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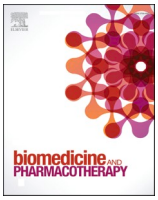

Influence of Ibuprofen on glycerophospholipids and sphingolipids in context of Alzheimer's Disease

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ABSTRACT

Alzheimer's disease (AD) is a multifactorial disorder associated with neuroinflammation, elevated oxidative stress, lipid alterations as well as amyloid-deposits and the formation of neurofibrillary tangles. Ibuprofen, a globally used analgesic, is discussed to influence disease progression due to its anti-inflammatory effect. However, changes in lipid-homeostasis induced by Ibuprofen have not yet been analyzed. Here we investigate the effect of Ibuprofen on lipid classes known to be associated with AD. Ibuprofen treatment leads to a significant increase in phosphatidylcholine, sphingomyelin and triacylglyceride (TAG) species whereas plasmalogens, which are highly susceptible for oxidation, were significantly decreased. The observed alterations in phosphatidylcholine and sphingomyelin levels in presence of Ibuprofen might counteract the reduced phosphatidylcholine- and sphingomyelin-levels found in AD brain tissue with potential positive aspects on synaptic plasticity and ceramide-induced apoptotic effects. On the other hand, Ibuprofen leads to elevated TAG-level resulting in the formation of lipid droplets which are associated with neuroinflammation. Reduction of plasmalogen-levels might accelerate decreased plasmalogen-levels found in AD brains. Treatment of Ibuprofen in terms of lipid-homeostasis reveals both potentially positive and negative changes relevant to AD. Therefore, understanding the influence of Ibuprofen on lipid-homeostasis may help to understand the heterogeneous results of studies treating AD with Ibuprofen.

1. Introduction

Ibuprofen (IBU) is a globally used analgesic with additional antipyretic and anti-inflammatory effects [1,2], that belongs to the group of non-steroidal anti-inflammatory drugs (NSAID) [2,3]. After its introduction to the market in 1969, it was included in the WHO list of essential medicines in 1977 because of its analgesic effect [2]. As non-opioid analgesics, NSAID represent the first stage of the WHO's graded scale for the use of analgesics in the treatment of pain. Originally developed for the treatment of tumor pain, the stepwise approach is now also used for the treatment of acute and chronic pain [4]. The use of non-opioid analgesics such as NSAID represents the basic therapy and can be modified by co-analgesics and opioids depending on the intensity and type of pain [4].

Chemically, NSAID are a heterogeneous group of drugs typically consisting of an acidic moiety (enol or carboxylic acid) with an attached aromatic functional group [2,5]. The alpha-methyl substitution of the phenylacetic acid derivative leads to an arylpropionic acid derivative, to which IBU can be assigned [5] (Fig. 1a).

NSAID exert their effect by inhibiting cyclooxygenases 1 and 2

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(COX1 and COX2) [1], which leads to an inhibition of prostaglandin (PG) synthesis (Fig. 1b). PG are tissue hormones that are ubiquitous in the body and act as pain mediators. In addition to mediating inflammatory response, they are also known to increase core body temperature [6,7]. IBU competitively, reversibly, and non-selectively inhibits COX1 and COX2, which catalyze the conversion of arachidonic acid (AA) to PG [8–10]. COX is a membrane-bound homodimeric enzyme complex that forms a hydrophobic channel, with the active site for cyclooxygenase activity located at the end of its membrane-binding region [9].

NSAID inhibit the conversion of AA into PG by blocking the access of AA to the catalytic site by occupying the binding pockets [11]. Phylogenetically, the two enzyme complexes differ on the one hand in the structure of this binding pocket (due to the replacement of the amino acid isoleucine by the slightly smaller valine, the channel of COX1 is somewhat narrower than that of COX2) [8]. On the other hand, it has been shown that the two proteins differ within their expression behavior [12]. While COX1 is continuously expressed in almost all tissues and is probably largely responsible for PG synthesis with housekeeping function, the expression of COX2 seems to be mainly coupled to inflammatory processes [9,12,13], although continuous expression in kidney and central nervous system (CNS) has also been demonstrated [14].

This explains the adverse effects of NSAID. While primarily COX1 inhibition increases bleeding tendency by inhibiting thromboxane A2 synthesis [10,15], selective COX2 inhibition increases the incidence of cardiovascular events, which is why some coxibs have been withdrawn from the market [1,8,9]. The side-effect profile of non-selective NSAID thus depends on the COX specificity in question; gastrointestinal complaints such as nausea, vomiting, or the formation of gastric and intestinal ulcers may occur especially with prolonged use [1,8,9]. In particular, a combination therapy with NSAID and glucocorticoids increases the risk of ulcers by a factor of 15 [16]. Additionally, central nervous complaints such as headache, dizziness, tinnitus, or visual disturbances may occur, indicating permeability of the blood-brain barrier to NSAID [17,18].

Increased cardiovascular risk may be mediated by selective COX2 inhibitors [19]. Both selective and non-selective COX inhibitors induce renal dysfunction as the renal blood flow is PG-dependent [20]. Contraindications derived from the side effect profile include previously experienced hypersensitivity reactions, an unexplained bleeding tendency, existing ulcers, inflammatory bowel diseases, cardiovascular disease or the last trimester of pregnancy [21].

As already described, IBU crosses the blood-brain barrier and thus enters the CNS [17,18]. Furthermore, the influence of IBU on neurodegenerative diseases such as Alzheimer's disease (AD) or Parkinson's disease has been described [22,23]. A link between neuroinflammation and cell death has been postulated several times, which could be slowed down by anti-inflammatory drugs such as IBU [22–27].

In addition to the well-described involvement of COX in neurodegenerative processes and thus IBU as a potential therapeutic approach, there also appears to be a link between IBU and an altered lipid metabolism. In mouse models, inhibition of β-oxidation by IBU has been shown to result in steatosis in various tissues such as liver, heart or kidney [28–32].

Particularly in the context of AD, lipid changes and their influence on the pathophysiology of AD have been widely discussed and several lipidomics studies have shown a deficit of glycerophospholipids as well as sphingolipids in brain tissue of AD patients, to name just a few examples [33–36]. That IBU might have an influence on AD has been postulated several times [23], and a common explanation relates to IBU-mediated anti-inflammation [22–27]. Nevertheless, there is few data regarding the influence of IBU on cellular lipid composition, which, given the altered lipid composition in the AD brain, could represent another explanatory approach for the effect of IBU in AD patients.

To address the question of whether and what influence IBU has on the lipid composition, we incubated the human neuronal cell line SH-SY5Y with 25 μM IBU and analyzed the lipidome by targeted shotgun mass spectrometry, focusing on glycerophospholipids and sphingolipids.

2. Results

2.1. Experimental design

To investigate the effects of IBU on cellular lipid metabolism, human neuroblastoma cells (SHSY5Y wt) were incubated with 25 μM IBU or

