Free Radical Biology and Medicine 204 (2023) 374–384

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Geometrical isomerization of arachidonic acid during lipid peroxidation interferes with ferroptosis

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ARTICLE INFO

Keywords:
Trans-fatty acids
Polyunsaturated fatty acids
Free radicals
Lipid peroxidation
Cis-trans isomerization
Ferroptosis
NADPH oxidase

ABSTRACT

Geometrical mono-trans isomers of arachidonic acid (mtAA) are endogenous products of free radical-induced cis-trans double bond isomerization occurring to natural fatty acids during cell metabolism, including lipid peroxidation (LPO). Very little is known about the functional roles of mtAA and in general on the effects of mono-trans isomers of polyunsaturated fatty acids (mtPUFA) in various types of programmed cell death, including ferroptosis. Using HT1080 and MEF cell cultures, supplemented with 20 μM PUFA (i.e., AA, EPA or DHA) and their mtPUFA congeners, ferroptosis occurred in the presence of RSL3 (a direct inhibitor of glutathione peroxidase 4) only with the PUFA in their natural cis configuration, whereas mtPUFA showed an anti-ferroptotic effect. By performing the fatty acid-based membrane lipidome analyses, substantial differences emerged in the membrane fatty acid remodeling of the two different cell fates. In particular, during ferroptosis mtPUFA formation and their incorporation, together with the enrichment of SFA, occurred. This opens new perspectives in the role of the membrane composition for a ferroptotic outcome. While pre-treatment with AA promoted cell death for treatment with H_2O_2 and RSL3, mtAA did not. Cell death by AA supplementation was suppressed also in the presence of either ferroptosis inhibitors, such as the lipophilic antioxidant ferrostatin-1, or NADPH oxidase (NOX) inhibitors, including diphenyleneiodonium chloride and apocynin. Our results confirm a more complex scenario for ferroptosis than actually believed. While LPO processes are active, the importance of environmental lipid levels, balance among SFA, MUFA and PUFA in lipid pools and formation of mtPUFA influence the membrane phospholipid turnover, with crucial effects in the occurrence of cell death by ferroptosis.

1. Introduction

After the discovery of programmed cell death, named apoptosis [1], multiple modes of non-apoptotic/necrotic programmed cell death have been identified, including necroptosis, pyroptosis, and most recently, ferroptosis, which are governed by different molecular mechanisms [2]. Ferroptosis is a caspase-independent cell death triggered by iron-dependent lipid peroxidation (LPO) [3,4]. Peroxidation of phosphatidylethanolamine (PE) containing arachidonic acid (AA) is considered a crucial triggering pathway [5,6], and Fig. 1a summarizes three molecular pillars (iron, thiols and phospholipids) and their metabolism in ferroptosis. Notably, GPX4 (glutathione peroxidase 4) enzymatically reduces toxic phospholipid hydroperoxides (LOOH) to non-toxic phospholipid alcohols (LOH) at the expense of glutathione (GSH) [7]; indirect or direct inhibition of GPX4, by depleting GSH or blocking GPX4 activity, respectively, induces ferroptosis [3,4,7]. Ferroptosis serves as a tumor-suppressing mechanism, or can be at the onset of neurodegenerative and cardiovascular diseases [8,9]. Accordingly, pharmacological inducers (e.g., RSL3) and inhibitors (e.g., Ferrostatin-1) have been developed and applied to the treatment of cancer and ferroptosis-related diseases [8,9]. Fig. 1b shows the non-enzymatic LPO of polyunsaturated

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https://doi.org/10.1016/j.freeradbiomed.2023.05.026
Received 26 April 2023; Received in revised form 24 May 2023; Accepted 29 May 2023
Available online 29 May 2023

Abbreviations

AA	arachidonic acid
Apo	apocynin
CHX	cycloheximide
DFO	deferoxamine
DHA	docosahexaenoic acid
DPI	diphenyleneiodonium chloride
EPA	eicosapentaenoic acid
FAME	fatty acid methyl ester
Fer-1	Ferrostatin-1
GC	gas chromatography
GSH	glutathione
GPX4	glutathione peroxidase 4
LOOH	phospholipid hydroperoxides
LOH	phospholipid alcohols
LPO	lipid peroxidation
MEF	mouse embryonic fibroblast
mt	mono-trans
MUFA	monounsaturated fatty acids
PE	phosphatidylethanolamine
PL	phospholipids
PUFA	polyunsaturated fatty acids
ROS	Reactive oxygen species
TFA	trans-fatty acid
NOX	NADPH oxidase

fatty acids (PUFA) residues of phospholipids (PL). It is widely believed, although actively discussed, that during ferroptosis LPO occurs by both enzymatic and non-enzymatic reactivity [5,10,11]. Free radical-mediated oxidation of PUFA gives random, racemic products, while 15-LOX oxidizes AAs regio-, stereo-, and enantiospecifically [12,13].

Enzymatically introduced carbon-carbon double bonds in the fatty acid hydrocarbon chain are only in the cis configuration in the human body [14]. Trans-fatty acids (TFAs), that contain at least one double bond in the trans configuration, are known as side products of industrial oil processing and can enter the body through dietary consumption [15]. TFA intake has been associated with various pathologies including ischemic heart disease [16], Alzheimer [17], and bowel cancer [18]. On the other hand, TFAs are formed during metabolism by an endogenous free radical-catalyzed isomerization of natural cis lipids [19]. This process occurs independently of foods, as formerly demonstrated for mono-trans AA (mtAA) in rats fed a TFA-free diet [20]. In the last twenty years [15,19,21–23], thiyl radicals ($RS^{\bullet}$) were mainly individuated as

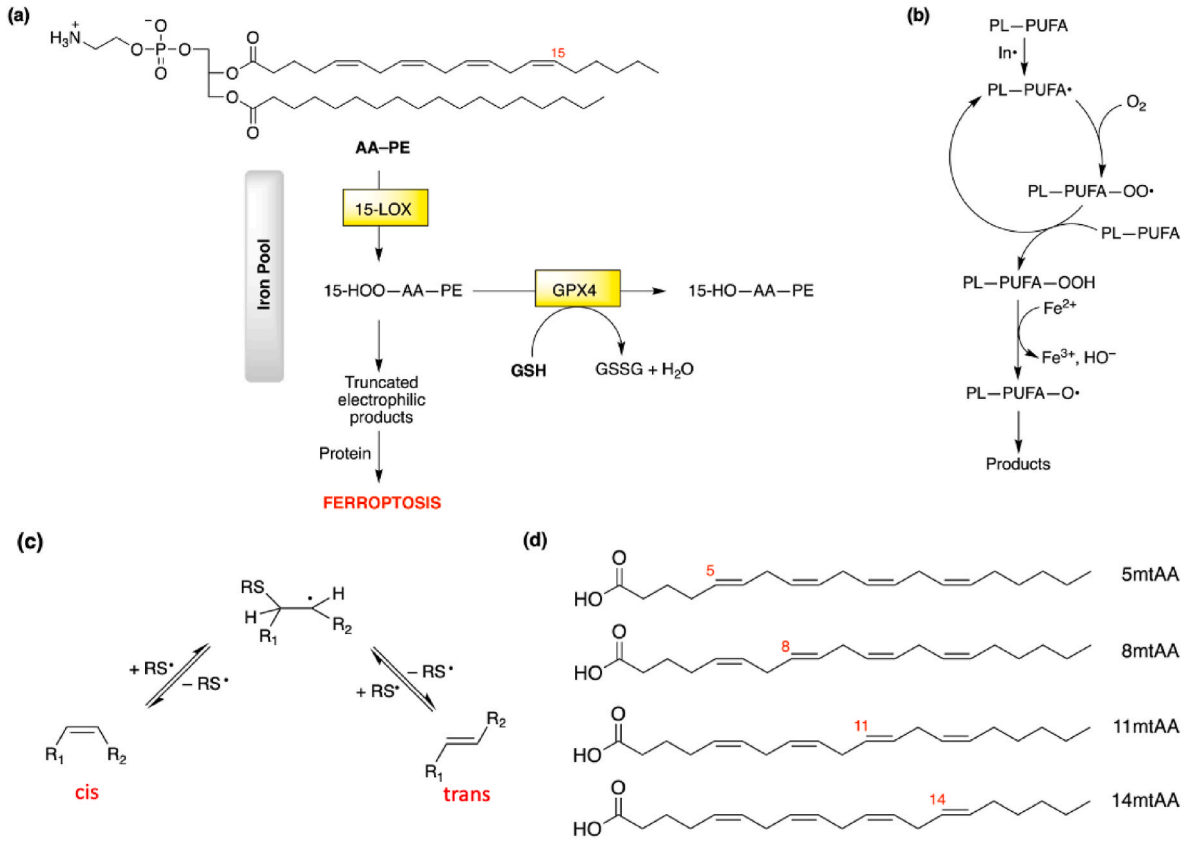

Fig. 1. (a) Schematic representation of enzymatic lipid peroxidation (LPO)-induced ferroptosis with its three molecular pillars: iron, thiols and polyunsaturated phospholipids. AA–PE: esterification of arachidonic acid (AA) into phosphatidylethanolamine (PE). The enzymatic AA–PE peroxidation yielding a specific hydroperoxide (15-HOO–AA–PE), a pro-ferroptotic metabolite. Glutathione peroxidase 4 (GPX4) uses glutathione (GSH) to reduce lipid peroxides with formation of the corresponding alcohols. 15-HOO–AA–PE is subsequently degraded to form adducts with target proteins, eventually leading to cell death. **(b)** Non-enzymatic mechanism of free radical-initiated lipid peroxidation (LPO) of polyunsaturated fatty acid (PUFA) moieties incorporated in phospholipids (PL), depicted as free radical chain reaction ($In^{\bullet}$, radical initiation). **(c)** Mechanism of thiyl radical catalyzed cis-trans double bond isomerization of unsaturated fatty acid moieties in phospholipids depicted as consecutive addition-elimination process. **(d)** The four monotrans isomers of arachidonic acid (mtAA) structures, as product mix obtained from the thiyl radical-catalyzed isomerization of AA.

responsible reactive species causing the endogenous TFAs formation [15,19,24], with the mechanism shown in Fig. 1c. In the case of AA, a mixture of four mtAA isomers is produced (Fig. 1d) [25,26]. They were synthetically obtained and such library of mtAA used as molecular standards to check their endogenous metabolic formation in cells [27,28], animals [29], and humans [27,28]. An example is the treatment of cells with bleomycin, an anticancer drug known to generate $RS^{\bullet}$, which induces cis–trans isomerization of unsaturated fatty acids in membrane phospholipids [29]. It is important to underline that gas chromatography (GC) is the gold standard methodology for lipid analysis concerning fatty acid structures and related modifications, in particular those without change of molecular mass. Synthetic TFA library expanded to eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) ensures accurate identification and calibration of the main mtPUFA isomers in various metabolic situations [26,27].

