extracts were resuspended in 100 μL methanol containing 20 ng of the internal standard 1-cyclohexyluriedo-3-dodecanoic acid (CUDA) (Cayman Pharma, Neratovice, Czech Republic) to control the stability of the platform.

FAHFA purification was performed using solid-phase extraction (SPE) with HyperSep SPE columns (500 mg/10 mL, 40–60 μm, 70 Å; Thermo Scientific). FAHFAs were eluted with ethyl acetate and dried using a SpeedVac (Brezinova et al., 2018). The dry extracts were resuspended in 50 μL of methanol before analysis.

### 2.3. Lipidomic profiling

Lipidomic profiling of mushroom extracts was conducted using liquid chromatography-mass spectrometry (LC-MS) systems consisting of a Vanquish ultra-high performance liquid-chromatography (UHPLC) System (Thermo Fisher Scientific, Bremen, Germany) coupled to a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) operated in the negative ion mode, as described previously (Paluchova, Vik, et al., 2020). Two internal standards were used: (i) [$^{13}C_4$]-9-PAHSA for quantification of FAHFAs based on the chemical similarity between analytes, and (ii) CUDA to control the stability of the platform and possible differences in injection volumes (Cajka, Smilowitz, & Fiehn, 2017). FAHFAs were annotated using an in-house retention time–$m/z$ library and MS/MS libraries (NIST20, MassBank, MoNA, LipidBlast) (Ma et al., 2015). The amount of each FAHFA was quantified by normalizing its peak height (obtained from MS-DIAL 4.8) to the peak height of the internal standard [$^{13}C_4$]-9-PAHSA and the total mushroom tissue weight. A minimum of 12 points per peak and a minimum peak intensity of 10,000 counts were used. Native FAHFAs exist as multiple isomers, and no baseline separation is currently possible. We used a signal-to-noise ratio of 10 for the cluster of isomers and calculated the peak heights versus the internal standard (Brejchova et al., 2022).

### 2.4. FAHFA regioisomer analysis

FAHFA regioisomer analysis was performed using an LC-MS system consisting of a UHPLC Ultimate 3000 RSLC (Thermo Scientific) coupled with a QTRAP 5500/SelexION, a hybrid triple-quadrupole, and a linear ion trap mass spectrometer equipped with an ion mobility cell (SCIEX, Massachusetts, USA). A targeted method based on pre-calculated multiple reaction monitoring (MRM) ion pairs with one quantifier (FA) and two qualifier transitions (HFA, HFA-$H_2O$) per FAHFA was used (Kuda, 2018; Kuda et al., 2016; Syed et al., 2018). The quantifier ion was used as a survey scan for information-dependent acquisition in a linear ion trap for enhanced-resolution MS/MS and second-generation (MS/MS/MS) product ion spectra (Kuda et al., 2016). This approach was used to identify FAHFA regioisomers based on the branching position of the backbone HFA using MS/MS/MS, as previously described (Kuda et al., 2018).

### 2.5. Phylogenetic tree construction

Phylogenetic analysis was performed using the NCBI Genome Workbench (Kuznetsov & Bollin, 2021) for comparative genomics using nucleotide sequences consisting of concatenated protein-coding mitochondrial genes. We applied fast Fourier transforms (MAFFT) in a multiple sequence alignment tool to generate the distance matrix

required for the phylogenetic tree builder tool. A phylogenetic tree was constructed using the tukes-Cantor distance method through neighbor-joining.

### 2.6. Data analysis and statistics

Data processing and FAHFA annotation were performed using MS-DIAL 4.8 (Tsugawa et al., 2015) for untargeted lipidomic analysis and Multiquant 3.0 (SCIEX) for regioisomer analysis. MetaboAnalyst 5.0 (Pang et al., 2021) was used for constructing FAHFAs heatmaps, clustering, and statistical analysis using log data transformation and Pareto scaling for sample value normalization. GraphPad Prism was used to construct bar graphs and for statistical analyses. FAHFA comparisons across all mushroom species, dendrograms, and heat maps were performed using one-way analysis of variance (ANOVA; adjusted $p$-value cutoff = 0.05) with Fisher's LSD post hoc analysis. T-test two-tailed analysis was used to compare pairs with highly related FAHFA content; $p$-values are provided with the results.

## 3. Resu1ts

### 3.1. FAHFA content in mushrooms

We used LC-MS-based profiling to conduct lipidomic analysis of seven edible mushroom species. We found 34 different FAHFAs from the mushrooms analyzed; the acyl chain length ranged 2–26 carbon atoms, and several acyl chains were unsaturated (Fig. 1 & Fig. 2).

The most representative FAHFAs were those containing LA. The FAHFA content in mushrooms was processed to create a heat map (Fig. 2) representing the normalized abundance of each type of FAHFA according to the mushroom species. The heatmap was clustered through similarities across the three replicates of the samples for each mushroom species. In addition, a phylogenetic tree based on mtDNA was constructed and connected in a clustering dendrogram to show the ontogeny connection between the mushroom species and their FAHFA profiles.