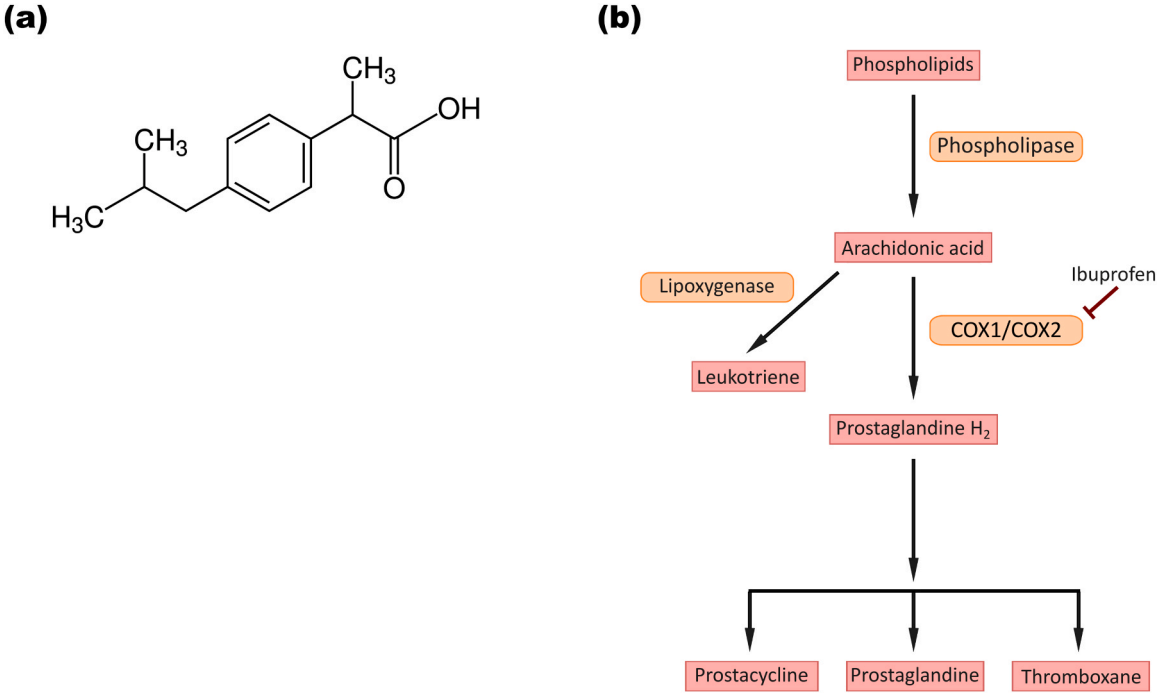

Fig. 1. Chemical structural formula of Ibuprofen (a). Synthesis of cyclooxygenase-dependent tissue hormones. Isoforms of the heterodimeric cyclooxygenases are reversibly blocked by Ibuprofen (b).

solvent as control for 72 hours. The cell homogenates were then adjusted to a uniform protein concentration of 8 mg/mL, and, after solid-liquid extraction of the lipids, the samples were subjected to targeted shotgun semi-quantitative mass spectrometric analysis. The data obtained were normalized to standards added prior to lipid extraction, to exclude influences of lipid extraction. Alterations in the lipid profile due to IBU treatment were expressed as percentage change compared to the control group. The results of phosphatidylcholine species (PCaa), phosphatidylcholine-plasmalogen species (PCae), sphingomyelin species (SM) and triacylglyceride species (TAG) are presented below.

In order to establish a more robust correlation between the IBU-induced changes in lipid homeostasis and AD, SH-SY5Y cells stably expressing the familial amyloid precursor protein swedish mutation (APPswe) [37], leading to increased amyloid-β levels and representing a widely used cellular model of AD were treated with 25 μM IBU for 72 hours [38–41]. The treatment was followed by protein adjustment and lipid extraction.

2.2. Analysis of phosphatidylcholine species

Phosphoglycerides chemically consist of a glycerol linked to a phosphated and esterified alcohol as well as to two fatty acids, with the characteristic structure as follows: Fatty acid chains are attached to a glycerol-3-phosphate at sn-1 and sn-2 positions, leaving sn-3 for alternating substitutes. The lipid molecule is then defined by the substituent at the sn-3 position. In addition, both the length of the acyl chain and the degree of saturation have an influence on the membrane properties [42,43]. During degradation by phospholipases A1, A2, C2 or D, second messengers such as arachidonic acid or diacylglycerol are formed [44]. As the most important lipid class in relation to the cell membrane, phosphoglycerides are involved in numerous processes at the boundary between the intracellular and extracellular compartments, e.g., signal transduction [45]. They are also present as part of lipid droplets (LD) by forming monolayers, acting as mediators between different compartments by using their amphiphilic character [46]. Phosphatidylcholines are one of the most important lipid classes within the phosphoglycerides family, accounting for up to 35 % in the human brain [47]. As suggested by the name phosphatidylcholine, the bound alcohol is a choline compound linked to glycerol via a phosphate group.

After incubation with IBU, 41 of the 43 PCaa species examined were increased, while only the 2 species C42:1 and C42:2 were decreased, C42:2 not significantly (99.9 % ± 2.1 %, $p = 0.971$) (Fig. 2a). 35 of the elevated species showed significant changes and 1 species was significantly reduced (C42:1). This resulted in an increase in the total amount of PCaa to 117.4 % (± 2.8 %, $p \leq 0.001$, Fig. 2b). In the volcano plots the area of the bubbles symbolizes the proportion of each species within the lipid class. Including this information showed that all species with a proportion greater than 1 % were significantly elevated.

Additional analysis focusing on the chain length of the bound fatty acids showed a consistent increase compared to the control, limited by the non-significant effects for C20:X-C26:X (Fig. 2c). The significant upregulation for all saturations (Fig. 2d) indicated that the observed increase was independent of chain saturation. Overall, the PCaa species showed a global upregulation after treatment with IBU.

The APPswe cells showed an increase of 25 PCaa species, 4 species increased significantly (C38:4, C32:2, C36:4 and C34:2), while 18 species decreased, none of them significantly, including C42:1 and C42:2 (Figure S2). All species with a proportion greater than 2.0 % of total PCaa increased, adding up to 94.5 % of total PCaa.

2.3. Analysis of phosphatidylcholine-plasmalogen species

Phosphatidylcholine plasmalogens, which belong to the glycerophospholipid family, promote the differentiation and maturation of neurons in the central nervous system. In addition, they may also act as endogenous antioxidants and reduce neuroinflammation [48].

Under the experimental conditions chosen in this study, the incubation with IBU had detrimental effects on PCae species compared to PCaa species. Treatment with IBU reduced the total cellular PCae content compared to control. This can be deduced from the fact that out of 39 species measured, 27 were downregulated, 19 significantly. At the same time, however, there were also 6 significantly increased species (Fig. 3a). Considering the proportion of these upregulated species in the PCae lipid class, it can be observed that these species account for only 1.8 % of the PCae total. So, the bar graph in Fig. 3b shows a significant decrease in the total amount to 93.9 % (± 0.5 %, $p \leq 0.001$). Thus, incubation with IBU also alters the ratio of PCae to PCaa, which decreases to 79.9 % (± 3.3 %, $p \leq 0001$, Fig. 3c).

PCae species grouped by their chain length revealed that shorter chain lengths were downregulated, while longer chain lengths showed a slight tendency towards upregulation (Fig. 3e). The individual changes were as follows: C30:X was downregulated to 90.0 % (± 0.8 %, $p \leq 0.001$), C32:X to 90.3 % (± 0.7 %, $p \leq 0.001$), C34:X to 93.4 % (± 0.6 %, $p \leq 0.001$), C36:X to 93.3 % (± 0.6 %, $p \leq 0.001$), and C38:X to 95.6 % (± 0.5 %, $p \leq 0.001$). C40:X (99.0 % ± 0.9 %, $p = 0.493$), C42:X (102.4 % ± 1.6 %, $p = 0.264$) and C44:X (104.1 % ± 1.8 %, $p = 0.064$) did not reach significance but continued the trend. To further support these results, a linear regression of chain length and effect was calculated, resulting in a regression coefficient of 0.9547 ($p \leq 0.001$, Fig. 3e). To identify differential effects between specific chain length groups, the measured PCae species were grouped in four categories accordingly: short (C<32:X), medium (C32–36:X), long (C38–40:X) and very long (C>40:X) chain length. The results shown in Fig. 3d also indicate a significant downregulation of the short and medium groups. The long group was still downregulated, but with lower significance, up to the very long group, where no significance could be detected. To analyze the differences between these groups, a multi comparison analysis was performed. All groups differed significantly from each other in their effect, as shown by the horizontal brackets in Fig. 3d. This leads to the conclusion that the observed effect is strongly dependent on chain length, resulting in an increased probability of downregulation in PCae species bound to a fatty acid with a shorter chain length.

When considering the saturation of the bound fatty acid, Fig. 3f shows a significant downregulation for all different saturation levels. Therefore, it could be concluded that the effect of downregulation does not seem to be related to the degree of saturation, which is supported by a correlation coefficient of 0.0074 ($p = 0.856$).

In the APPswe cells, a decrease in 38 of the 39 measured species was observed after IBU incubation. 28 species decreased significantly, one species increased non-significantly (C42:5 to 109.4 % ± 12.2 %, $p = 0.632$) (Figure S3).

2.4. Analysis of sphingomyelin species

Chemically, sphingolipids are based on the amino alcohol sphingosine. The complex lipids sphingomyelin and glycosphingolipids, including gangliosides, belong to the sphingolipid family. These lipids are considered to be bioactive, regulating various mechanisms such as cell growth, cell death, immune response, inflammation or cellular signaling [49]. Sphingomyelins (SM) are a ubiquitous component of the biological cell membrane, and their importance extends to several areas. For example, their dynamic modification in lipid rafts in the plasma membrane influences the development of fatty liver and obesity as well as insulin resistance or atherosclerosis [50,51].