In this study, we performed a comprehensive analysis to examine the effect of PUFA and mtPUFA on various types of programmed cell deaths in cultured cells, identifying peculiar behavior of mtPUFA in ferroptosis. Indeed, while AA, EPA and DHA promoted a strong pro-ferroptotic activity by increasing lipid peroxidation, their corresponding mtPUFA did not. For the first time membrane remodeling was followed-up during RSL3-induced ferroptosis evidencing that mtAA formation occurs during increased lipid peroxidation, and the mtAA content in HT1080 cell membranes is similar to when these isomers are exogenously supplied at 20 μM in the medium. The fatty acid-based membrane lipidome analysis identified the complete membrane remodeling after 20 μM of PUFA and mtPUFA supplementations contributes to the scenario of this important membrane-dependent process.

2. Materials and Methods

2.1. Reagents

ATP, tunicamycin, and H_2O_2 were purchased from Wako (Tokyo, Japan). LPS was purchased from Invivogen (San Diego, CA, USA). Cycloheximide, (1*S*,3*R*)-RSL3, and ferrostatin-1 were purchased from Sigma-Aldrich (Burlington, MA, USA). TNF-α was purchased from Enzo Life Sciences (Farmingdale, NY, USA). Z-VAD-fmk was purchased from Peptide Institute (Osaka, Japan). Necrostatin-1 was purchased from Santa Cruz (Dallas, TX, USA). Rucaparib was purchased from Selleck Chemicals (Houston, TX, USA). 1-Methyl-3-nitro-1-nitrosoguanidine (MNNG) and doxorubicin were purchased from Tokyo Chemical Industry (Tokyo, Japan). phorbol 12-myristate 13-acetate (PMA), and nigericin were purchased from Santa Cruz (Dallas, TX, USA). MCC950, Deferoxamine, Triacsin C, all *cis*-AA, EPA and DHA were purchased from Cayman (Ann Arbor, MI, USA). Apocynin was purchased from Tocris Bioscience (Bristol, UK). Diphenyleneiodonium chloride (DPI) was purchased from Abcam (Cambridge, UK). Sapienic acid (6c-16:1), 8c-18:1 and sebaleic acid (5c, 8c-18:2) methyl esters were purchased from Lipidox (Lidingö, Sweden); cis and trans FAME were purchased from Merck (Darmstadt, Germany) and used without further purification; chloroform, methanol, isopropanol, diethyl ether and n-hexane were purchased from Baker (Phillipsburg, NJ, USA) (HPLC grade) and used without further purification. Silica gel analytical thin-layer chromatography (TLC) was performed on Merck silica gel 60 plates, 0.25 mm thickness, and spots were detected by spraying the plate with cerium ammonium sulfate/ammonium molybdate reagent.

2.2. Cell culture

HT1080 and mouse embryonic fibroblast (MEF) cells were cultured in Dulbecco's Modified Eagle Medium (Nakalai Tesque, Kyoto, Japan) containing 10% heat-inactivated fetal bovine serum (FBS, Nichirei Bioscience, Tokyo, Japan) and 1% penicillin-streptomycin solution (Nakalai Tesque) in 5% CO_2 at 37 °C. THP-1 cells were cultured in RPMI 1640 (Nakalai Tesque) containing 10% heat-inactivated FBS (Nichirei Bioscience), 1% penicillin-streptomycin solution, and Plasmocin (Invivogen) at 37 °C under a 5% CO_2 atmosphere. For experiments, THP-1 cells were differentiated for 3 h with 100 nM phorbol 12-myristate 13-acetate (PMA) on the day before stimulation.

2.3. Synthesis of monotrans polyunsaturated fatty acids (mtPUFA)

The synthesis of mono-trans isomers of arachidonic acid (mtAA), eicosapentaenoic acid (mtEPA), and docosahexaenoic acid (mtDHA), followed thiyl radical-catalyzed isomerization reaction starting from the commercially available all-cis PUFA methyl esters in alcohol solvent followed by isolation of mono-trans isomers mixture of the corresponding methyl esters and hydrolysis to free fatty acids. Under these conditions the preparation of mtPUFAs as free acids can reach the mg scale, thus fostering more applications to biochemical and biological studies. A detailed description of our protocol has been recently provided [30]. It is worth underlining that the four mtAA isomers (for structures see Fig. 1d) were isolated in high purity (>98%) and analyzed by GC to be in the following isomeric ratio: 5 mt: 8 mt + 11 mt: 14 mt = 35 : 36: 29. Similarly, mtEPA and mtDHA were obtained in high purity (>98%) and they consist of a mix of five and six isomers, respectively, in approximately equal amounts (for details see Ref. [1]).

2.4. Preparation and treatment of fatty acids

Fatty acids were prepared as described previously [31]. Briefly, fatty acids were dissolved in 0.1 N NaOH at 70 °C, and then conjugated with fatty acid-free BSA (Wako, pH 7.4) at 55 °C for 10 min to make 5 mM BSA-conjugated fatty acid stock solutions containing 10% BSA. Various concentrations of BSA-labeled fatty acids were added to the culture medium containing fetal bovine serum to achieve a final concentration of 1% BSA.

2.5. Phospholipid extraction and fatty acid analysis

HT1080 cells were seeded on 10 cm dish, and treated with or without 20 μM mtPUFAs and 10 μM RSL3 for 6 h. The method of phospholipid extraction and fatty acid analysis was described recently in some details [32] and followed the well-established Folch method [33]. Briefly, a sample of ca. 1×10^6 of HT1080 cells suspended in 1 mL PBS was added to 0.5 mL triple-distilled H_2O and centrifuged at 14,000 rpm for 5 min at 4 °C; lipid extraction was performed with 2:1 chloroform/methanol (4 × 4 mL). The organic layers were dried on anhydrous Na_2SO_4 and evaporated to dryness. The total lipid extracts (0.9–1.1 mg), composed by phospholipids and cholesterol analysis (by TLC n-hexane:diethylether 9:1) were converted to FAMEs by adding 0.5 M KOH in MeOH (0.5 mL); the transesterification reaction was stirred for 10 min and quenched by brine (0.5 mL). FAMEs were extracted with n-hexane (4 × 2 mL), dried on anhydrous Na_2SO_4, evaporated to dryness and analyzed by GC in comparison with standard references. The GC (Agilent 6850, Milan) was used in splitless mode, equipped with a 60 m × 0.25 mm × 0.25 μm (50%-cyanopropyl)-methylpolysiloxane column (DB23, Agilent, USA), and a flame ionization detector with the following oven program: temperature started from 165 °C, held for 3 min, followed by an increase of 1 °C/min up to 195 °C, held for 40 min, followed by a second increase of 10 °C/min up to 240 °C, and held for 10min. A constant pressure mode (29 psi) was chosen with helium as carrier gas. Methyl esters were identified by comparison with the retention times of authentic samples and trans fatty acid references obtained as described elsewhere [30,32,34].

2.6. Cell viability assay

Cell viability was assayed as described previously [35]. Briefly, cells were seeded on 96-well plates, and after stimulation/treatment, cell viability was determined using Cell Titer 96 Cell Proliferation Assay

(Promega, Madison, WI, USA), according to the manufacturer's protocol. The absorbance was read at 490 nm using a microplate reader (iMark microplate reader, Biorad). Data are normalized to control without stimulus, unless noted otherwise.

2.7. Cell death assay

Cell death was monitored by using LDH-Cytotoxic Test Kit (Wako) according to the manufacturer's protocol. The activity level of lactate dehydrogenase (LDH) released into the culture media was quantified as a percentage of the total activity level of LDH.

2.8. Enzyme-linked immunosorbent assay (ELISA)

IL-1β released to culture medium was measured by the IL-1β ELISA kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

2.9. Quantification of lipid peroxidation

Lipid peroxidation was assessed using a flow cytometer as described previously with modifications [36]. For measurement of lipid peroxidation, HT1080 cells were incubated with 1 μM Liperfluo (Dojindo, Kumamoto, Japan) for 30 min before collection. The fluorescence intensity of cells was measured by a flow cytometer (CytoFLEX, Beckman Coulter, Brea, CA, USA) with the excitation wavelength at 488 nm and the emission wavelength at 525 nm. Data were analyzed by CytoExpert (Beckman Coulter).

2.10. Statistics

All the values are expressed as means ± SD, and statistical analyses were performed using GraphPad Prism software (v.9.3.0). Two groups were compared using two-tailed Student's t-test. Multiple-group comparisons were conducted using either the one-way or two-way ANOVA analysis followed by Tukey-Kramer or Dunnett's test. NS, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (compared to control unless stated otherwise).

3. Results and discussion

3.1. PUFA and mtPUFA supplementations evaluating fatty acid-based membrane lipidome remodeling

We initially examined whether single treatment of PUFAs or mtPUFAs causes cytotoxicity in HT1080 and MEF cells. We chose the 20 μM concentration since a physiological concentration of PUFA in cells with a biological effect begins at around 5–10 μM and can reach a maximal response at 50–100 μM [37]. Furthermore, possibility for lipoapoptosis and formation of lipid droplets due to lipid accumulation can occur at higher concentrations. It is also known that AA concentrations of 50–100 μM could be toxic for many cell types [38]. Fig. 2a and b shows that there was no significant change in cell viability after 24 h of treatment with 20 μM of AA, mtAA, EPA, mtEPA, DHA or mtDHA in HT1080 and MEF cells, indicating also that mtPUFA behave as PUFA concerning cell viability.