The phylogenetic tree represents the construction of a phylogenetic relationship in the mtDNA between the seven species in the five taxa. As expected, the maximum likelihood tree demonstrated that *H. tessulatus* (shimeji brown), *H. ulmarius* (shimeji white), *A. campestris* (champignon), and *A. bisporus* (cremini) were closely related. Interestingly, the dendrogram sortation of FAHFA profiles created the same cluster pattern as the mtDNA information.

The diversity of FAHFAs varies among the various types of mushrooms (Fig. 2). However, similarities in the type and amount exist between the nearer clades of species. These similarities were noticeable between the pairs of champignon and cremini from the Agaricaceae family and shimeji brown and shimeji white from the Lyophyllaceae family. In contrast, shiitake (*L. edodes*) and enokitake (*F. velutipes*) mushrooms exhibited the most divergent FAHFA profiles rich in short-chain FAHFAs and very long-chain FAHFAs, respectively.

It was found that some specific FAHFAs were present at high concentrations in all mushrooms (Fig. 3). For instance, the most abundant FAHFAs were those with short carbon chains (Fig. 3A–D) (Gowda et al., 2020), followed by LA derivatives, namely, LAHPA, LAHSA, LAHOA, LAHLA, and LAHARA (Fig. 3E–I). LAHLA, a known anti-inflammatory lipid (Kolar et al., 2019), was present mainly in the champignon and cremini samples.

Targeted analysis of the FAHFA regioisomer composition revealed several structural patterns. Mushrooms contained fewer regioisomers than the mammalian FAHFA profile, and the branching position was closer to the carboxylic function (Fig. 3t). However, we were unable to determine the regioisomers of short-chain FAHFA, which are likely C2 HFAs (Gowda et al., 2020).

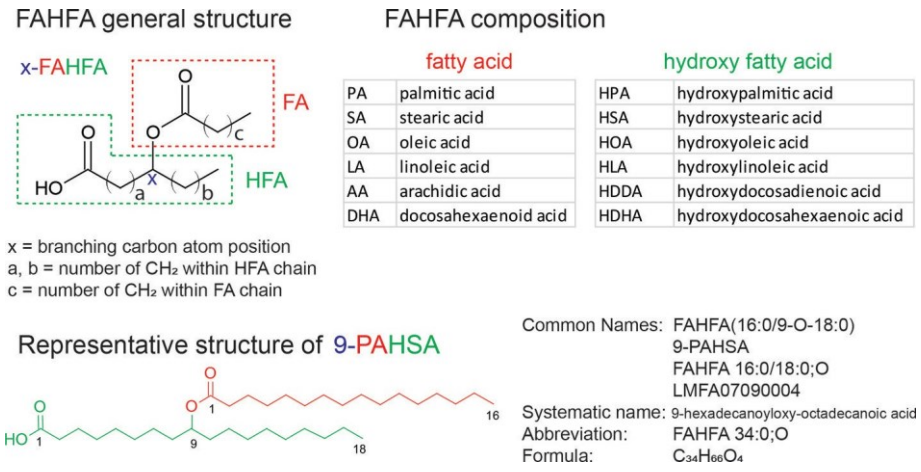
Without the short-chain FAHFA regioisomers, the clustering algorithm failed to separate mushrooms according to taxonomy (Fig. 3t). Shimeji brown and shimeji white presented the closest relationship with FAHFA regioisomer content, followed by cremini and champignon. However, a closer relationship exists between cremini and champignon, with nearly identical profiles. Clustering between shimeji brown and shimeji white mushrooms was better observed for FAHFA isomers from the PAHSA family. The maximum amount of FAHFA isomers was observed in enokitake, with 7- and 9-PAHPA, 10-AAHDDA, and 14/17-DHAHDHA as major representatives.