In the present observation, all SM species in the treatment group were increased with an effect greater than the mean standard error of the mean (mean SEM), 10 species reached significance (C14:1 OH, C16:1, C18:0, C18:1, C20:2, C22:1 OH, C22:3, C24:1, C24:1 OH and C26:1, Fig. 4a). As a result, the total amount of SM species was significantly increased to 110.3 % (± 3.1 %, $p = 0.017$, Fig. 4b). Calculation of the ratio of all measured SM and PCaa species compared to the control showed a significant decrease to 93.7 % (± 1.6 %, $p = 0.007$, Fig. 4c),

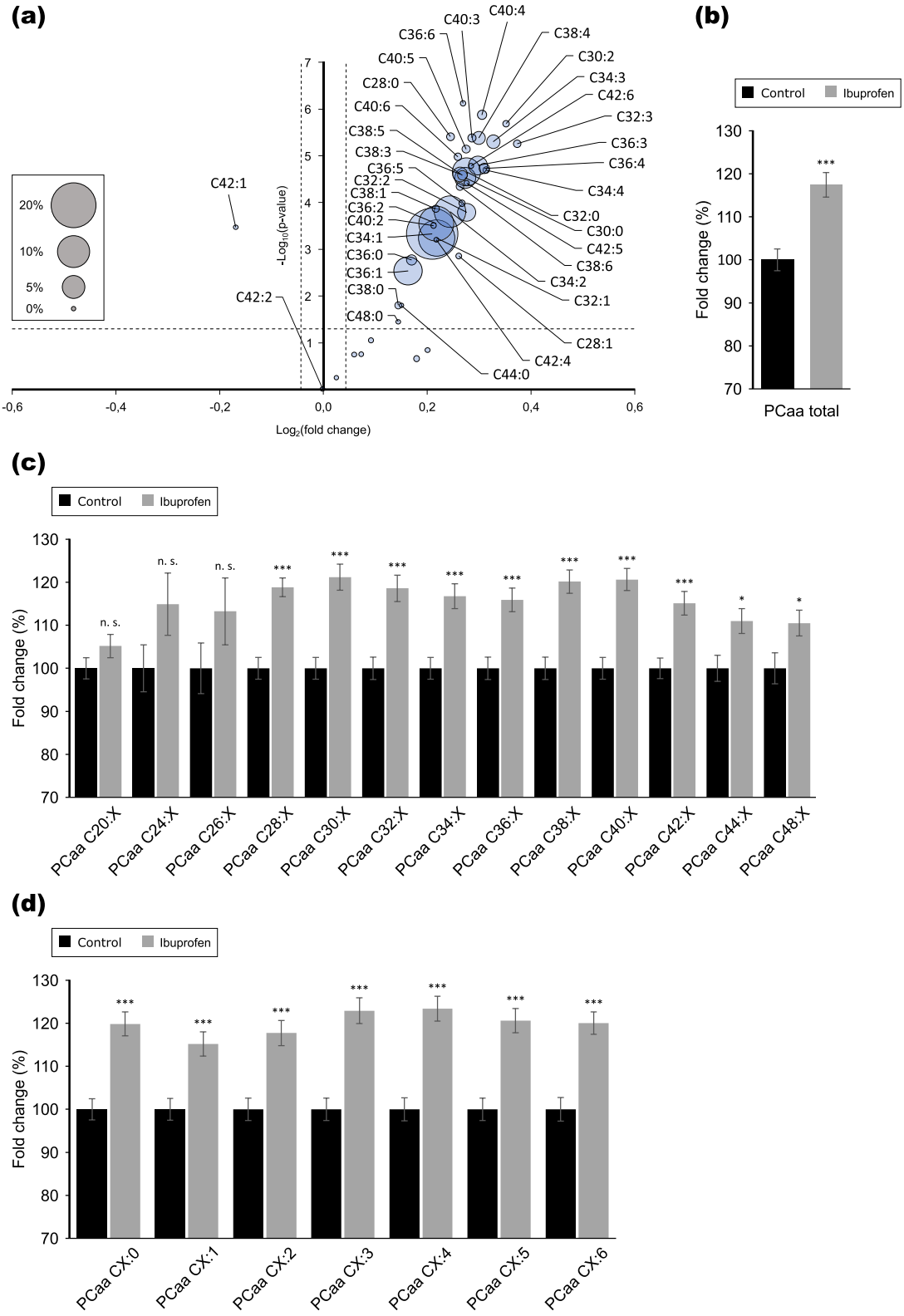

Fig. 2. Phosphatidylcholine (PCaa) levels in homogenates of SH-SY5Y wt cells incubated with 25 μM Ibuprofen for 72 hours compared to solvent control. (a) Fold changes of the individual PCaa species are shown as a volcano plot. The bubble area represents the proportion of each species in the PCaa lipid class under incubation conditions. Grey bubbles show reference proportions. The vertical line represents the mean standard error of the mean (mean SEM), the horizontal line marks a significance level of 0.05. Significantly changed and highlighted species are named. (b-e) Bar charts showing the relative fold change of all measured PCaa species (b), the relative fold changes of PCaa species grouped by their chain length (c) and the relative fold changes of PCaa species grouped by their saturation (d). Error bars represent the SEM and statistical significance was set as * $p \leq 0.05$ and * * * $p \leq 0.001$, n. s. = non-significant. The statistical analyses applied are described in detail in Section 4.8.