We next performed the fatty acid-based membrane lipidome analysis of HT1080 cells treated with PUFAs or mtPUFAs at 20 μM for 6 h to investigate the fatty acid remodeling which is a natural process ensuring the membrane lipid turnover. On the basis of the above-mentioned viability tests, in 6 h cells are perfectly alive, therefore the metabolic turnover of fatty acids in cell membranes can be observed in the cultures at physiological levels of fatty acids provided by the supplementation, plus the lipids in the medium [37].

Comparing the control cells with those added with AA, no significant changes in the fatty acid composition of membrane phospholipids are detectable (Tables S1 and S2), except for a significant DGLA increase. This can be attributed to the negative metabolic feedback given by the supplemented AA, slowing down DGLA transformation in the omega-6 cascade (i.e., delta-5 desaturase enzyme to AA) and increasing DGLA availability for insertion in the membrane phospholipids. It is interesting to evaluate the effect of omega-3 EPA and DHA supplementations (Tables S1 and S2) since in both cases the fatty acid remodeling revealed a clear inhibition of delta-9 desaturase metabolism, with significant diminution of MUFA (9c-16:1, 9c-18:1, 11c-18:1). The interference of omega-3 with delta-9 desaturase (i.e., stearoyl-CoA desaturase) has been reported in breast cancer patients [39]; however, in the case of tumoral cell cultures this interference has not yet fully assessed, whereas the interference with delta-6 and delta-5 desaturase enzymes is much more known, since they are directly involved in PUFA metabolism [40]. As a consequence of the MUFA decrease, the SFA/MUFA ratio was significantly increased in the cell membrane of EPA- and DHA-treated cultures. Both EPA and DHA had significant incorporation in membrane phospholipids in comparison with AA that did not increase its level in membrane lipids. Besides, in the case of DHA, the total trans isomers were increased significantly.

Supplementation of mtPUFA gave distinct results of membrane remodeling compared to the above-described PUFA supplementation without affecting cell viability as in the case of PUFA (Tables S3 and S4). This is the first time that membrane lipidome remodeling is compared for the three main PUFA and their geometrical mtPUFA isomers. With 20 μM mtAA, this isomer was incorporated in the membrane phospholipid after 6 h (0.26 ± 0.10, increased significantly vs controls, see Table 1) accompanied by a significant increase of SFA. In the supplementations of mtEPA and mtDHA, the total SFA increase and MUFA decrease are significant compared to the control. Particularly in the case of mtDHA, significant increase in the omega-6 PUFA (basically, due to more linoleic acid incorporation) and decrease in the omega-3 PUFA

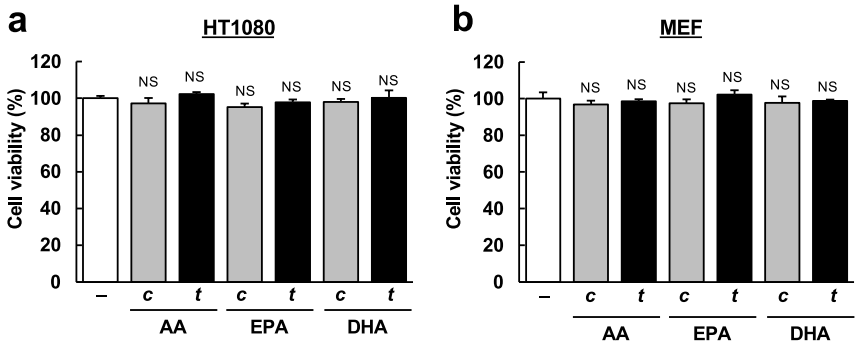

Fig. 2. PUFAs or mtPUFAs are not cytotoxic. HT1080 (**a**) and MEF (**b**) cells were treated with the indicated fatty acids for 24 h at 20 μM, and assayed for cell viability. Data are shown as mean ± SD (n = 3). NS: not significant (vs control without fatty acid). *c*, PUFA; *t*, mtPUFA.

Table 1
Trans-fatty acids of PUFA residues quantified by GC analysis (% rel. quant.) measured under PUFA and mtPUFA supplementation (20 μM for 6 h).

FAME[a]	control	+AA	+EPA	+DHA	+mtAA	+mtEPA	+mtDHA
mtAA[b]	0.05 ± 0.03	0.15 ± 0.04	0.11 ± 0.02	0.12 ± 0.05	0.26 ± 0.10[e]	c	0.04 ± 0.03
mtEPA[b]	–	–	–	–	–	0.84 ± 0.13[f]	–
mtDHA[b]	–	–	–	0.14 ± 0.05 [d]	–	–	0.75 ± 0.07 [g]

[c] Traces; Significance of comparisons between control and treated cells.
[d] vs. control, p = 0.008.
[e] vs. control, p = 0.04.
[f] vs. control, p = 0.0004.
[g] vs. control, p = 0.0001.
[a] Quantified by GC as fatty acid methyl ester (FAME) and represented as mean ± SD (n = 3) (taken from Tables S1 and S3.
[b] mt, mix of monotrans isomers.

(DPA and DHA) were observed as well. Importantly, both mtEPA and mtDHA were significantly incorporated in membrane phospholipids (see Table 1). In Table 1, we summarized the mtPUFA incorporation in membranes upon PUFA and mtPUFA supplementations comparing with controls. Fatty acid levels in membranes are quantified and expressed as relative percentage (% rel. quant.) over the whole FAME recognized in the analysis (cf., Supplementary Data). In controls, only mtAA was present in traces (0.05%). With 20 μM of mtAA, mtEPA and mtDHA, the incorporation of these isomers occurred (0.26, 0.84 and 0.75, respectively).

From these results, a first indication can be obtained on possible inhibitory effects of TFA for desaturase enzymes. In literature, TFA coming from hydrogenated foods or from MUFA are known to influence this important lipid metabolism [41]. For mtPUFA there are no specific studies yet, and we can hypothesize that delta-9 desaturase metabolism of palmitic acid toward vaccenic acid (palmitic→ palmitoleic→vaccenic) can be impaired, as it is reported for elaidic acid (9t-18:1, the trans isomers of oleic acid) in HepG2 cell lines [42]. In our previous work we showed that elaidic acid supplementation has a profound effect on gene and protein regulations, therefore more insights are needed on "-omics" profiles that can depend on the specific TFA used. It is worth mentioning that both the increase of SFA/MUFA ratio and the trans isomer incorporation have immediate effects on the structural organization of membrane phospholipids, the molecular packing and cholesterol incorporation, with a tendency of decreasing the fluidity, reducing calcium influx [43,44].

3.2. Phenotypic screen on various types of programmed cell death for identifying a mtAA-specific role in ferroptosis

We previously demonstrated that food-derived TFAs, such as elaidic acid and linolelaidic acid (LEA), facilitated DNA damage-induced apoptosis by augmenting generation of mitochondrial ROS and subsequent activation of c-jun N-terminal kinase (JNK) [31]. To test whether mtPUFAs also promote DNA damage-induced apoptosis, we pretreated HT1080 cells with 20 μM mtAA followed by treatments with the DNA-damaging reagent doxorubicin, and then assessed cell viability. Comparison with AA was provided. As shown in Fig. 3a, no effect on cell death induced by doxorubicin was seen, indicating that mtAA does not have a pro-apoptotic role in DNA damage-induced cell death as it occurs with elaidic acid [31]. We also tested tunicamycin, a typical ER stress inducer well known to trigger apoptosis [45], observing again any significant effect of mtAA on tunicamycin-induced cell death (Fig. 3b). There are two major pathways in apoptosis, i.e., the intrinsic and extrinsic pathways: (i) intrinsic pathway is activated in response to stimuli causing mitochondrial damage, such as DNA damage (Fig. 3a) and ER stress (Fig. 3b), which leads to release of cytochrome *c* that induces formation of a complex, called apoptosome, to activate caspases; (ii) extrinsic pathway is triggered by activation of death receptors on the cell surface, including tumor-necrosis factor receptor (TNFR), in response to their ligands, leading to the formation of death-inducing signal complexes and activation of caspases [46]. The effect of mtAA on apoptosis through an extrinsic pathway was tested by TNF-induced apoptosis in mouse embryonic fibroblast (MEF) cells. We found that 20 μM of mtAA did not differ from AA and did not significantly affect cell death upon treatment of TNF-α along with cycloheximide (CHX), commonly used in combination with TNF-α to effectively induce apoptosis by suppressing nuclear factor-κB (NF-κB)-dependent cell survival signal (Fig. 3c) [36]. Taken together, these results suggest that mtAA does not promote apoptosis, similarly to AA.

We next sought to determine the role of mtAA in other types of programmed cell death, such as necroptosis, parthanatos, and pyroptosis. To this end, we treated MEF cells with TNF-α+CHX + Z-VAD and HT1080 cells with MNNG, which are typical stimuli used for inducing necroptosis and parthanatos, respectively [47,48]. However, no significant effect of mtAA was observed in both conditions, as in the case of AA. Specific inhibitors for necroptosis, necrostatin-1 (Nec-1), and parthanatos, rucaparib (Ruc), clearly prevented cell death (Fig. 3d and e). We also tested nigericin to induce pyroptosis, a pro-inflammatory necrotic cell death triggered by activation of NLRP3 inflammasomes [49], in a human monocytic cell line THP-1, differentiated into macrophage-like cells by PMA treatment, which is commonly utilized to effectively induce NLRP3 inflammasome activation in response to various stimuli including nigericin (K^+ ionophore). Whereas a specific inhibitor for NLRP3, MCC950, abolished nigericin-induced lytic cell death (Fig. 3f) and IL-1β release (Fig. 3g), mtAA did not have any effect on both. These results collectively suggest that mtAA does not affect non-apoptotic cell death pathways, including necroptosis, parthanatos, and pyroptosis, similarly to AA.