### 3.2. FAHFA food content

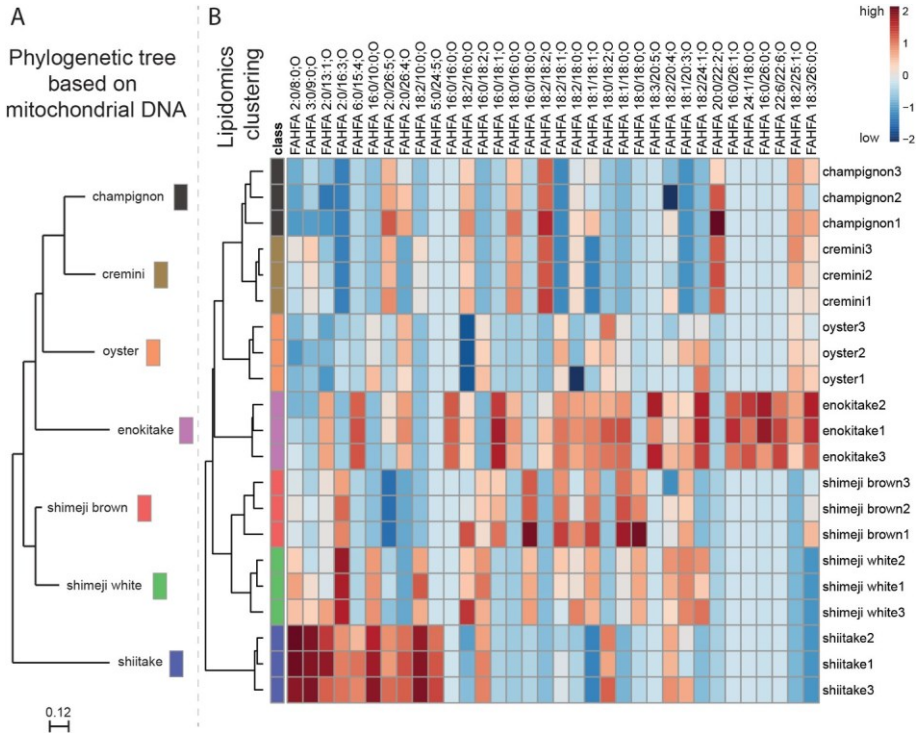
Edible mushrooms contain FAHFA regioisomers that are relevant to human health, such as PAHSAs in shimeji and 13-LAHLA in cremini and champignon. To relate the FAHFA content in mushrooms to the known nutritional sources of FAHFAs, we reviewed published concentrations in foods. The results were expressed in absolute amounts, including vegetables, fruits, grains (Liberati-Cizmek et al., 2019), Fu brick, green and black tea (Zhu et al., 2022), fermented brown rice (FBRA), rice bran (Watanabe et al., 2022), and oils (Syed et al., 2018; Takumi et al., 2021). The overall FAHFA content in edible mushrooms was found to be lower ($0.01–0.4 \times 10^{-7}$ g/g tissue fresh weight) than that in common human foods and oils (approximately $1–30 \times 10^{-7}$ g/g tissue fresh weight, apple to walnut), except for shiitake, which had a higher FAHFA content than FBRA and black and green teas due to short-FAHFAs (Fig. 3).

## 4. Discussion

Mushrooms are a rich source of valuable natural products and novel




**/ig. 1.** General structure of fatty acid esters of hydroxy fatty acids found in mushrooms. FAHFAs belong to a family of estolides that are defined as oligomers of a hydroxy fatty acid and a fatty acid. Hence, FAHFA is a monoestolide in which the carboxylic acid group of a fatty acid (FA) is esterified to the hydroxyl group of a hydroxy fatty acid (HFA). Depending on the length and saturation of their acyl chains and the position of the branching carbon, they are grouped into various super−/sub-families.




**/ig. 2.** Relative abundance of FAHFAs in mushrooms based on species. (A) The dendrogram illustrates the taxonomic closeness between the various mushroom classes. (B) The heatmap represents FAHFA abundance clustered using the complete linkage method and Euclidean distance measure. FAHFA X1:Y1/X2:Y2;O. X1 and Y1 define the FA, and X2 and Y2 represent the HFA. X defines the number of carbon atoms and Y defines the number of double bonds.

It was found that some specific FAHFAs were present at high concentrations in all mushrooms (Fig. 2). For instance, the most abundant FAHFAs were those with short carbon chains (Fig. 2A–D) (Gowda et al., 2020), followed by LA derivatives, namely, LAHPA, LAHSA, LAHOA, LAHLA, and LAHARA (Fig. 2E–I). LAHLA, a known anti-inflammatory lipid (Kolar et al., 2019), was present mainly in the champignon and cremini samples.

compounds with important biological activities, including nutritional products that are largely untapped (Zhong & Tang, 2004). In this study, it was found that FAHFA content changed across mushroom samples of the same order, but was maintained between species of the same family.

The nutritional value of mushrooms is often neglected compared to that of familiar sources of lipids. Compared to other foods, FAHFAs contained in mushrooms may also exert higher bioavailability, supporting the premise that their high fiber and protein content may aid in fatty acid absorption (Karine et al., 2006). The intake of FAHFAs in humans has been previously studied in vegetarians and omnivores, reflecting how the inclusion of well-known sources of fatty acids has a predominant impact on nutrition levels (Kellerer et al., 2021).

The dendrogram clustering of FAHFA concentrations across mushroom samples highly matched the phylogenetic tree for the species analyzed. This result suggests that a genetic link creating species-specific molecules provides a mechanistic approach for understanding the origins and implications of species interactions and diversity (Raguso et al., 2015). It has been stated that a heterogeneous class of metabolites often mediates the interaction among species (Reynolds et al., 2018). The evidence pointed out here suggests that bioactive nutraceuticals detected in mushrooms, such as oysters (*P. ostreatus*) (G. X. Ma et al., 2018), shiitake (*L. edodes*), and enokitake (*F. velutipes*) (Fukushima-Sakuno, 2020) are highly preserved in fungi. The findings presented here may aid in identifying taxa using bioactive metabolites such as FAHFAs.