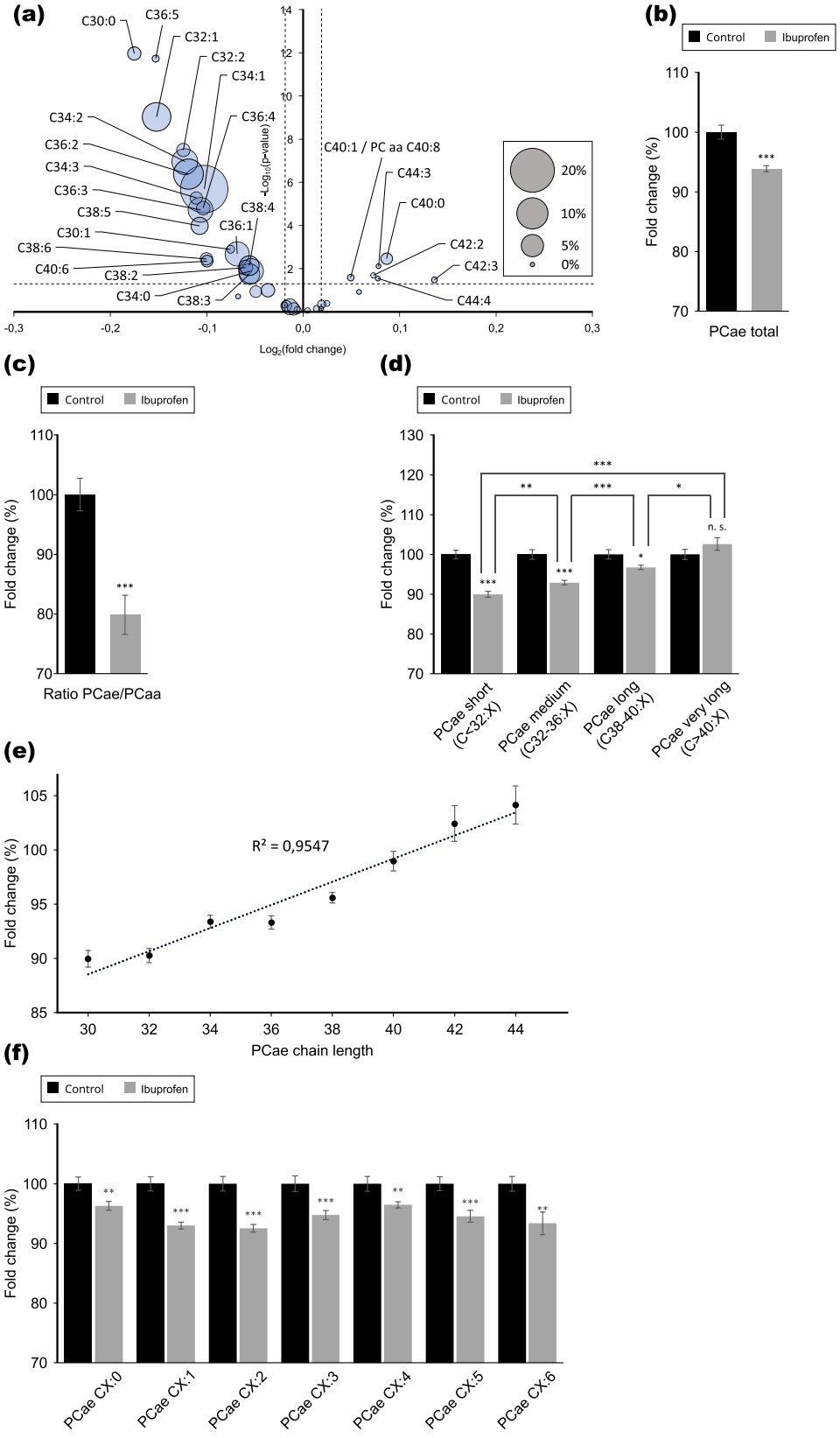

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Fig. 3. Phosphatidylcholine-plasmalogen (PCae) levels in homogenates of SH-SY5Y wt cells incubated with 25 µM Ibuprofen for 72 hours compared to solvent control. (a) Fold changes of the individual PCae species are shown as a volcano plot. The bubble area represents the proportion of each species in the PCae lipid class under incubation conditions. Grey bubbles show reference proportions. The vertical line represents the mean standard error of the mean (mean SEM), the horizontal line marks a significance level of 0.05. Significantly changed species are named. (b-d, f) Bar charts showing the relative fold change of all measured PCae species (b), the relative fold change of the ratio of all measured PCae species and all measured PCaa species (c), the relative fold changes of PCae species grouped by classes of chain length (d) and the relative fold changes of PCae species grouped by their saturation (f). (e) Diagram showing the relative fold changes of PCae species grouped by their chain length. Error bars represent the SEM and statistical significance was set as * $p \leq 0.05$, * * $p \leq 0.01$ and * * * $p \leq 0.001$, n. s. = non-significant. The statistical analyses applied are described in detail in Section 4.8.

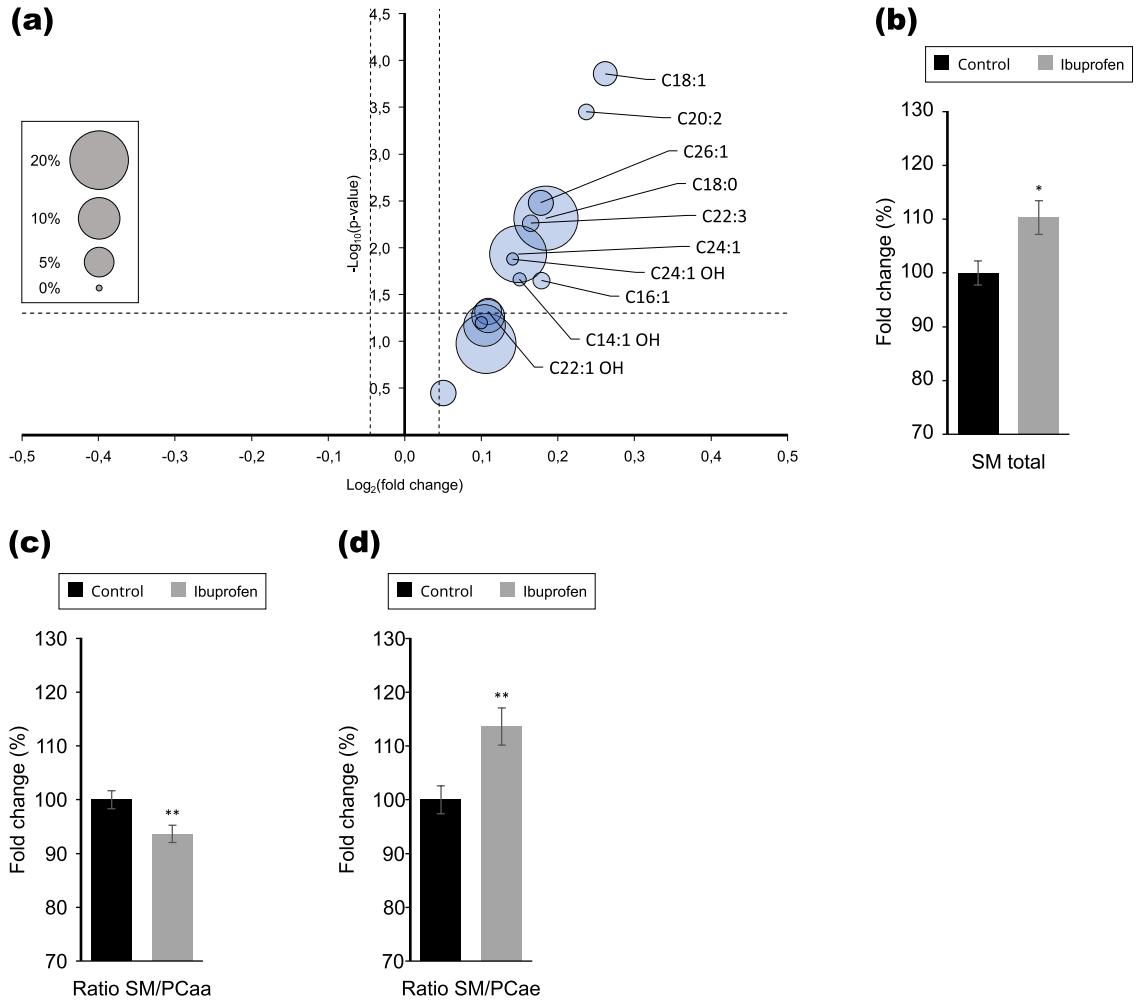

Fig. 4. Sphingomyelin (SM) levels in homogenates of SH-SY5Y wt cells incubated with 25 µM Ibuprofen for 72 hours compared to solvent control. (a) Fold changes of the individual SM species are shown as a volcano plot. The bubble area represents the proportion of each species in the SM lipid class under incubation conditions. Grey bubbles show reference proportions. The vertical line represents the mean standard error of the mean (mean SEM), the horizontal line marks a significance level of 0.05. Significantly changed species are named. (b-d) Bar charts showing the relative fold change of all measured SM species (b), the relative fold change of the ratio of all measured SM species and all measured PCaa species (c) and the relative fold change of the ratio of all measured SM species and all measured PCae species (d). Error bars represent the SEM and statistical significance was set as * $p \leq 0.05$ and * * $p \leq 0.01$, n. s. = non-significant. The statistical analyses applied are described in detail in Section 4.8.

while the ratio of all SM and PCae species increased to 113.6 % (± 3.4 %, $p = 0.007$, Fig. 4d). These data could indicate a global upregulation of SM species and suggest detectable changes in the ratio of PCaa, PCae and SM lipid classes.

IBU also caused an increase in all 15 SM species measured in APPswe cells. Six species, C16:0, C16:1, C16:1 OH, C24:1 OH, C26:0 and C26:1, increased with an effect strength greater than the mean SEM, including the main species C16:0 with a proportion of 36.3 % of total analyzed SM species. SM C16:0 revealed an increase to 117.8 % (± 13.0 %, $p = 0.344$) (Figure S4).

2.5. Analysis of triacylglycerol species

Triacylglycerides (TAG) are one of the most important lipid stores of the human body and are mainly ingested with food [52,53]. TAG are molecules consisting of three fatty acids esterified with a glycerol molecule and are stored in so-called lipid droplets (LD) [54]. In addition, TAG are involved in various metabolic processes, such as the biosynthesis of other molecules, the determination of the fatty acid oxidation rate or the regulation of the plasma levels of free fatty acids [55].

In this present study, 37 out of 39 TAG species were found to be upregulated after IBU administration. 32 of these species with an increase greater than the mean SEM and at 11 of them the elevation was

statistically significant (C50:1, C50:2, C52:1, C52:2, C52:3, C54:2, C54:3, C54:4, C56:1, C56:2 and C60:3). In contrast, only 2 TAG species were downregulated without reaching significance (C60:5 and C60:6, Fig. 5a). All TAG species accounting for more than 1 % of all species were increased. The total amount of TAG species increased to 130.2 % (± 8.7 %, p = 0.019, Fig. 5b).

The measurement of TAG species extracted from APPswe cells resulted in an increase of the seven species C50:1, C50:2, C50:3, C52:2, C52:3, C54:4, C54:5, including the two main species C52:2 (12.4 %) and C50:1 (12.3 %). 31 species decreased, 20 with an effect greater than the mean SEM, however none of these species with a proportion of more than 2.5 % of the total TAG amount (Figure S5).