Interestingly, when HT1080 cells were treated with 100 μM H_2O_2, we found a remarkable difference in cell viability between AA-pretreated cells and mtAA-pretreated cells; while AA markedly promoted cell death, mtAA hardly affected it (Fig. 3h). Ferroptosis is triggered by peroxidation of membrane phospholipids that contain PUFAs, including AA, susceptible to attack by ROS due to the existence of multiple bis-allylic hydrogen atoms, which can be blocked by lipophilic antioxidants, such as ferrostatin-1 (Fer-1) [3,4]. As expected, Fer-1 cancelled the AA-dependent increase in H_2O_2-induced cell death but no differences with and without Fer-1 were detected for mtAA (Fig. 3h), suggesting that mtAA does not synergize with H_2O_2 to induced ferroptotic cell death. In the light of the membrane lipidome changes observed with mtAA supplementation (Tables S3 and S4), it can be hypothesized that the higher SFA content in cell membrane phospholipids alters the membrane organization and functionality thus rendering the cells more resistant to the H_2O_2 oxidative effect.

3.3. mtPUFAs do not harbor pro-ferroptotic activities as do PUFAs when repair systems are impaired

Glutathione peroxidase 4 (GPX4) is primarily responsible for eliminating lipid peroxides to maintain cell survival by constitutively converting toxic hydroperoxidized phospholipids to non-toxic hydroxyl

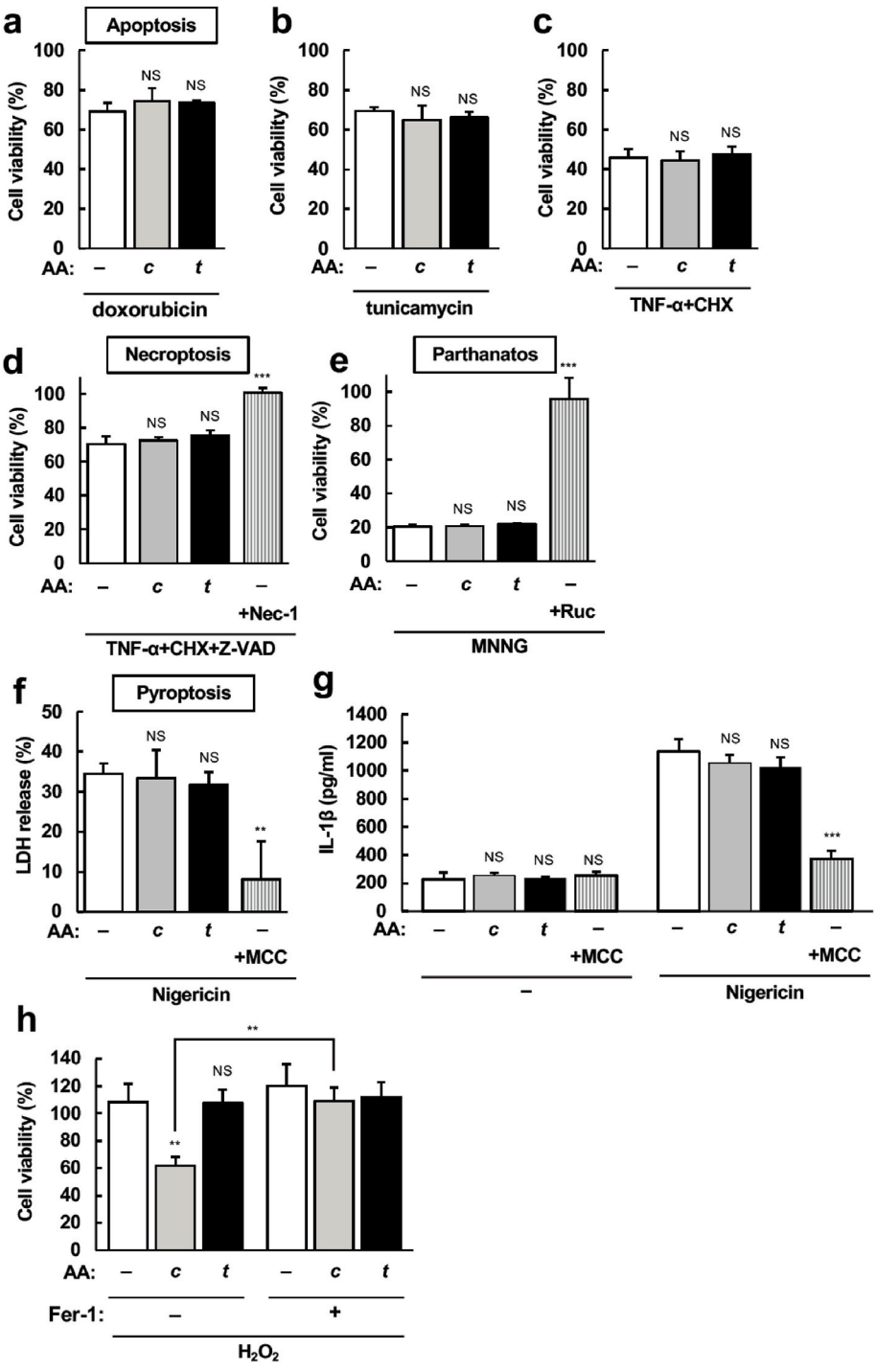

Fig. 3. Phenotypic screen for identifying a mtAA-specific effect on various types of programmed cell death; all data are shown as mean ± SD (n = 3); NS: not significant (vs control without fatty acid supplement). **(a, b)** HT1080 cells were pretreated with 20 μM AA (*c*) or mtAA (*t*) for 6 h, treated with 0.25 μg/mL doxorubicin or 20 μg/mL tunicamycin for 24 h, and assayed for cell viability. **(c, d)** MEF cells were pretreated with 20 μM AA (*c*) or mtAA (*t*) for 6 h; in figure **c**, cells were treated with 10 μg/mL CHX for 0.5 h, while in figure **d**, cells were treated with 10 μg/mL CHX, 20 μM Z-VAD and 10 μM Nec-1 for 0.5 h; in both cases, cells were subsequently stimulated with 50 ng/mL TNF-α for 12 h, and assayed for cell viability. **(e)** HT1080 cells were pretreated with 20 μM AA (*c*) or mtAA (*t*) for 6 h, treated with or without 1 μM Rucaparib for 0.5 h, stimulated with 20 μM MNNG for 12 h, and assayed for cell viability. **(f, g)** PMA-differentiated THP-1 cells were pretreated with 20 μM AA (*c*) or mtAA (*t*) for 20 h, treated with 1 μM MCC950 for 0.5 h, and then stimulated with 20 μM nigericin for 2 h; cell cytotoxicity was measured by LDH release assay, figure **f**, and IL-1β release was analyzed by ELISA, figure **g**. **(h)** HT1080 cells were pretreated with 20 μM AA (*c*) or mtAA (*t*) for 6 h, treated with 100 μM H_2O_2 in the presence or absence of 5 μM ferrostatin-1 (Fer-1), and assayed for cell viability.

phospholipids at the expense of reduced glutathione; inactivation of GPX4 causes accumulation of lipid peroxides, leading to ferroptosis [5]. (1*S*,3*R*)-RSL3 is a direct GPX4 inhibitor, most commonly used for ferroptosis studies [7]. We were interested to follow-up the effect of PUFA addition since it can cause membrane remodeling with effects such as membrane fluidity increase and lipid peroxidation, which are known to increase cell sensitivity to GPX4 inhibition and ferroptosis, as previously reported in the experiments using LNCaP cell lines treated with this compound [50].

To further characterize the role of mtPUFAs in ferroptosis, we first examined the pro-ferroptotic activity of AA and mtAA. Before analysis, we tested dose response of HT1080 cells to RSL3. Treatment of HT1080 cells with 1 μM RSL3 (sub-lethal dose) for 6 h hardly affected cell viability, and using 2 μM RSL3 (minimal lethal dose) a marginal effect was detected (Fig. 4a). Of note, while 1–2 μM RSL3 strongly induced cell death in HT1080 cells in the previous reports [3], it caused none or only mild cell death in this study due to BSA dissolved in the medium used for ensuring efficient cellular uptake of PUFAs (see Materials and Methods). As single treatment of AA or mtAA did not induce ferroptosis (Fig. 2a), we pretreated HT1080 cells with 20 μM AA (*c*) or mtAA (*t*) for 6 h and then treated with RSL3 at 1 or 2 μM, to assess pro-ferroptotic effect in a condition where GPX4 activity is mildly reduced. Fig. 4b shows that in both RSL3 concentrations AA pretreatment substantially decreased cell viability, in line with previous reports [51], whereas mtAA pretreatment