The variety of FAHFA regioisomers in the seven mushrooms differs from the typical families in other realms (Brejchova et al., 2020), including their abundance. This suggests that mushrooms have specific and/or related mechanisms to produce FAHFAs, including biochemical machinery for their biosynthesis. Adipose triglyceride lipase (ATGL, also known as patatin-domain-containing protein, EC:3.1.1.3) has been identified as a biosynthetic enzyme for FAHFAs in mammalian adipose tissue (Patel et al., 2022). ATGL is also involved in FAHFA metabolism (Brejchova et al., 2021; Paluchova, Oseeva, et al., 2020). Several orthologs of the ATGL enzyme, such as triacylglycerol (TG) lipases, have also been found in fungi, namely, Tgl3p (Athenstaedt & Daum, 2003), Tgl4p, and Tgl5p, which share homology with certain enzymatic specificities (Athenstaedt & Daum, 2005). These TG lipases were detected in the transcriptome of the club fungi shiitake (*L. edodes*) (Sakamoto et al., 2017), which suggests their presence in other Basidiomycetes. As previously stated, FAHFAs are preserved in fungi and mammals (Celis Ramirez et al., 2020; Yore et al., 2014). A possible explanation for this may be the existence of a similar lipase for FAHFA biosynthesis as a model mechanism extrapolated among the species. Additional studies should be conducted to further understand the relationship and mechanisms involved in the presence of FAHFA and TG lipase activity in edible mushrooms.

## 5. Conc1usion

In the present study, we reported the concentrations of FAHFAs in edible mushrooms as a potential nutritional source of FAHFAs with a low lipid content. FAHFA diversity can be used for chemotaxonomic classification of the mushroom family, similar to mtDNA information.

## /unding

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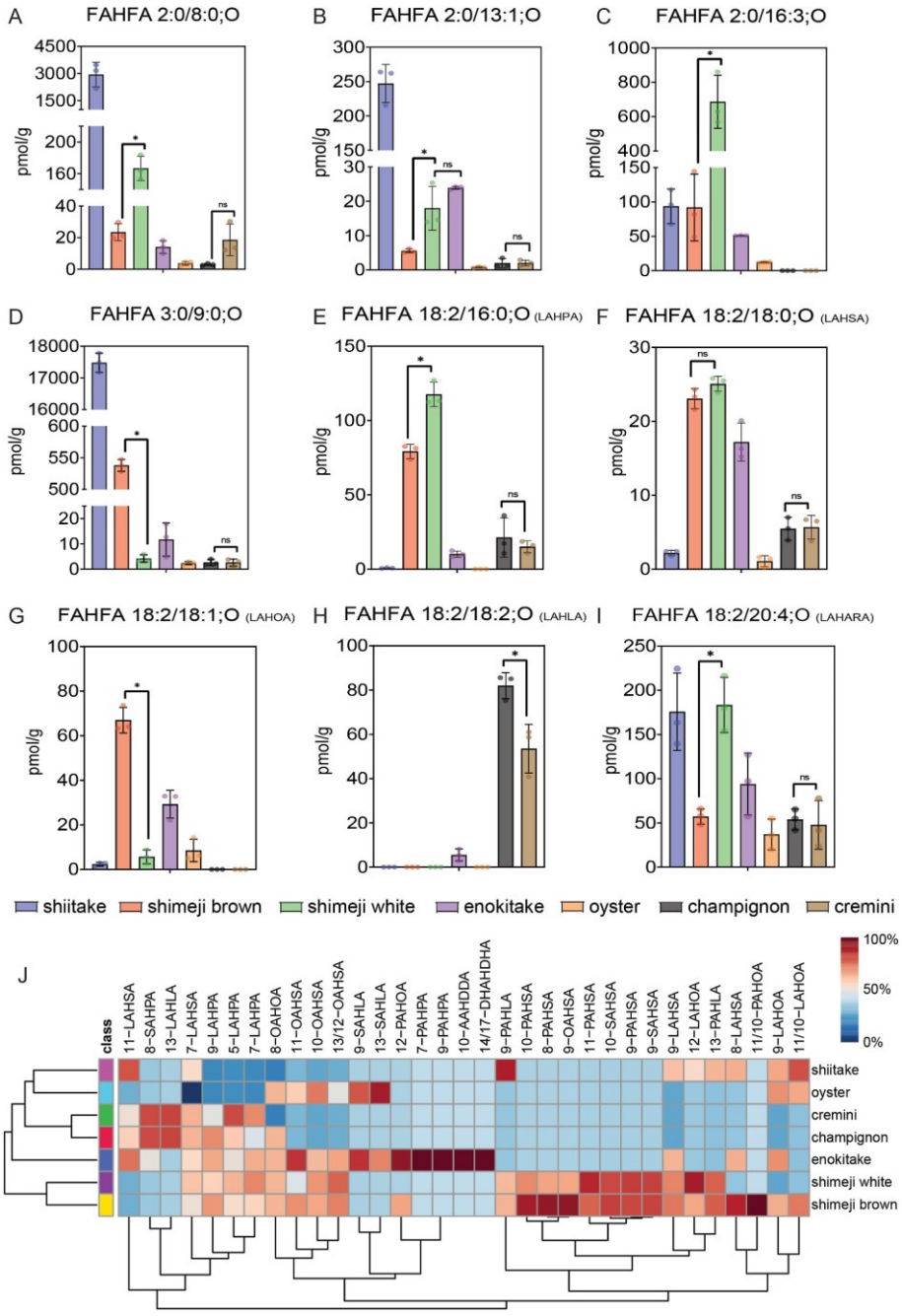
## CRediT authorship contribution statement

**/e1ipe Martínez-Ramírez:** Data curation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Martin Riecan:** Formal analysis, Methodology, Writing – review & editing. **Tomas Cajka:** Formal analysis, Methodology, Resources, Software, Writing – review & editing. **Ondrej Kuda:** Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing, Conceptualization.