3. Discussion

IBU is an analgesic prescribed worldwide with additional antipyretic and anti-inflammatory effects [1,2]. The main effects of IBU are well known and frequently described in literature [1,8,56], whereas the influence on cellular lipid homeostasis has not yet been studied in detail.

Particularly in the context of neurodegenerative diseases such as Alzheimer's disease (AD) or Parkinson's disease, IBU appears to be able to influence disease progression [22,23]. Several studies based on animal models also suggest that IBU could have a positive influence on AD [57–59]. Epidemiological studies are also indicative of a reduced risk of developing AD as a result of taking NSAID (Table 1). For example, the Rotterdam Study, a prospective cohort study that has been running since 1990, suggests a protective effect of NSAID in relation to the development of AD, with IBU accounting for around 20 % of all prescribed NSAID over an eight-year study period [60,61]. Similar findings were provided by a case-control study conducted with US veterans, which analyzed data on the use of NSAID and the progression of AD over a period of seven years. IBU in particular showed a strongly increasing protective effect with the duration of use [62].

Based on these promising results, a number of clinical studies were conducted to substantiate a link between the use of NSAID and the slowed progression of AD. To this end, Pasqualetti *et al.* conducted one of the most comprehensive studies with IBU alone, in which 132 patients were observed over a period of one year. A Mini Mental Status Examination (MMSE) and the Alzheimer's Disease Assessment Scale (ADAS) carried out at the end of the study showed no difference between the placebo group, consisting of 46 patients, and the IBU group, which comprised 51 patients [63]. In other clinical studies, which aimed to prove a connection between the use of NSAID and slower progression of AD, there was also no evidence of an NSAID-induced reduction in progression [64–66].

One explanation for the different results in epidemiological and clinical studies could be the study period. While clinical studies were often limited to one year or less [67], epidemiological studies such as the Rotterdam Study looked at a period extending over decades. A longer study period of clinical studies could therefore be an approach to re-evaluate the results.

The potential protective effect of IBU on neurodegenerative diseases could possibly be due to the anti-inflammatory effect of IBU [24–27]. The starting point of this consideration is a chronic inflammatory response mediated by microglia, which is characterized by increased cytokine and complement release, ultimately leading to pyroptotic cell death [68]. In an AD mouse model, a reduction in the inflammatory response and amyloid-β plaques has been shown in transgenic mice, fed with IBU [69], supporting the above-mentioned explanatory approach.

Nevertheless, alteration of cellular lipid composition also seems to be related to the pathophysiology of AD [33]. Referring to AD, lipid changes have been reported in *post mortem* AD brain tissue and in animal models, mainly affecting the lipid classes of phospho- and sphingolipids [70,71]. Vice versa, several lipids have been shown to alter the processing of the amyloid-precursor-protein (APP), leading to amyloid-β (Aβ) peptides that accumulate and aggregate as senile plaques in brains of AD-affected individuals [72]. Therefore, in this study, the effects of IBU on lipid homeostasis and the possible effects on AD were investigated in a neuronal cell model using the human neuroblastoma cell line SH-SY5Y.

In vitro methods allow a comparable and reproducible view of molecular mechanisms of action due to the controlled and standardized external influencing factors and therefore contribute to a fundamental understanding of the pharmacodynamics of a therapeutic agent and its possible effect on AD. A key limitation of this study is the use of undifferentiated SH-SY5Y cells, which do not fully recapitulate the physiological properties of mature neurons. These cells exhibit an immature neuronal phenotype, characterized by limited neurite outgrowth and differential expression of neuronal markers compared to their differentiated counterparts. Consequently, their lipid metabolism and response

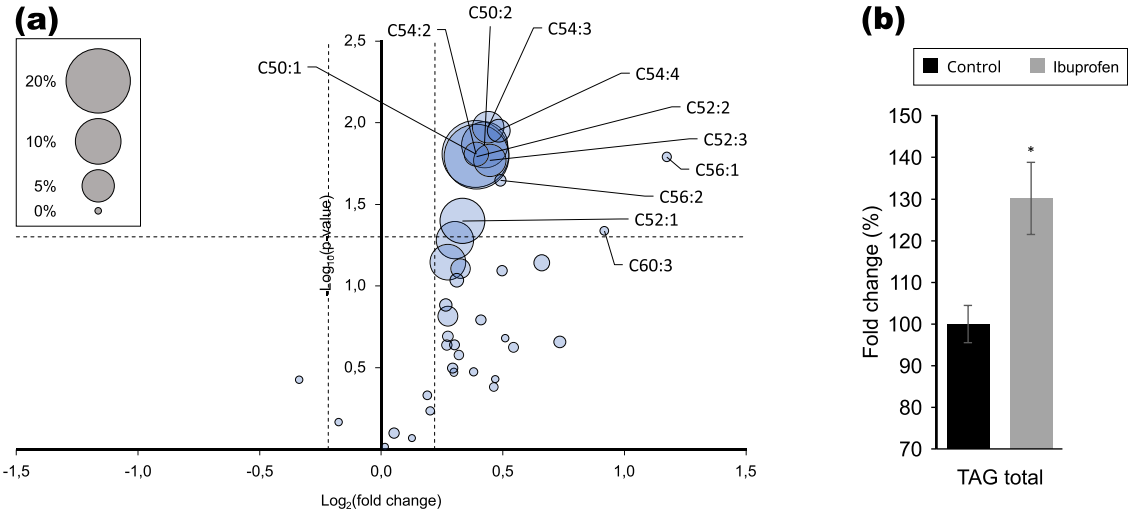

Fig. 5. Triacylglycerol (TAG) levels in homogenates of SH-SY5Y wt cells incubated with 25 μM Ibuprofen for 72 hours compared to solvent control. (a) Fold changes of the individual TAG species are shown as a volcano plot. The bubble area represents the proportion of each species in the TAG lipid class under incubation conditions. Grey bubbles show reference proportions. The vertical line represents the mean standard error of the mean (mean SEM), the horizontal line marks a significance level of 0.05. Significantly changed species are named. (b) Bar chart showing the relative fold change of all measured TAG species. Error bars represent the SEM and statistical significance was set as * $p \leq 0.05$ n. s. = non-significant. The statistical analyses applied are described in detail in Section 4.8.

Table 1
Clinical trials investigating connections between NSAID and AD. * Specified as "defined daily dose", i.e. the average dose of a medicinal product taken by adults for the main indication, according to the WHO.

Author	Pasqualetti *et al.*	Vlad *et al.*	Hofman *et al.*
Year	2009	10/1998–09/2005	Since 1990
Study type	Clinical trial	Case-control study	Prospective cohort study
Study duration	12 months	7 years	Ongoing
Dosage	2 x 400 mg/d	No data	1200 mg/d *
Test method	Alzheimer Disease Assessment Scale (ADAS), Mini-Mental-Status Examination (MMSE)	No data	Mini Mental State Examination (MMSE), Geriatric Mental Schedule (GMS), followed by the Cambridge Examination for Mental Disorders of the Elderly (CAMDEX) in screenpositives
Results	No efficacy in the tertiary prevention of AD	Long-term use of NSAID, especially Ibuprofen, could protect against AD	Long-term use of NSAID could protect against AD

to external stimuli, including drug treatments, may differ from that of mature neurons, potentially influencing the observed effects of IBU on lipid homeostasis. However, the choice of undifferentiated SH-SY5Y cells was deliberate and based on several methodological considerations. First, undifferentiated SH-SY5Y cells provide a robust and reproducible system for metabolic studies, minimizing variability introduced by differentiation protocols that can yield heterogeneous neuronal populations. Second, their high proliferative capacity facilitates the generation of sufficient biological material for comprehensive lipidomic analyses, ensuring reliable quantification of lipid species. Third, while differentiated SH-SY5Y cells more closely resemble post-mitotic neurons, undifferentiated cells still retain key neuronal properties and have been widely used in studies investigating fundamental aspects of lipid metabolism and neurodegenerative diseases [73–75]. Additionally, numerous previous studies have utilized SH-SY5Y cells to investigate lipidomic changes in response to various compounds, including neuroprotective agents and metabolic modulators [76–79]. The use of this model allows direct comparison with existing datasets and facilitates the assessment of whether IBU-induced lipid alterations align with or differ from those observed with other pharmacological interventions.