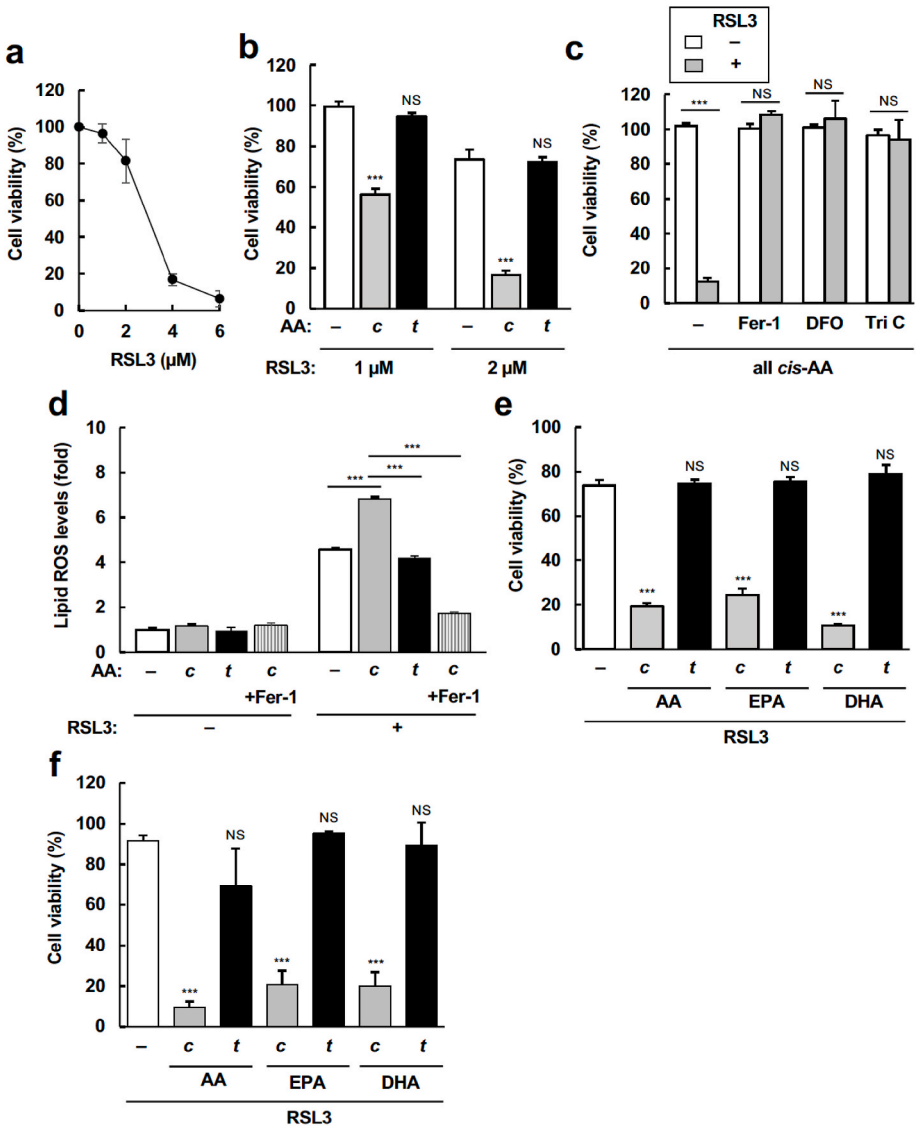

Fig. 4. mtPUFAs do not harbor pro-ferroptotic activities as do PUFAs. **(a)** HT1080 cells were pretreated with 1% BSA in the absence of fatty acid, and then treated with the indicated concentrations of RSL3 for 24 h, assayed for cell viability. Data are shown as mean ± SD (n = 3). **(b)** HT1080 cells were pretreated with 20 μM AA (*c*) or mtAA (*t*) for 6 h, treated with RSL3 at 1 μM or 2 μM, and assayed for cell viability. Data are shown as mean ± SD (n = 3). **(c)** HT1080 cells were pretreated with 20 μM AA (*c*) for 6 h, treated with 5 μM ferrostatin-1 (Fer-1), 100 μM deferoxamine (DFO), or 5 μM Triacsin C (Tri C) for 30 min, and then stimulated with 2 μM RSL3 for 24 h, subsequently assayed for cell viability. Data are shown as mean ± SD (n = 3). **(d)** HT1080 cells were pretreated with 20 μM AA (*c*) or mtAA (*t*) for 6 h, treated with or without 5 μM ferrostatin-1 (Fer-1) for 30 min, and then stimulated with 2 μM RSL3 for 4 h. Lipid ROS level was assessed by a flow cytometer after treatment of 1 μM Liperfluo for 30 min, and represented as fold change of fluorescence intensity (mean ± SD, n = 3). **(e)** HT1080 cells were pretreated with 20 μM PUFAs (*c*) or mtPUFAs (*t*) for 6 h, treated with 2 μM RSL3, and assayed for cell viability. Data are shown as mean ± SD (n = 3). **(f)** MEF cells were pretreated with 20 μM PUFAs (*c*) or mtPUFAs (*t*) for 6 h, treated with RSL3 at 0.7 μM for 24 h, and assayed for cell viability. Data are shown as mean ± SD (n = 3).

did not affect cell viability compared to the control. We also confirmed that AA-dependent reduction in cell viability was reversed in the presence of either Fer-1, deferoxamine (DFO, an iron chelator) [3], or Triacsin C (Tri C, a long-chain fatty acyl-CoA synthetase inhibitor) [51], commonly used ferroptosis inhibitors (Fig. 4c).

Next, we considered lipid ROS production (as detected by changes of fluorescence intensity) [52] in the absence and presence of RSL3. Fig. 4d (left side) shows that the levels of lipid ROS are low and similar when the HT1080 cells are pretreated with 20 μM AA (*c*) or mtAA (*t*) for 6 h, or when in the case of AA are further treated with 5 μM ferrostatin-1 (Fer-1) for 30 min. Fig. 4d (right side) shows a different picture of lipid ROS production upon 2 μM RSL3 treatment for 4 h. The level of lipid ROS increase of 4-fold in the control and 7-fold in the cells pretreated with 20 μM AA (*c*). On the other hand, the analogous experiment with 20 μM mtAA (*t*) shows lipid ROS similar to control. The enhanced lipid ROS level by addition of AA was cancelled by the treatment of 5 μM Fer-1. These results collectively suggest that mtAA does not possess pro-ferroptotic activity as does AA.

Fig. 4e illustrates that the omega-3 PUFA, like EPA and DHA, behave similarly to the omega-3 AA. Indeed, EPA and DHA had potent pro-ferroptotic activities whereas mtEPA and mtDHA did not affect cell viability compared to the control. We also repeated similar experiments in mouse embryonic fibroblast (MEF) cells and found similar results, i.e., PUFAs had potent pro-ferroptotic activities where no pro-ferroptotic activities were observed for mtPUFAs (Fig. 4f). We demonstrated for the first time that mtAA, mtEPA and mtDHA do not have a pro-ferroptotic effect as their corresponding all cis-isomers do when the cellular repair system by GPX4 is impaired. In the case of mtDHA and mtEPA they are present in natural oils treated by industrial processes such as deodorization, and can be incorporated in liver mitochondria of rats fed with processed fish oils [53,54]. So far, no evidence of their endogenous production in the human body is provided. On the other hand, mtAA isomers have been well characterized as endogenously produced TFA [19,55], starting from the seminal work reporting mtAA in tissues and plasma of rats fed a TFA-free diet [20].

3.4. *Lipid peroxidation facilitates cis-trans isomerization of AA possibly leading to NOX activity inhibition*

Lipid peroxidation could be triggered not only by ROS but also by reactive sulfur and nitrogen species, which are also known as potent inducers of cis-trans isomerization of PUFAs, including AA (see Fig. 1c and d) [15,19]. Therefore, we speculated that mtAA might be produced during lipid peroxidation upon RSL3 treatment. It is known that lipid peroxidation and isomerization can occur together [56,57], and an example is draw in Fig. 5. In order to understand better the influence of

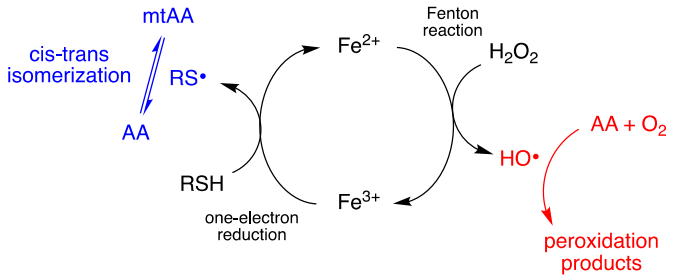

Fig. 5. Example of peroxidation and isomerization processes occurring under Fenton-like conditions to double bond AA moieties in the presence of thiol RSH.

AA and mtAA under GPX4 inhibition, we performed the fatty acid-based membrane lipidome analysis of HT1080 cells treated with RSL3 in the absence or presence of mtAA, having for the first time an insight of the fatty acid remodeling, which is a natural process ensuring the membrane lipid turnover.

We first examined the dose and treatment time of RSL3 to find an optimal condition for lipidomic analysis. As shown in Fig. S1, half of the cells died after 6 h of RSL3 treatment at 10 μM along with 1% BSA, and we, thus, determined to perform analysis under this condition, when the majority of cells are in the midst of lytic cell death. Subsequent fatty acid-based lipidomic analysis showed that RSL3 treatment significantly increased cellular mtAA level to the same extent as single treatment of 20 μM mtAA (Fig. 6a). On the other hand, the influence of mtAA formation on ferroptosis is supported by the fact that AA, mtAA and controls all give some ROS levels during RSL3 treatment (Fig. 4d, right side), and under this circumstances thiyl radical production occurs, triggering the catalytic cycle of the isomerization process, as shown in Fig. 5. Indeed, cellular mtAA level is higher (~0.6%) in the experiment where RSL3 and mtAA are together. It is also worth underlining at this point that mtAA has been reported to repress NADPH oxidase (NOX) activity by directly interacting with p67phos, one of the components of the NOX complex, whereas AA rather activates NOX [58]. Given the importance of NOX as a source of ROS to facilitate lipid peroxidation during ferroptosis [59], we propose that the distinct effects on NOX activity of monotrans and all cis-AAs could account for their different roles in ferroptotic conditions. As shown in Fig. 6b, NOX inhibitors, including diphenyleneiodonium chloride (DPI) and apocynin (Apo), strongly suppressed AA-mediated enhancement of RSL3-induced cell death; therefore, it cannot be ruled out that mtAA increase, as a consequence of lipid ROS and thiyl radical production, suppresses ferroptosis through inhibition of NOX activities.

The role of other membrane fatty acid components, changing with the membrane remodeling, must be also remarked. In the case of RSL3 alone, PUFA (omega-6 and omega-3) increased, with the remodeling effect of SFA diminution, as expected for the consequent ferroptosis outcome (Tables S5 and S6). When 20 μM of AA or mtAA pre-treatment was performed before RSL3, the same membrane remodeling of RSL3 alone, i.e., SFA diminution and PUFA increase (omega-6 and omega-3), was observed. However, in the presence of RSL3 a higher incorporation of mtAA was detected compared with mtAA alone (i.e., 0.61 vs 0.21, respectively), and the total trans fatty acids content reached 1% of the total fatty acid quantities in membranes (Fig. 6a and Table S6).