## Dec1aration of competing interest

All authors declare that they have no conflicts of interest.




**/ig. 3.** Concentrations of the major FAHFAs in mushrooms with the difference between the pair of mushrooms that are most highly taxonomically related. (A) FAHFA 2:0/8:0;O, (B) FAHFA 2:0/13:1;O, (C) FAHFA 2:0/16:3;O, (D) FAHFA 3:0/9:0;O, (E) LAHPA, (F) LAHSA, (G) LAHOA, (H) LAHLA, and (I) LAHARA; *$p < 0.05$, ns = not significant using t-test. Data are means ± S.E., $n = 3$. (t) LC-MS analysis depicting percentage content of FAHFA regioisomers in mushrooms represented as a heat map. The data were normalized to 100% as the maximum abundance value using $\log_{10}$ data transformation and Pareto scaling, and clustered using the complete linkage method and Euclidean distance measure. The data were averaged using classes of mushrooms ($n = 3$).

## Data avai1abi1ity

Data will be made available on request.

## Acknow1edgments

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## Appendix A. Supp1ementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2022.114311.

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LETTER OPEN ACCESS

# Tetraspanin CD53 Promotes Inflammation but Restrains Mucus Production in a Mouse Model of Allergic Airway Inflammation

Amy T. Hsu[1] | Viktor Bugajev[2] | Timothy A. Gottschalk[1] | Livia Demkova[2] | Lucie Potuckova[2] | Lubica Draberova[2] | Monika Bambouskova[2] | Philipp Hagemann[3] | Caitlin A. O'Brien[1] | Martin Riecan[4] | Ondrej Kuda[4] | Evelyn Tsantikos[1] | Petr Draber[2] | Mark D. Wright[1] | Annemiek B. van Spriel[3] | Margaret L. Hibbs[1] | Ivana Halova[2]

[1]Department of Immunology, Alfred Research Alliance, Monash University, Melbourne, Victoria, Australia | [2]Laboratory of Signal Transduction, Institute of Molecular Genetics of the Czech Academy of Sciences, Prague, Czech Republic | [3]Department of Medical BioSciences, Radboud University Medical Center, Nijmegen, The Netherlands | [4]Laboratory of Metabolism of Bioactive Lipids, Institute of Physiology of the Czech Academy of Sciences, Prague, Czech Republic

**Correspondence:** Margaret L. Hibbs (margaret.hibbs@monash.edu) | Ivana Halova (ivana.halova@img.cas.cz)

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To the Editor,

CD53 is a pan-leukocyte-expressed tetraspanin that is implicated in controlling immune cell function, including leukocyte trafficking [1]. CD53 is associated with several inflammatory diseases such as asthma, where genetic studies report that a promotor polymorphism that reduces CD53 expression is associated with higher asthma risk [2]. Herein, the role of CD53 in mast cell responses and Type 2 inflammation was investigated.

Bone marrow-derived mast cells (BMMC) from wild-type (WT) and *Cd53*$^{-/-}$ mice activated with IgE antigen did not differ in early activation events (Figure S1A–H). Analysis of the late phases of IgE-antigen activation revealed that neither prostaglandins nor leukotrienes were increased in the absence of CD53 (Figure 1A). However, proinflammatory cytokine production (Figure S1I) and secretion (Figure 1B) were significantly increased by CD53 deficiency. Expression of CD53 in *Cd53*$^{-/-}$ and WT BMMCs rescued TNF-α overproduction (Figure 1C) directly implicating CD53 in this phenotype. Elevated cytokine production was FcεRI-dependent, as no differences in cytokine production were observed in cells activated with thapsigargin (Figure S1J). Further analyses showed that CD53 deficiency augmented nuclear translocation of NF-κB (Figure 1D,E) and prolonged phosphorylation of c-Jun (Figure 1F).

Given these findings, we assessed the role of CD53 in allergic airway disease by immunizing and challenging mice with PBS (sham) or ovalbumin (OVA) (Figure 1G). In contrast to the exaggerated signaling and cytokine production observed in *Cd53*$^{-/-}$ BMMCs, components of the immune response were dampened in OVA-challenged *Cd53*$^{-/-}$ mice, including a significant reduction in leukocyte recruitment to the lungs and airspaces, particularly eosinophils (Figure 1H,I, Figure S2A,B). While steady-state IgE levels were not affected

Petr Draber, Mark D. Wright, Annemiek B. van Spriel, Margaret L. Hibbs and Ivana Halova share senior authorship.