Despite the advantages of this in vitro model, it is important to acknowledge that cell culture systems lack the complexity of the *in vivo* environment, where interactions between neurons, glial cells, and systemic metabolic factors play a crucial role in lipid homeostasis and neuroinflammation. To fully elucidate the impact of IBU on lipid metabolism in the context of AD, future studies should incorporate *in vivo* models that better reflect the multicellular and systemic dynamics of lipid regulation. These models will help clarify whether the observed lipid changes translate to functional alterations in brain physiology and disease progression. Furthermore, longitudinal clinical studies are needed to investigate whether long-term IBU intake will influence lipid metabolism in patients at risk for AD.

The lipidomic approach used in this study is suitable for identifying and quantifying the different lipid species of the lipidome [80]. Therefore, lipidomics is a suitable method to investigate the influence of IBU on lipid homeostasis.

After treatment with IBU, PCaa, SM and TAG levels were significantly increased, whereas PCae levels were significantly decreased.

Phosphatidylcholines (PCaa) are essential components of the cell membrane [81,82]. Reduced PCaa levels are thought to be associated with reduced neuronal plasticity through decreased formation of synapses [83,84]. We showed that under the influence of IBU, 41 of the 43 PCaa species examined were increased irrespective of their saturation, 35 of them significantly. Since a reduction of phospholipids has been observed in *post mortem* AD brain tissue [70,85], (long-term) therapy with IBU (or other NSAID) could counteract this loss and thus slow down disease progression (Table 2). However, the exact mechanism that ultimately induces phospholipid elevation has not yet been conclusively elucidated.

Examining TAG, a global increase in synthesis was found. 32 of the 39 TAG species examined were upregulated and outside the SEM, 11 of which were also significant. Intracellularly, TAG are stored in lipid droplets (LD) [78]. LD are mainly composed of TAG and cholesterol esters surrounded by a phospholipid monolayer, with PCaa forming the bulk of these phospholipids [78,86,87]. Therefore, the previously discussed increase in PCaa could also be related to increased TAG synthesis. Due to their influence on numerous processes such as energy or lipid metabolism, current research considers LD to be multifunctional cell organelles in their own right [31,87]. Thus, intracellular accumulation of LD has been described in a variety of disorders [88,89], including AD (Table 2).

As reported in the introduction, IBU affects lipid and energy metabolism by inhibiting β-oxidation [31,78], leading to intracellular accumulation of acyl-CoA. Accumulated acyl-CoA is either present as a free fatty acid in the cytosol or is esterified to TAG, whereby inhibition of β-oxidation can be considered as a possible reason for the results obtained here. This assumption could be supported by the tendential reduction of carnitine C02, which is a final product of β-oxidation, to 92.8 % ($\pm$ 6.7 %, $p = 0.306$). Thus, in the context of AD, two factors could influence negative disease progression. First, LD are associated with neuroinflammation and thus represent a cause of neurodegenerative disease [78,90], and second, inhibited β-oxidation leads to a decrease in acetyl-CoA, which serves as a substrate for the citrate cycle of energy production. It is well known that in the brain damaged by AD, glucose metabolism is reduced [91], which is why neurons rely on β-oxidation for energy production. As this pathway appears to be inhibited by IBU, this potential mode of ATP synthesis might be disturbed.

In contrast to PCaa, we have shown that incubation with IBU lead to a global downregulation of PCae. In this context, 27 of the 39 PCae species examined were downregulated, 19 of them significantly, with the subspecies with short (C<32:X) to medium chain length (C32–38:X) being significantly downregulated, whereas the significance decreases with increasing chain length. This suggests that the observed effect is strongly dependent on chain length.

Plasmalogens can be detected ubiquitously in membranes and in the brain the concentration of plasmalogens is highest, accounting for 20 % of the total phospholipid content [92,93]. Nevertheless, the function of plasmalogens has not been conclusively clarified [92]. Current research suggests that plasmalogens are involved in cell membrane processes, as well as signal transduction [94]. Moreover, due to their structure, which is characterized by a vinyl ether group in sn1 position, they can scavenge reactive oxygen species (ROS) as endogenous antioxidants [95,96]. It has been repeatedly reported that neurodegenerative diseases such as AD or Parkinson's disease are associated with altered cellular plasmalogen composition [92,93,97] and oxidative stress has been recognized as a contributing factor in the progression of multiple neurodegenerative diseases, including AD and Parkinson's disease [98,99].

While some studies report unchanged PCae concentrations in AD

Table 2
Effects of Ibuprofen on lipid metabolism in SH-SY5Y cells in context of Alzheimer's disease (AD). Changes in the lipid class total are shown by thick arrows, altered species are counted as follows: ↑: number of increased species; ↑SEM: number of increased species with an effect strength greater than the mean standard error of the mean; ↑*: number of significantly increased species; ↓: number of decreased species; ↓SEM: number of decreased species with an effect strength greater than the mean standard error of the mean; ↓*: number of significantly decreased species.

Lipid class	Effect of IBU in		Effect in Alzheimer's disease
	SH-SY5Y wt cells	SH-SY5Y APPswe cells	
PCaa	↑ ↑: 41 ↑SEM: 40 ↑* : 35	↑ ↑: 25 ↑SEM: 5 ↑* : 4	**↓ PCaa levels observed in AD patients**: Reduced in plasma and brain tissues of AD patients [111,112]. **Critical for synaptic function and membrane integrity**: Reduction linked to impaired neurotransmission [83]. **Associated with cognitive decline:** Lower PCaa levels correlate with memory loss and executive dysfunction [113]. **DHA-containing PC deficiency linked to increased dementia risk:** Essential for neuronal survival; deficiency increases AD risk [114]. **Dietary PC intake correlates with better cognitive performance:** Higher intake linked to improved cognition, suggesting a protective role [113].
PCae	↓ ↓: 27 ↓SEM: 23 ↓* : 19	↓ ↓: 38 ↓SEM: 36 ↓* : 28	**↓ Plasmalogen levels observed in AD patients:** Significant reductions in plasmalogen levels detected in both brain and serum samples of AD patients [97]. **Correlation with cognitive impairment:** Lower plasmalogen levels are associated with cognitive deficits and AD severity [97]. **Potential therapeutic benefits of plasmalogen supplementation:** Studies suggest plasmalogen supplementation may improve cognitive function in mild AD cases [115]. **Mechanistic insights:** Plasmalogens may reduce γ-secretase activity, decreasing Aβ production, and prevent neuronal cell death by activating AKT and ERK signaling pathways [97]. **Association with synaptic function:** Plasmalogens are crucial for synaptic vesicle fusion and neurotransmitter release; their deficiency may contribute to synaptic dysfunction in AD [97].
SM	↑ ↑: 15 ↑SEM: 15 ↑* : 10	↑ ↑: 15 ↑SEM: 6 ↑* : 0	**↓ SM levels observed in AD patients:** Significant reductions in SM levels have been detected in the middle frontal gyrus of AD patients, a region vulnerable to AD pathology [36]. **Aβ influences SM metabolism:** Aβ has been shown to activate neutral sphingomyelinase (nSMase), leading to increased hydrolysis of SM and contributing to oligodendrocyte cell death [116]. **Role in neurodegeneration:** Decreased SM levels may disrupt membrane integrity and myelin sheath maintenance, potentially accelerating neurodegenerative processes in AD [117,118]. **Therapeutic potential:** Modulating SM metabolism, possibly through inhibition of nSMase activity, could offer a therapeutic strategy to preserve SM levels and protect against Aβ-induced cytotoxicity [116].
TAG	↑ ↑: 37 ↑SEM: 32 ↑* : 11 ↓: 2 ↓SEM: 1 ↓* : 0	~ ↑: 7 ↑SEM: 1 ↑* : 0 ↓: 31 ↓SEM: 20 ↓* : 0	**↑ midlife TAG levels predict AD pathology:** Increased triglyceride levels during midlife are associated with higher brain Aβ and tau deposition two decades later, suggesting a link between early lipid metabolism and subsequent AD pathology [119]. **LD accumulation in AD brains:** Studies have observed an accumulation of LD in the brains of AD patients, particularly within microglia, which may contribute to neurodegeneration [120]. **APOE4 genotype linked to LD accumulation:** The presence of the APOE4 allele is associated with increased LD formation in microglia, leading to cellular dysfunction and elevated AD risk [109,121]. **Aβ induces LD formation:** Aβ peptides have been shown to trigger LD accumulation in microglia, resulting in impaired cellular function and contributing to AD progression [121].

with significantly reduced phosphatidylethanolamines [100], other studies indicate a reduction in total plasma levels also with respect to PCae [70,85,92] (Table 2).