In the experiments of RSL3 with mtAA, also the levels of AA in membrane phospholipids increased (Table S5), indirectly indicating its higher presence in the lipid pool, possibly due to the rescue from peroxidation process. It is also worth noting that, when AA and EPA (as well as with their mt isomers) are used without RSL3, no increases of these PUFA were observed, but significant increases were seen with DHA and mtDHA (Tables S1 and S3, respectively). This is an interesting difference of PUFA effects, to be deepened in further studies distinguishing AA, EPA and DHA for specific biological, biophysical and physiological effects [60–62]. The mtEPA and mtDHA pre-treatments gave the same effect of mtAA for inhibiting RSL3-induced ferroptosis; however, the remodeling was completely different, as previously discussed, with significant total SFA increase and MUFA decrease together with total TFA of 1.25 and 1.11, respectively. Intriguingly, we have recently reported that lipid peroxidation increases cell volume and membrane tension, which opens mechanosensitive channels including Piezo1 and induces sodium influx and potassium efflux due to their ion concentration gradients, thereby promoting ferroptosis [52]. SFA have been shown to suppress Piezo1 gating and channel activity by increasing membrane bending stiffness and decreasing membrane fluidity [63]. Therefore, SFA increase observed when cells were treated with mtPUFAs, but not with PUFA, may also contribute to ferroptosis suppression in synergy with mtPUFA.

Due to the precise analysis of each mtAA isomer by GC, we could also examine in detail the regiochemistry of the fatty acid isomerization process. Due to the supramolecular disposition of the hydrophobic fatty acid chains in membrane phospholipids, it can be expected that the free radicals diffuse and reach the double bond closer to the carbonyl group with a higher probability than the other three double bond positions, as demonstrated in biomimetic models [26,64], as well as in cell [29] and murine models [20]. Indeed, 5mtAA resulted to be the prevalent isomer formed in membranes of HT1080 cells triggered by RSL3 (Table 2). It is interesting to observe that the mtAA isomers ratio in HT1080 membranes reflected the supplemented isomer mix (i.e., 35:36:29, see section

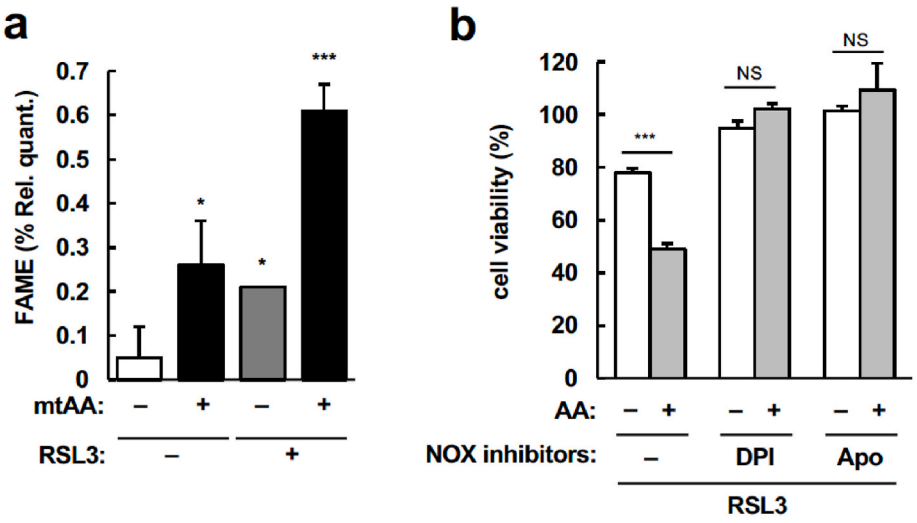

Fig. 6. Lipid peroxidation facilitates *cis*-trans isomerization of AA and ferroptosis in a NOX-dependent manner. **(a)** HT1080 cells were pretreated with 20 μM mtAA and/or 10 μM RSL3 for 6 h. Cells were then collected and subjected to fatty acid-based lipidomic analysis. Data are shown as mean ± SD (n = 3). **(b)** HT1080 cells were pre-treated with 20 μM all *cis*-AA for 6 h, treated with 5 μM DPI or 100 μM apocynin (Apo) for 30 min, and then stimulated with 2 μM RSL3 for 6 h, subsequently assayed for cell viability. Data are shown as mean ± SD (n = 3).

Table 2
Total mtAA formation (% rel. quant.) and mtAA isomeric distribution (% rel) quantified by GC analysis in the experiments with RSL3.

Sample	Total mtAA	5 mt:8 mt + 11 mt:14 mt
control	0.05	29:39:32
+RSL3	0.21	53:25:22
+mtAA[a]	0.26	36:34:30
+RSL3+mtAA[a]	0.61	45:30:24

[a] The composition of mtAA supplementation determined by GC analysis gave the following percentual isomeric distribution 5 mt:8 mt + 11 mt:14 mt = 35:36:29.

2.3.) in the absence of RSL3, whereas in case of (RSL3 and RSL3+mtAA), the total mtAA content increased and the prevalence of 5mtAA over the other mtAA isomers is evident.

More details of the mt-isomers incorporation in specific lipid classes of phospholipids (PC, PE) will be important to understand the ferroptosis inhibition. Further work, using knowledge of the trans-PUFA library and corresponding analytical protocols developed by our group, will contribute to fully understand the role of geometrical isomerism and their biological effects.

Pro-ferroptotic effect of AA was blocked in the presence of NOX inhibitors, such as DPI and Apo (Fig. 6b), implying a significant role of NOX in the enhancement of ferroptosis by AA. It is of note that more mtAA was incorporated into cells by exogenous addition than AA (% rel quant: AA vs mtAA = 0.15 vs 0.26, cfr. Table S1 and Table S3) and that the amount of incorporated mtEPA and mtDHA (% rel quant: 0.84 and 0.75) was much higher than mtAA (Table 1), indicating that in the time frame of the 6 h of our experiments exogenously added mtPUFAs can enter membrane acylation/reacylation remodeling cycle (Lands's cycle) influencing membrane contents more than AA supplementation, blocking one of the most potent pro-ferroptotic fatty acids. Importantly, it has been reported that PUFAs possess a potency to activate NOXs [65], whereas mtAA directly interacts with p67phox subunit of NOXs and functions as suppressor [58], differently from PUFA which are known to potentiate NOX activity [66].

Our results give complementary information to those of a recent report on PUFA structures modified at the bis-allylic positions with deuterium, instead of hydrogen, that display anti-ferroptotic effect in the same conditions used in our experiments, i.e., HT1080 cell lines and 20 μM d_6-AA, d_6-EPA and d_6-DHA [67]. The deuterated compounds are exclusively obtained by synthesis and cannot simulate naturally occurring processes. Instead, in our case, mtPUFA can be formed by radical-based mechanism triggered by the iron cycle, as shown in Fig. 5, accompanying lipid peroxidation, thus offering a novel pathway in the scenario so far known for ferroptosis.

4. Conclusions

Our results confirm a more complex scenario for ferroptosis than actually believed. While LPO processes are active, the importance of environmental lipid levels and balance among SFA, MUFA and PUFA in lipid pools and membrane phospholipids is crucial to the occurrence of cell death by ferroptosis [68,69]. Additionally, when in the PUFA pool there are mtPUFA, whose production and levels depend on the redox balance and thiyl radical production during Fenton process [70], the possible interference with peroxidation inducers, such as NOX, can occur, thus slowing down degradative pathways and allowing cell rescue. Further work will be needed to completely unveil the scenario of mtPUFA in ferroptosis, also deepening the different AA, EPA and DHA metabolism or incorporation for the final balance of lipid classes, such as phospholipids containing ethanolamine or sphingolipids [71,72]. We also showed that there is another element that is synergically crucial for an anti-ferroptosis effect, i.e., the membrane remodeling with SFA increase in the phospholipids, such as described in the palmitic acid-rich diets inhibiting ferroptosis [73], which is known to change membrane fluidity and oxidation sensitivity, rendering this compartment more resistant to stress. Our results integrate the known paradigm of the PUFA enrichment in membrane phospholipids to render cells susceptible to ferroptosis, also through the activation of desaturase enzymes [12], with the new aspect of the integrity of the natural cis configuration of PUFA double bonds and its conversion to the trans geometry by intervention of thiyl radicals. In conclusion, we advance the hypothesis of "resistance" to ferroptosis induced by the lipid peroxidation microenvironment based on the membrane enrichment of the combined SFA and TFA during remodeling.

Funding sources

This work was supported by JSPS/MEXT KAKENHI Grant Numbers JP20K07011, JP20KK0361 (Y·H.); JP21K19325, JP21H02620 and JP21H00268 (A.M.). This work was also supported by Mitsubishi Foundation, the Fugaku Trust for Medicinal Research, Takeda Science Foundation, and Lotte Research Promotion Grant. This research was also funded by Fondazione Di Bella, Bologna, Italy (C·F.) and by Lipinutragen srl, Bologna, Italy (C·C.) for a research grant to F·V.

Author's contributions

Y·H., C·C., A.M. and C.F. designed experiments, Y·H., Y·Y., A.I., A.S., F·V., W.S. and S.T. performed and analyzed experiments, Y·H., C·C., T. N., A.M. and C.F. interpreted results, Y·Y, C·C., A.M. and C.F. wrote the manuscript. All authors read and approved the final manuscript.

Declaration 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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.freeradbiomed.2023.05.026.