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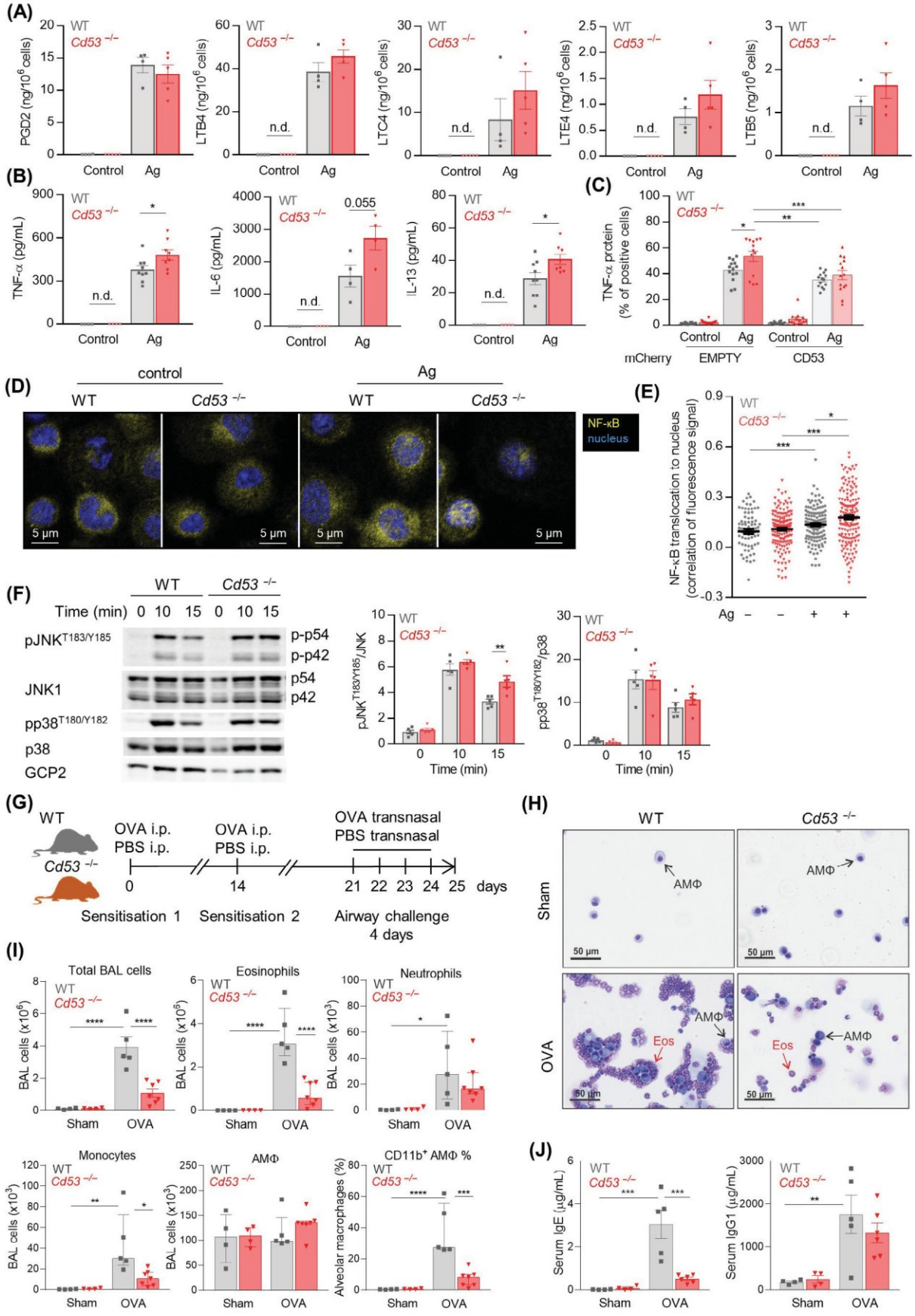


**FIGURE 1** | Legend on next page.



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**FIGURE 1** | CD53 deficiency enhances FcεRI-mediated cytokine production in BMMCs but suppresses cellular inflammation and IgE production in OVA-challenged mice. (A–E) BMMCs were left unstimulated (Control) or activated with IgE-Ag (Ag). (A) Prostaglandins and leukotrienes production. (B) Cytokine production. (C) TNF-α production in WT and *Cd53*$^{-/-}$ BMMCs transfected with empty mCherry expression vector or mCherry expression vector with hCD53. (D, E) NF-κB p65 subunit localization to the nucleus (15–50 cells per cell type and condition, $n = 3$); (F) Representative image and evaluation of JNK and p38 phosphorylation. GCP2 is independent loading-control. (G–J) Evaluation of inflammation in sham or OVA-challenged WT and *Cd53*$^{-/-}$ mice ($n = 4$–7). (G) Experimental design. (H) Cytospins of cells from bronchoalveolar lavage (BAL), and immune cell counts in BAL from flow cytometry and cell counts (I). (J) Serum IgE and IgG1 levels. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