However, it has not been conclusively established whether down-regulated plasmalogen levels are a cause or consequence of AD [101]. On the one hand, some lipidomic studies suggest that altered neuronal lipid composition may be partly responsible for the occurrence of AD [97]. On the other hand, ROS and Aβ plaques typical for AD appear to inhibit the expression of an enzyme relevant to plasmalogen synthesis, alkyl dihydroxyphosphate synthase, which reduces plasmalogen levels [97].

Overall, AD brain tissue is strongly characterized by oxidative damage, resulting from an imbalance between ROS formation and antioxidant defense [102,103]. Therefore, the fact that IBU leads to a global reduction of PCae should be critically evaluated in the context of AD.

In contrast, in all SM species studied, we observed an upregulation greater than the mean SEM under IBU treatment, which is equivalent to global upregulation. In synopsis of the previously discussed reduction of PCae, a transfer of the choline group of phosphatidylcholine to a ceramide by sphingomyelin synthase could be hypothesized as a possible cause [104]. Abnormal sphingolipid metabolism has been reported in AD [71,105] (Table 2). Strikingly, increased expression of sphingomyelinase, which catalyzes the degradation of SM to ceramides, was found in AD brain tissue [105,106]. As second messengers, ceramides regulate cell growth and apoptosis, among other functions [106,107]. It could therefore be suggested that IBU interferes with sphingolipid metabolism by inhibiting degradation, thereby reducing the accumulation of ceramides with apoptotic effects.

To further substantiate the relevance of our findings in the context of AD, we extended our analysis to SH-SY5Y cells stably expressing the APP Swedish mutation (APPswe). This mutation enhances β-secretase cleavage of APP, leading to increased Aβ production, and is widely recognized as a well-characterized *in vitro* AD model [39–41]. By incorporating this model, we aimed to determine whether IBU-induced lipid alterations are also present in a cellular system with increased Aβ burden, which better reflects key aspects of AD pathology. Our results demonstrate that IBU treatment led to similar lipid alterations in APPswe cells, including an increase in PCaa and SM, as well as a reduction in PCae. Given that PCaa and SM are typically reduced in AD brain tissue, their elevation could indicate a beneficial effect (Fig. 6). However, the decrease in plasmalogens, known for their antioxidant properties, might contribute to increased oxidative susceptibility, a key feature in AD pathology. These findings highlight the necessity of further studies to elucidate the potential impact of IBU on lipid metabolism in the AD brain and its functional consequences.

Interestingly, in contrast to wild-type SH-SY5Y cells, IBU-induced TAG accumulation was not observed to the same extent in APPswe cells. Given that AD pathology is associated with increased LD accumulation in neurons, largely composed of TAG species [108–110], this difference suggests that the baseline elevation of LD in APPswe cells may already reach a point, limiting further IBU-induced accumulation. This observation aligns with the notion that neuroinflammation and metabolic dysfunction in AD brains contribute to intrinsic lipid storage dysregulation, which may not be further exacerbated by IBU treatment in this model. In contrast, in wild-type cells, where LD formation is not inherently upregulated, IBU-induced TAG accumulation is more pronounced (Fig. 6 and Table 2). This suggests that the mechanisms underlying IBU-mediated LD formation may be particularly relevant in non-AD conditions but less so in an AD-like metabolic environment. Further studies are required to determine whether this differential response is related to Aβ-associated alterations in lipid metabolism or compensatory lipid homeostasis mechanisms specific to AD pathology.

In summary, our cell culture study shows that IBU has serious effects on lipid metabolism with potential positive but also negative aspects in respect to AD. The potential beneficial IBU-induced changes in lipid homeostasis include increased SM and PCaa level, that could counteract synaptic loss and ceramide-induced apoptotic effects. On the other hand, IBU treatment resulted in elevated TAG levels, maybe caused by the IBU-induced inhibition of β-oxidation, which has to be considered critically as glucose metabolism is reduced in AD and neurons therefore rely on β-oxidation for energy production. Furthermore, increased TAG level may result in the formation of LD, that are associated with neuroinflammation. Notably, IBU-induced TAG accumulation was observed in wild-type SH-SY5Y cells but not in APPswe cells. This suggests that in AD conditions, the pre-existing elevation of LD and TAG may reach a threshold beyond which additional accumulation is mitigated, potentially due to homeostatic or saturation effects in lipid storage pathways. The decrease in plasmalogens, known for their antioxidant properties, might contribute to increased oxidative susceptibility, a key feature in AD pathology. Due to the reduction also in the cellular AD model, the antioxidant capacity could be additionally limited. Therefore, despite the positive effects of IBU, which also include the general and well-known anti-inflammatory effect, IBU treatment should be considered critical in respect to AD, as some lipid changes induced by IBU might accelerate the progression of the disease.

4. Materials and methods

4.1. Chemicals, reagents, standards

All chemicals were purchased from Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA), unless stated otherwise.

4.2. Drug preparation

Ibuprofen with a HPLC-grade higher than 98 %, shipped as powder, was procured from Merck KGaA, (Darmstadt, Germany). Stock solutions of 100, 50, 25 and 5 mM IBU were prepared using HPLC-grade ethanol and stored in a −25°C freezer and before being used in any cell treatment, warmed to 37°C and thoroughly vortexed. Reaching the final concentrations of 100, 50, 25 and 5 μM was achieved by a 1:1000 dilution upon preparation of incubation media. The final target concentration of 25 μM was chosen due to reports from Ronly Har-Even *et al.* and A. Mannila *et al.* showing plasma peak levels of about 100 μM and cerebrospinal fluid peak levels of about 1 μM after administration of 10 mg/kg body weight [122,123]. Furthermore, this concentration based on our results from the cell viability assay, in which 25 and 5 μM showed no toxic effects after 72 hours of incubation

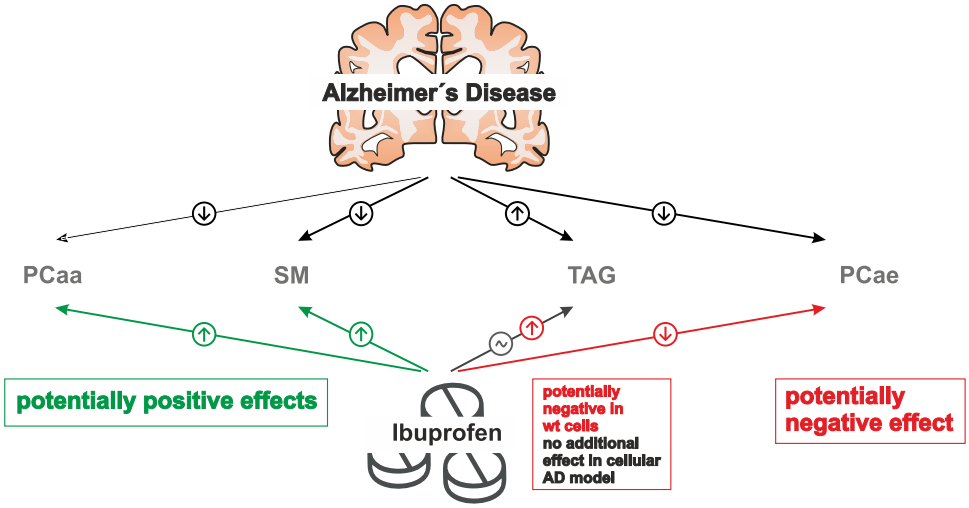

Fig. 6. Schematic summary of the effects of Ibuprofen on lipid metabolism in the context of Alzheimer's disease (AD). Lipid alterations associated with AD are indicated with the arrows above (decrease in phosphatidylcholine (PCaa), sphingomyelin (SM), and plasmalogens (PCae), and an increase in triacylglycerols (TAG)). Ibuprofen treatment modulated these lipid classes in SH-SY5Y cells, shown with the arrows below, with increases in PCaa and SM (potentially beneficial) and a reduction in PCae (potentially detrimental). Notably, Ibuprofen-induced TAG accumulation was observed in wild-type (wt) SH-SY5Y cells but not in APPswe cells. This suggests that in AD conditions, the pre-existing elevation of lipid droplets and TAG may reach a threshold beyond which additional accumulation is mitigated, potentially due to homeostatic or saturation effects in lipid storage pathways.