References

[1] J.F.R. Kerr, A.H. Wyllie, A.R. Currie, Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics, Br. J. Cancer 26 (1972) 239–257.
[2] D. Tang, R. Kang, T.V. Berghe, P. Vandenabeele, G. Kroemer, The molecular machinery of regulated cell death, Cell Res. 29 (2019) 347–364.
[3] S.J. Dixon, K.M. Lemberg, M.R. Lamprecht, R. Skouta, E.M. Zaitsev, C.E. Gleason, D.N. Patel, A.J. Bauer, A.M. Cantley, W.S. Yang, B. Morrison, B.R. Stockwell, Ferroptosis: an iron-dependent form of nonapoptotic cell death, Cell 149 (2012) 1060–1072.
[4] B.R. Stockwell, Ferroptosis turns 10: emerging mechanisms, physiological functions, and therapeutic applications, Cell 185 (2022) 2401–2421.
[5] H. Bayir, T.S. Anthonymuthu, Y.Y. Tyurina, S.J. Patel, A.A. Amoscato, A. M. Lamade, Q. Yang, G.K. Vladimirov, C.C. Philpott, V.E. Kagan, Achieving life through death: redox biology of lipid peroxidation in ferroptosis, Cell Chem. Biol. 27 (2020) 387–408.
[6] J.-Y. Lee, W.K. Kim, K.-H. Bae, S.C. Lee, E.-W. Lee, Lipid metabolism and ferroptosis, Biology 10 (2021) 184.
[7] W.S. Yang, R. SriRamaratnam, M.E. Welsch, K. Shimada, R. Skouta, V. S. Viswanathan, J.H. Cheah, P.A. Clemons, A.F. Shamji, C.B. Clish, L.M. Brown, A. W. Girotti, V.W. Cornish, S.L. Schreiber, B.R. Stockwell, Regulation of ferroptotic cancer cell death by GPX4, Cell 156 (2014) 317–331.
[8] X. Jiang, B.R. Stockwell, M. Conrad, Ferroptosis: mechanisms, biology and role in disease, Nat. Rev. Mol. Cell Biol. 22 (2021) 266–282.
[9] C. Bartolacci, C. Andreani, Y. El-Gammal, P.P. Scaglioni, Lipid metabolism regulates oxidative stress and ferroptosis in RAS-driven cancers: a perspective on cancer progression and therapy, Front. Mol. Biosci. 8 (2021), 706650.
[10] D.A. Stoyanovsky, Y.Y. Tyurina, I. Shrivastava, I. Bahar, V.A. Tyurin, O. Protchenko, S. Jadhav, S.B. Bolevich, A.V. Kozlov, Y.A. Vladimirov, A.A. Shvedova, C. C. Philpott, H. Bayir, V.E. Kagan, Iron catalysis of lipid peroxidation in ferroptosis: regulated enzymatic or random free radical reaction? Free Radic. Biol. Med. 133 (2019) 153–161.

[11] M.K. Foret, R. Lincoln, S. Do Carmo, A.C. Cuello, G. Gonzalo Cosa, Connecting the "dots": from free radical lipid autoxidation to cell pathology and disease, Chem. Rev. 120 (2020) 12757–12787.
[12] H. Yin, L. Hu, N.A. Porter, Free radical lipid peroxidation: mechanisms and analysis, Chem. Rev. 111 (2011) 5944–5972.
[13] E. Niki, Lipid peroxidation, in: C. Chatgilialoglu, A. Studer (Eds.), Encyclopedia of Radicals in Chemistry, Biology and Materials, vol. 3, Wiley, Chichester, UK, 2012, pp. 1577–1597.
[14] J.G. Wallis, J.L. Watts, J. Browse, Polyunsaturated fatty acid synthesis: what will they think of next? Trends Biochem. Sci. 27 (2002) 467–473.
[15] C. Chatgilialoglu, C. Ferreri, M. Melchiorre, A. Sansone, A. Torreggiani, Lipid geometrical isomerism: from chemistry to biology and diagnostics, Chem. Rev. 114 (2014) 255–284.
[16] D. Mozaffarian, M.B. Katan, A. Ascherio, M.J. Stampfer, W.C. Willett, Trans fatty acids and cardiovascular disease, N. Engl. J. Med. 354 (2006) 1601–1613.
[17] M.C. Morris, D.A. Evans, J.L. Bienias, C.C. Tangney, D.A. Bennett, N. Aggarwal, J. Schneider, R.S. Wilson, Dietary fats and the risk of incident alzheimer disease, Arch. Neurol. 60 (2003) 194–200.
[18] M.L. Slattery, J. Benson, K.-N. Ma, D. Schaffer, J. Potter, Trans-fatty acids and colon cancer. D, Nutr. Cancer 39 (2001) 170–175.
[19] W.-L. Hung, L.S. Hwang, F. Shahidi, M.-H. Pan, Y. Wang, C.-T. Ho, Endogenous formation of trans fatty acids: health implications and potential dietary intervention, J. Funct.Foods 25 (2016) 14–24.
[20] L. Zambonin, C. Ferreri, L. Cabrini, C. Prata, C. Chatgilialoglu, L. Landi, Occurrence of trans fatty acids in rats fed a trans-free diet: a free radical-mediated formation? Free Radic. Biol. Med. 40 (2006) 1549–1556.
[21] C. Chatgilialoglu, C. Ferreri, Trans lipids: the free radical path, Acc. Chem. Res. 38 (2005) 441–448.
[22] C. Chatgilialoglu, C. Ferreri, M. Guerra, A. Samadi, V.W. Bowry, The reaction of thiyl radical with methyl linoleate: completing the picture, J. Am. Chem. Soc. 139 (2017) 4704–4714.
[23] C. Chatgilialoglu, C. Ferreri, Reductive stress of sulfur-containing amino acids within proteins and implication of tandem protein-lipid damage, Int. J. Mol. Sci. 22 (2021), 12863.
[24] C. Chatgilialoglu, C. Ferreri, I.N. Lykakis, P. Wardman, Trans fatty acids and radical stress: what are the real culprits? Bioorg. Med. Chem. 14 (2006) 6144–6148.
[25] C. Ferreri, M.R. Faraone Mennella, C. Formisano, L. Landi, C. Chatgilialoglu, Arachidonate geometrical isomers generated by thiyl radicals: the relationship with trans lipids detected in biological samples, Free Radic. Biol. Med. 33 (2002) 1516–1526.
[26] C. Ferreri, A. Samadi, F. Sassatelli, L. Landi, C. Chatgilialoglu, Regioselective cis-trans isomerization of arachidonic double bonds by thiyl radicals: the influence of phospholipid supramolecular organization, J. Am. Chem. Soc. 126 (2004) 1063–1072.
[27] C. Ferreri, C. Chatgilialoglu, Role of fatty acid-based functional lipidomics in the development of molecular diagnostic tools, Expert Rev. Mol. Diagn. 12 (2012) 767–780.
[28] C. Ferreri, C. Chatgilialoglu, Membrane Lipidomics for Personalized Health, Wiley, Chichester, UK, 2015.
[29] A. Cort, T. Ozben, M. Melchiore, C. Chatgilialoglu, C. Ferreri, A. Sansone, Effects of bleomycin and antioxidants on the fatty acid profile of testicular cancer cell membranes, Biochim. Biophys. Acta 1858 (2016) 434–441.
[30] F. Vetica, A. Sansone, C. Ferreri, C. Chatgilialoglu, A convenient route to mono-trans polyunsaturated free fatty acids, J. Chem. Res. (2022) 46, https://doi.org/10.1177/17475198221090908.
[31] Y. Hirata, A. Inoue, S. Suzuki, M. Takahashi, R. Matsui, N. Kono, T. Takuya Noguchi, A. Matsuzawa, trans-Fatty acids facilitate DNA damage-induced apoptosis through the mitochondrial JNK-Sab-ROS positive feedback loop, Sci. Rep. 10 (2020) 2743.
[32] C. Ferreri, A. Sansone, M.G. Krokidis, A. Masi, B. Pascucci, M. D'Errico, C. Chatgilialoglu, Effects of oxygen tension for membrane lipidome remodeling of cockayne syndrome cell models, Cells 11 (2022) 1286.
[33] J. Folch, M. Lees, G.H. Sloane Stanley, A simple method for the isolation and purification of total lipids from animal tissues, J. Biol. Chem. 226 (1957) 497–509.
[34] R. Scanferlato, M. Bortolotti, A. Sansone, C. Chatgilialoglu, L. Polito, M. De Spirito, G. Maulucci, A. Bolognesi, C. Ferreri, Hexadecenoic fatty acid positional isomers and de novo PUFA synthesis in colon cancer cells, Int. J. Mol. Sci. 20 (2019) 832.
[35] Y. Hirata, M. Takahashi, Y. Yamada, R. Matsui, A, R. Ashida, T. Noguchi, A. Matsuzawa, trans-Fatty acids promote p53-dependent apoptosis triggered by cisplatin-induced DNA interstrand crosslinks via the Nox-RIP1-ASK1-MAPK pathway, Sci. Rep. 11 (2021), 10350.
[36] T. Shimada, Y. Yabuki, T. Noguchi, M. Tsuchida, R. Komatsu, S. Hamano, M. Yamada, Y. Ezaki, Y. Hirata, A. Matsuzawa, The distinct roles of LKB1 and AMPK in p53-dependent apoptosis induced by cisplatin, Int. J. Mol. Sci. 23 (2022), 10064.
[37] A.R. Brash, Arachidonic acid as a bioactive molecule, J. Clin. Invest. 107 (2001) 1339–1345.
[38] H. Tallima, R. El Ridi, Arachidonic acid: physiological roles and potential health benefits - a review, J. Adv. Res. 11 (2017) 33–41.
[39] A. Manni, J. Richie, S. Schetter, A. Calcagnotto, N. Trushin, C. Aliaga, K. El-Bayoumy, Stearoyl-CoA desaturase-1, a novel target of omega-3 fatty acids for reducing breast cancer risk in obese postmenopausal women, Eur. J. Clin. Nutr. 71 (2017) 762–765.
[40] U. Bhagat, U.N. Das, State of the art paper. Potential role of dietary lipids in the prophylaxis of some clinical conditions, Arch. Med. Sci. 11 (4) (2015) 807–818, https://doi.org/10.5114/aoms.2015.53302.
[41] M. Mahfouz, Effect of dietary trans fatty acids on the delta 5, delta 6 and delta 9 desaturases of rat liver microsomes in vivo, Acta Biol. Med. Ger. 40 (1981) 1699–1705.
[42] L. Vendel Nielsen, T.P. Krogager, C. Young, C. Ferreri, C. Chatgilialoglu, O. Nørregaard Jensen, J.J. Enghild, Effects of elaidic acid on lipid metabolism in HepG2 cells, investigated by an integrated approach of lipidomics, transcriptomics and proteomics, PLoS One 8 (9) (2013 Sep 13), e74283, https://doi.org/10.1371/journal.pone.0074283.
[43] C. Ferreri, S. Pierotti, C. Chatgilialoglu, A. Barbieri, F. Barigelletti, Probing the influence of cis-trans isomers on model lipid membrane fluidity using cis-parinaric acid and a stop-flow technique, Chem. Commun. (2006) 529–531.
[44] F.A. Kummerow, Q. Zhou, M.M. Mahfouz, Effect of trans fatty acids on calcium influx into human arterial endothelial cells, Am. J. Clin. Nutr. 70 (1999) 832–838.
[45] H. Nishitoh, A. Matsuzawa, K. Tobiume, K. Saegusa, K. Takeda, K. Inoue, S. Hori, A. Kakizuka, H. Ichijo, ASK1 is essential for endoplasmic reticulum stress-induced neuronal cell death triggered by expanded polyglutamine repeats, Genes Dev. 16 (2002) 1345–1355.
[46] B.A. Carneiro, W.S. El-Deiry, Targeting apoptosis in cancer therapy, Nat. Rev. Clin. Oncol. 17 (2020) 395–417.
[47] Y. Wang, N.S. Kim, J.-F. Haince, H.C. Kang, K.K. David, S.A. Andrabi, G.G. Poirier, V.L. Dawson, T.M. Dawson, Poly(ADP-ribose) (PAR) binding to apoptosis-inducing factor is critical for PAR polymerase-1-dependent cell death (parthanatos), Sci. Signal. 4 (2011) ra20.
[48] J. Zhao, S. Jitkaew, Z. Cai, S. Choksi, Q. Li, J. Luo, Z.-G. Liu, Mixed lineage kinase domain-like is a key receptor interacting protein 3 downstream component of TNF-induced necrosis, Proc. Natl. Acad. Sci. USA 109 (2012) 5322–5327.
[49] T. Noguchi, Y. Sekiguchi, Y. Kudoh, R. Naganuma, T. Kagi, A. Nishidate, K. Maeda, C. Ishii, T. Toyama, Y. Hirata, G. Hwang, A. Matsuzawa, Gefitinib initiates sterile inflammation by promoting IL-1beta and HMGB1 release via two distinct mechanisms, Cell Death Dis. 12 (2021) 49.
[50] K.D. Tousignant, A. Rockstroh, B.L.J. Poad, A. Talebi, R.S.E. Young, A. Taherian Fard, R. Gupta, T. Zang, C. Wang, M.L. Lehman, J.V. Swinnen, S.J. Blanksby, C. C. Nelson, M.C. Sadowski, Therapy-induced lipid uptake and remodeling underpin ferroptosis hypersensitivity in prostate cancer, Cancer Metabol. 8 (2020), https://doi.org/10.1186/s40170-020-00217-6 article 11.
[51] S. Doll, B. Proneth, Y.Y. Tyurina, E. Panzilius, S. Kobayashi, I. Ingold, M. Irmler, J. Beckers, M. Aichler, A. Walch, H. Prokisch, D. Trümbach, G. Mao, F. Qu, H. Bayir, J. Füllekrug, C.H. Scheel, W. Wurst, J.A. Schick, V.E. Kagan, J. P. Friedmann Angeli, M. Conrad, ACSL4 dictates ferroptosis sensitivity by shaping cellular lipid composition, Nat. Chem. Biol. 13 (2017) 91–98.
[52] Y. Hirata, R. Cai, A. Volchuk, E.B. Steinberg, Y. Saito, A. Matsuzawa, S. Grinstein, A.S. Freeman, Lipid peroxidation increases membrane tension, Piezo1 gating and cation permeability to execute ferroptosis, Curr. Biol. 33 (2023) 1–13.
[53] C. Ferreri, S. Grabovskiy, M. Aoun, M. Melchiorre, N. Kabal'nova, C. Feillet-Coudray, G. Fouret, C. Coudray, C. Chatgilialoglu, Trans fatty acids: chemical synthesis of eicosapentaenoic acid isomers and detection in rats fed a deodorized fish oil diet, Chem. Res. Toxicol. 25 (2012) 687–694.
[54] G. Menounou, G. Giacometti, R. Scanferlato, P. Dambruoso, A. Sansone, I. Tueros, J. Amézaga, C. Chatgilialoglu, C. Ferreri, Trans lipid library: synthesis of docosahexaenoic acid (DHA) monotrans isomers and regioisomer identification in DHA-containing, Chem. Res. Toxicol. 31 (2018) 191–200.
[55] M. Balazy, S. Chemtob, Trans-arachidonic acids: new mediators of nitro-oxidative stress, Pharmacol. Ther. 119 (2008) 275–290.
[56] I. Tartaro-Bujak, B. Mihaljević, C. Ferreri, C. Chatgilialoglu, The influence of antioxidants in the thiyl radical induced lipid peroxidation and geometrical isomerization in micelles of linoleic acid, Free Radic. Res. 50 (2016) S18–S23.
[57] B. Mihaljević, I. Tartaro, C. Ferreri, C. Chatgilialoglu, Linoleic acid peroxidation vs cis–trans isomerization: a biomimetic model of free radical reactivity in the presence of thiols, Org. Biomol. Chem. 9 (2011) 3541–3548.
[58] H. Souabni, V. Thoma, T. Bizouarn, C. Chatgilialoglu, A. Siafaka-Kapadai, L. Baciou, C. Ferreri, C. Houée-Levin, M.A. Ostuni, Trans Arachidonic acid isomers inhibit NADPH-oxidase activity by direct interaction with enzyme components, Biochim. Biophys. Acta Biomembr. 1818 (2012) 2314–2324.
[59] X. Chen, J. Li, R. Kang, D.J. Klionsky, D. Tang, Ferroptosis: machinery and regulation, Autophagy 17 (2021) 2054–2081.
[60] W.C. Chang, J. So, S. Lamon-Fava, Differential and shared effects of eicosapentaenoic acid and docosahexaenoic acid on serum metabolome in subjects with chronic inflammation, Sci. Rep. 11 (2021), 16324.
[61] R. Gorjão, A.K. Azevedo-Martins, H.G. Rodrigues, F. Abdulkader, M. Arcisio-Miranda, J. Procopio, R. Curi, Comparative effects of DHA and EPA on cell function, Pharmacol. Ther. 122 (2009), 56–6.
[62] L.O. Romero, A.E. Massey, A.D. Meta-Daboin, F.J. Sierra-Valdez, S.C. Chauhan, J. F. Cordero-Morales, V. Vásquez, Dietary fatty acids fine-tune Piezo1 mechanical response, Nat. Commun. 10 (2019) 1200.
[63] M.L. Jacobs, H.A. Faizi, J.A. Peruzzi, P.M. Vlahovska, N.P. Kamat, EPA and DHA differentially modulate membrane elasticity in the presence of cholesterol, Biophys. J. 120 (2021) 2317–2329.
[64] I. Tremi, D. Anagnostopoulos, E. Spyratou, P. Gkeka, A.G. Georgakilas, C. Chatgilialoglu, Z. Cournia, Effect of 5-trans isomer of arachidonic acid on model liposomal membranes studied by a combined simulation and experimental approach, J. Membr. Biol. 251 (2018) 475–489.
[65] A. Rossary, K. Arab, J.P. Steghens, Polyunsaturated fatty acids modulate NOX 4 anion superoxide production in human fibroblasts, Biochem. J. 406 (2007) 77–83.