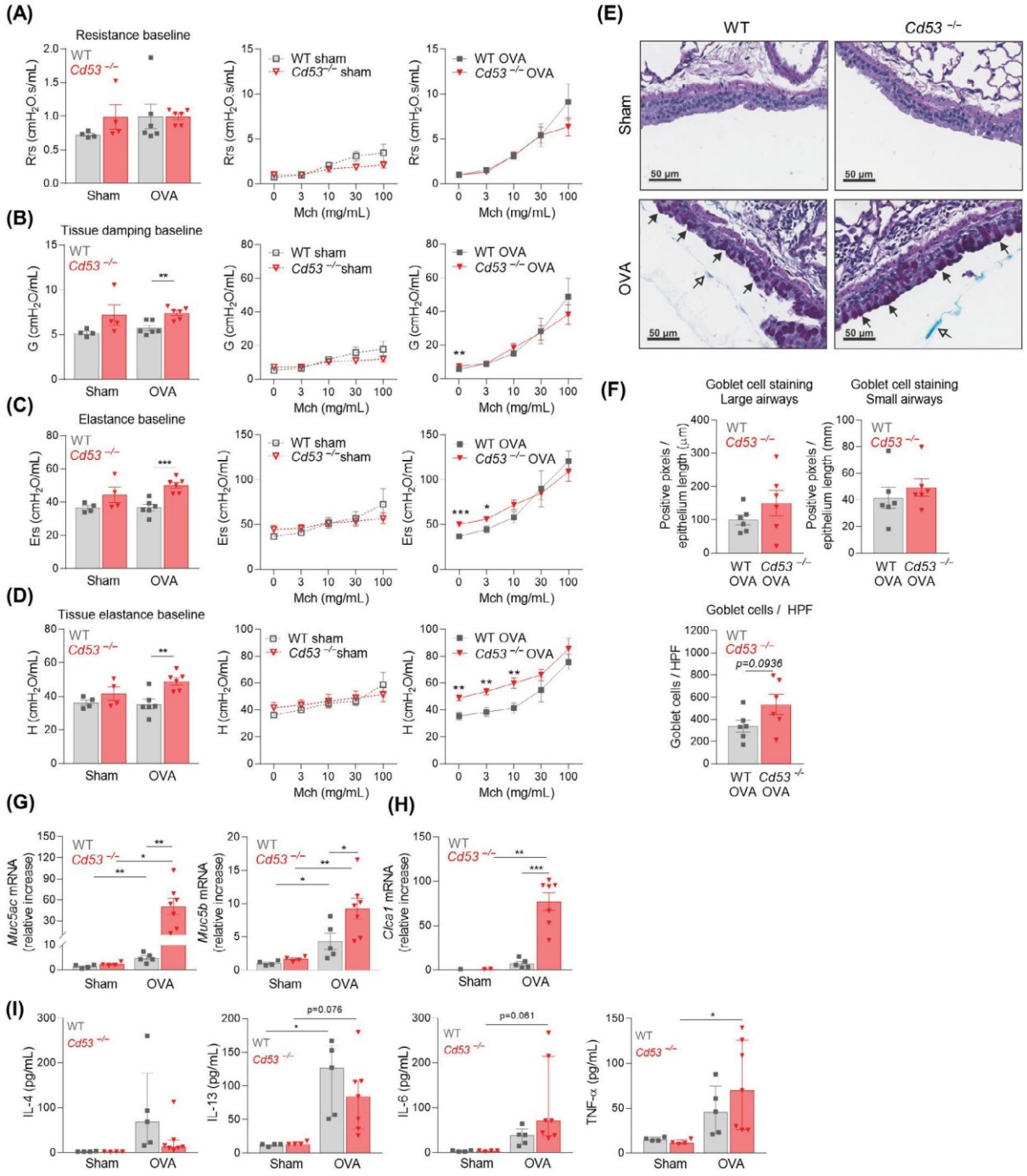


**FIGURE 2** | CD53 deficiency results in altered lung function at baseline and mucin production in allergen-challenged mice. (A–D) Lung function parameters at baseline (no methacholine) in sham and OVA-challenged mice (left graphs) and postmethacholine (Mch) challenge in sham (middle graphs) vs. OVA-challenged mice (right graphs) assessing (A) respiratory system resistance [Rrs], (B) tissue damping [G], (C) respiratory system elastance [Ers], and (D) tissue elastance [H]. (E) Goblet cell metaplasia and mucus in the large airways. Closed arrows = goblet cells, open arrows =mucus. (F) Goblet cell quantification in large and small airways and per high-power field (HPF). Expression of (G) mucin genes and (H) *Clca1* in lung tissue by qPCR. (I) Protein concentrations of mucin-stimulating cytokines in BAL fluid measured by multiplex assay. $n$=4–7, *$p<0.05$, **$p<0.01$, ***$p<0.001$.



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by CD53-deficiency, IgE responses were increased > 200-fold in OVA-challenged WT mice but this response was markedly dampened in OVA-challenged *Cd53*$^{-/-}$ mice, although conversely IgG1 levels were unchanged, suggesting impaired sequential class-switching from IgG1 to IgE (Figure 1J, Figure S2D).