(Figure S1a).

4.3. Cell culture and Ibuprofen incubation

The potential influence of IBU on lipid metabolism was examined in SH-SY5Y wild-type cells, an immortalized human neuroblastoma cell line, in this study. Additionally, SH-SY5Y cells stably transfected with the APP familial Swedish mutation (APPswe) were analyzed. Dulbecco's modified Eagle medium (DMEM) enriched with 10 % fetal bovine serum (FBS) from GE Healthcare Life Sciences (Chalfont St. Giles, UK) and 1 % non-essential amino acids (NEAA) from Sigma-Aldrich Cor. (St. Louis, Missouri, USA), was used to cultivate the cells under a 5 % CO_2 gas fraction in a humidified 37°C environment. For stably transfected cells, Hygromycin B (400 μg/mL) was added to the culture medium. Once the confluency of seeded cells reached 80 %, medium was changed to reduce the FBS content from 10 % to 2,5 % for the remain of the incubation. After a further 12 hours, incubation was started with 25 μM IBU and, for the solvent control, with one part per thousand HPLC-grade ethanol. A total incubation time of 72 h was chosen with two additional incubation medium changes each after a period of 24 h. Every cell dish underwent an optical light microscopical control to verify cell morphology during and at the end of the incubation period.

4.4. Cell viability

To eliminate the risk of possible toxic effects on the cells interfering with any of the described experiments, a lactate-dehydrogenase assay (LDH-assay) in conjunction with a bicinchoninic acid assay (BCA-assay), relying on the method as described in Kumar *et al.* and Theiss *et al.* [79,124], was conducted. The results are provided in the supplementary (Figure S1a and b). The samples were analyzed using the protocol from the cytotoxicity detection kit from Roche Holding AG (Basel, Switzerland) and a toxicity of less than 5 % per incubation condition was tolerated. All steps of the BCA assay were performed following the manual from the Pierce™ BCA Protein Assay kit by Thermo Fisher Scientific Inc., after harvesting the cells in 50 μL HPLC-H_2O. Triplicates were used to calculate the standard deviation, giving information about the homogeneity of the sample. The whole method is described in Smith *et al.* [125]. The possibility of measuring the protein content is important, as it might be the case that LDH is not elevated in the cell medium, although apoptotic cell death is existent. Under these conditions cytoplasmatic content and therefore also enzymes are packed into membrane enclosed compartments not sensitive to the LDH-assay kit. To sufficiently interpret the results of both experiments, a ratio of toxicity and protein content was formed. None of the analyzed samples showed an elevated toxicity nor a significant decrease in protein level after incubation with 25 μM IBU (Figure S1c).

4.5. Sample preparation

At the end of incubation period, cells were stored at 4°C on ice until further processed. Medium was removed, and cells were washed twice with HPLC-H_2O. Then 180 μL H_2O were added and cells were scraped from the cell dish bottom. The cell suspension was placed in MiniLys tubes each containing seven ceramic beads. To mechanically lysate the cells, those tubes were placed in a MiniLys from PEQLAB (Erlangen, Germany) for 30 seconds at maximum setting. To equilibrate possible differences due to different protein content, samples were adjusted to equal protein level, using the same BCA-assay as described above.

4.6. Lipid extraction

Adjacent to the steps described above, lipid extraction was performed as solid/liquid method. A detailed description of the method is given in Grimm *et al.* and Lauer *et al.* [37,126]. Briefly summarizing the important steps of the extraction procedure: A filter plate (0,45 μm; Merck) is positioned on a deep well plate. Whatman paper measuring 6 mm in diameter is then placed at the bottom of the filter plate. Standards for each lipid class are added with a total volume of 6 μL for each well. The following standards from Avanti Polar Lipids were used for normalization: 06:0 PC (DHPC), 19:0 Lyso PC, 06:0 SM (d18:1/6:0), and Splash II Lipidomix Mass Spec Internal Standard. A sample volume of 10 μL is finally added and samples are dehydrated under a nitrogen flow (1,5 bar) for 45 min to avoid oxidation. 20 μL phenylisothiocyanate solution, containing phenylisothiocyanate (Merck) / ethanol / water / pyridine (Merck) (3:19:19:19, v/v/v/v), is then added to every well and the plate is being incubated for 20 min at room temperature, followed by another 45 min under nitrogen flow. Ammonium acetate (4.93 mM in methanol, Merck), 300 μL per well, is finally added and the plate is incubated on a shaker for 30 min at 450 rpm. The extraction process is terminated by a 2 min centrifugation with 500 g, forcing the samples through the filter into the deep well. Prior usage in any mass spectrometry measurement, 600 μL running buffer containing ammonium acetate in methanol / water (97:3, v/v) is added to every well.

4.7. Targeted shotgun mass spectrometry

A 4000-quadrupole linear-ion trap (QTrap) with a Turbo Spray ion source (AB Sciex, Darmstadt, Germany) in conjunction with an autosampler unit of the Agilent HPLC 1200 series (Santa Clara, CA, USA) was used to perform the lipid analysis as previously described in [37] with the parameters defined in [127] and an Orbitrap ID-X Mass Spectrometer (Thermo Fisher Scientific Inc.) in conjunction with an autosampler unit of the Vanquish neo system (Thermo Fisher Scientific Inc.). A validation of the mass-spectrometry method was conducted in Lauer *et al.* [128]. Positive mode was used for the analysis of six different lipid classes, which include diacyl-phosphatidylcholines (PCaa), phosphatidylcholine-plasmalogens (PCae), lyso-phosphatidylcholines (Lyso-PC), carnitines (C), sphingomyelins (SM) and triacylglycerides (TAG). Samples were measured as triplicate. Results of the measurement were processed, using the analyst 1.4.2 software from AB Sciex and the Trace finder 5.2 software from Thermo Fisher Scientific Inc. to finally decode the counts per second for every single multi reaction monitoring (MRM) pair.

4.8. Statistical analysis of lipidomics data

The measured counts per second were normalized on the lipid class-corresponding standard and the arithmetic mean was formed over the triplicates, guided by a visual cross check of the graphs from each measurement to identify possible artefacts. Furthermore, for one lipid class, all samples were excluded, exceeding the standard deviation by more than two thirds of all measured species of this lipid class. To compare measurements from different incubations, samples were normalized on the primarily measured control group by comparison of the arithmetic mean of each control group. For one species inside a lipid class, the arithmetic mean was calculated over the samples of one incubation condition (solvent control or IBU). The calculated fold change for the intervention group, as percent of the received values from the control group, was plotted as binary logarithm on the axis of abscissas in the shown volcano plots. Here the axis of ordinate represents the p-values of two-sample *t*-test also being plotted logarithmically as negative common logarithm. To visualize the quantity of every species based on their fraction of the whole lipid class under IBU incubation condition, percentage values were calculated and drawn as surface area into the volcano plot. A minimum bubble size was set, and an amplification factor was used, to increase visibility to the reader's eye. Bar charts show the arithmetic mean of the intervention group in relation to the arithmetic mean of the control group. Significance was calculated using the two-sample *t*-test. Multi-comparison analyses were performed with ANOVA and following Tukey-HSD test in case of equality of variance and Games-Howell test in case of variance inequality. Significance was

defined as follows: * $p \leq 0.05$, * * $p \leq 0.01$, * * * $p \leq 0.001$.

Institutional review board statement

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Informed consent statement

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CRediT authorship contribution statement

Radermacher Juliane: Writing – original draft, Investigation. **Erhardt Vincent Konrad Johannes:** Writing – original draft, Visualization, Investigation, Formal analysis. **Walzer Oliver:** Writing – original draft, Visualization, Investigation, Formal analysis. **Haas Elodie Christiane:** Investigation. **Kuppler Konstantin Nicolas:** Investigation, Formal analysis. **Zügner Jill Sven René :** Writing – original draft, Investigation. **Lauer Anna Andrea:** Writing – review & editing, Investigation. **Hartmann Tobias:** Funding acquisition. **Grimm Heike Sabine:** Writing – review & editing, Supervision, Conceptualization. **Grimm Marcus Otto Walter:** Writing – review & editing, Supervision, Project administration, Conceptualization.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest regarding this manuscript.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2025.117969.

Data availability

Not applicable.

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