[66] M. Szori, T. Abou-Abdo, C. Fittschen, I.G. Csizmadia, B. Viskolcz, Allylic H-abstraction hydrogen abstraction from 1,4 type polyalkenes as a model for free radical trapping by polyunsaturated fatty acids (PUFAs), Phys. Chem. Chem. Phys. 9 (2007) 1931–1940.

[67] A.N. von Krusenstiern, R.N. Robson, N. Qian, B. Qiu, F. Hu, E. Reznik, N. Smith, F. Zandkarimi, V.M. Estes, M. Dupont, T. Hirschhorn, M.S. Shchepinov, W. Min, K. A. Woerpel, B.R. Stockwell, Identification of essential sites of lipid peroxidation in ferroptosis, Nat. Chem. Biol. (2023), https://doi.org/10.1038/s41589-022-01249-3.

[68] L. Magtanong, P.-J. Ko, M. To, J.Y. Cao, C.C. Forcina, A. Tarangelo, C.C. Ward, K. Cho, G.J. Patti, D.K. Nomura, J.A. Olzmann, S.J. Dixon, Exogenous monounsaturated fatty acids promote a ferroptosis-resistant cell state, Cell Chem. Biol. 26 (2019) 420–432.

[69] U.N. Das, Saturated fatty acids, MUFAs and PUFAs regulate ferroptosis, Cell Chem. Biol. 26 (2019) 309–311.

[70] M.G. Krokidis, M. Louka, E.K. Efthimiadou, C. Ferreri, C. Chatgilialoglu, Fatty acid remodeling of membrane glycerophospholipids induced by bleomycin and iron oxide nanoparticles in human embryonic kidney cells, Chem. Res. Toxicol. 33 (2020) 2565–2572.

[71] V. Kagan, G. Mao, F. Qu, J.P. Friedmann Angeli, S. Doll, C.St Croix, H.H. Dar, V. A. Tyurin, et al., Oxidized arachidonic and adrenic PEs navigate cells to ferroptosis, Nat. Chem. Biol. 13 (2017) 81–90.

[72] Y. Liu, L. He, B. Liu, Y. Ying, J. Xu, M. Yu, J. Dang, K. Liu, Pharmacological inhibition of sphingolipid synthesis reduces ferroptosis by stimulating the HIF-1 pathway, iScience 25 (2022), 104533.

[73] Y. Zhang, S. Li, F. Li, C. Lv, Q. Yang, High-fat diet impairs ferroptosis and promotes cancer invasiveness via downregulating tumor suppressor ACSL4 in lung adenocarcinoma, Biol. Direct 16 (2021) 10, https://doi.org/10.1186/s13062-021-00294-7.