Airway hyperresponsiveness to inhaled methacholine revealed that respiratory system elastance and tissue elastance were increased in OVA-challenged *Cd53*$^{-/-}$ mice at baseline and to low doses of methacholine, while baseline tissue damping was increased (Figure 2A–D). Airway mucus staining showed no significant differences between genotypes, but revealed a trend towards increased goblet cell numbers in OVA-challenged *Cd53*$^{-/-}$ mice (Figure 2E,F). Expression of mucin genes and regulators (*Muc5ac*, *Muc5b, Clca1*) were markedly upregulated in OVA-challenged *Cd53*$^{-/-}$ mice (Figure 2G,H), correlating with mildly enhanced mucus deposition in the large airways and dysregulated lung function. The classical mucin-inducing cytokines, IL-4 and IL-13 [3], were variably increased in OVA-challenged mice, but not significantly different between WT and *Cd53*$^{-/-}$ mice (Figure 2I). Two other mucin-inducing cytokines, IL-6 and TNF-α [3], were trending or significantly increased in the lung wash of OVA-challenged *Cd53*$^{-/-}$ mice (Figure 2I), although not to the levels expected to drive a strong mucus response, but this combination may underlie the observed phenotype in *Cd53*$^{-/-}$ mice. Other cytokines and chemokines were elevated in OVA-challenged mice, but with the exception of IL-1β and IL-12p40, which were significantly reduced in the absence of CD53, no major differences were observed between WT and *Cd53*$^{-/-}$ mice (Figure S2C).

Although CD53 expression is thought to be restricted to hematopoietic cells [4], a recent report shows an important role for CD53 in metabolic signaling in hepatocytes [5]. This raises the possibility that CD53 may have a non-immune cell function in the lung. According to the Human Protein Atlas, CD53 is expressed in type II alveolar epithelial cells and by ciliated and club cells in the airways. Thus, deficiency of CD53 may directly promote mucin gene expression in airway epithelium. Interestingly, there is a precedent for another tetraspanin, TSPAN8, regulating goblet cell metaplasia and mucus secretion [6]. Future studies should focus on assessing the role of CD53 in these key non-immune cells.

Despite numerous studies, the role of mast cells in mouse models of allergic asthma remains controversial [7, 8]. Our results, showing that CD53-deficiency led to reduced airway inflammation in vivo but increased proinflammatory cytokine production in mast cells in vitro, may seem contradictory. However, they suggest a multifunctional role for CD53 in different cell types and processes. Increased proinflammatory cytokine production in vitro aligns with previous finding in a macrophage cell line after CD53 knockdown [2]. Although reduced cellular inflammation was apparent in the lungs of OVA-challenged *Cd53*$^{-/-}$ mice, allergen-induced cytokines in BAL fluid, including those produced by mast cells, were not largely different. Diminished leukocyte migration into inflamed lungs corroborates previous reports of impaired *Cd53*$^{-/-}$ leukocyte recruitment [1, 9], while impaired IgE responses correlates with reduced humoral immunity in *Cd53*$^{-/-}$ mice [9]. No significant differences in mast cell numbers between sham and OVA-challenged mice of either genotype was observed (Figure S2B), which may be due to the chosen immunization schedule. Mast cells are one of several sources of cytokines in vivo, and whether CD53 also regulates proinflammatory cytokine production in Th2 cells, eosinophils or basophils remains to be determined.

In conclusion, this study demonstrates that CD53 has a complex and multifaceted role in asthma, being required for inflammatory leukocyte recruitment to asthmatic lungs and the production of IgE, yet negatively regulating mast cell signaling, cytokine and mucus production.

### Author Contributions

A.T.H.: methodology, formal analysis, investigation, writing. V.B., T.A.G., Li.D., L.P., Lu.D., M.B., P.H., C.A.O., E.T., M.R. and O.K.: methodology, formal analysis, investigation. P.D., M.D.W., A.B.S. and M.L.H.: conceptualization, funding acquisition, supervision, writing. I.H.: conceptualization, methodology, formal analysis, investigation, funding acquisition, writing.


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### Ethics Statement

All mouse studies complied with European legislation (directive 2010/63/EU of the European Commission) and were approved by local authorities (CCD, The Hague, the Netherlands), Institutional Animal Ethics Committee Review Board, IMG of the CAS guidelines (permit number 12135/2010–17210), and Czech national guidelines (2048/2004–1020). In vivo asthma studies were approved by the Alfred Research Alliance Animal Ethics Committee (E-1752-2017M, Australia). All experiments were conducted in accordance with the ARRIVE guidelines.

### Conflicts of Interest

The authors declare no conflicts of interest.

### Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.


Amy T. Hsu
Viktor Bugajev
Timothy A. Gottschalk
Livia Demkova
Lucie Potuckova
Lubica Draberova
Monika Bambouskova
Philipp Hagemann





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Caitlin A. O'Brien
Martin Riecan
Ondrej Kuda
Evelyn Tsantikos
Petr Draber
Mark D. Wright
Annemiek B. van Spriel
Margaret L. Hibbs
Ivana Halova

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## Supporting Information

Additional supporting information can be found online in the Supporting Information section.



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