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RESEARCH ARTICLE

Effects of Somatostatin and Vitamin C on the Fatty Acid Profile of Breast Cancer Cell Membranes

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Abstract: ***Background*:** Vitamin C (Vit C) is an important physiological antioxidant with growing applications in cancer. Somatostatin (SST) is a natural peptide with growth inhibitory effect in several mammary cancer models.

***Objective*:** The combined effects of SST and Vit C supplementation have never been studied in breast cancer cells so far.

***Methods*:** We used MCF-7 and MDA-MB231 breast cancer cells incubated with SST for 24h, in the absence and presence of Vit C, at their EC_{50} concentrations, to evaluate membrane fatty acid-profiles together with the follow-up of EGFR and MAPK signaling pathways.

***Results*:** The two cell lines gave different membrane reorganization: in MCF-7 cells, decrease of omega-6 linoleic acid and increase of omega-3 fatty acids (Fas) occurred after SST and SST+Vit C incubations, the latter also showing significant increases in MUFA, docosapentaenoic acid and mono-trans arachidonic acid levels. In MDA-MB231 cells, SST+Vit C incubation induced significant membrane remodeling with an increase of stearic acid and mono-trans-linoleic acid isomer, diminution of omega-6 linoleic, arachidonic acid and omega-3 (docosapentaenoic and docosadienoic acids). Distinct signaling pathways in these cell lines were studied: in MCF-7 cells, incubations with SST and Vit C, alone or in combination significantly decreased EGFR and MAPK signaling, whereas in MDA-MB231 cells, SST and Vit C incubations, alone or combined, decreased p-P44/42 MAPK levels, and increased EGFR levels.

***Conclusion*:** Our results showed that SST and Vit C can be combined to induce membrane fatty acid changes, including lipid isomerization through a specific free radical-driven process, influencing signaling pathways.

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1. INTRODUCTION

Breast cancer is the most common cancer among women, however, there are limitations in treatment options [1]. The etiology of breast cancer is multifactorial. Though many risk factors related to breast cancer, such as genetic inheritance, are not modifiable; others, for example, nutrients and dietary choices, physical activity, and behavioral factors are considered as modifiable factors to prevent development of breast cancer [2]. Various studies have shown that levels of circulating metabolites such as hormones, cytokines and adipokines might be involved in breast cancer cell proliferation and migration [3]. A few studies have demonstrated the potential role of diet on breast cancer risk and have shown that consumption of vegetables, fruits *i.e.*, might be protective due to antioxidant properties [4]. Gradual changes in molecular dynamics might be responsible for breast cancer initiation and progression. High levels of Estrogen Receptors (ERs), HER2/neu, PI3K/Akt, EGFR/VEGFR, MAPK and various growth factors and their receptors have been shown to be correlated with breast cancer development [5]. In this study, we compared the effects of SST and Vit C, alone and together, on membrane fatty acid profiles and two important biomarkers of signaling pathways (MAPK and EGFR) in two different breast cancer cell lines (MCF-7 and MDA-MB231). MCF-7 cells are ER-positive and have been used widely to study anti-hormonal therapies in breast cancer [6]. MDA-MB231 cells are ER-negative and resemble the recently characterized claudin-low tumor subtype of breast cancer [7]. Approximately 15-20% of breast cancer cells are triple negative (TNBC cells) lacking Estrogen Receptors (ERs), Progesterone Receptors (PRs) and Epidermal Growth Factor Receptor 2 (EGFR2) [8]. The expression of these receptors allows treatment with endocrine or targeted therapies in clinical cases [9], but these therapies are not applicable to clinical TNBC cases [10]. Therefore, it is important to develop new methods for suppressing TNBC cell growth and survival. In a study, Vit C was shown to attenuate the incidence of ER-positive breast cancer cells [11]. However, there is no evidence demonstrating that Vit C alone is useful for TNBC treatment. Ascorbic acid/Vit C is a common nutrient and potent antioxidative compound, which may exert che-

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mopreventive effects without significant toxic side effects. It clears toxic free radicals [12-14]. It is important to investigate whether a high concentration of Vit C associated autoxidation is critical for its anticancer effects. Vit C is very stable and barely autoxidizes alone [15]. Previous studies have reported that high concentrations of Vit C are able to induce autoxidation and thus reveal anticancer effects [16], while lower concentrations of Vit C failed to show similar effects [17]. Previous studies have also demonstrated that combined treatment of Vit C with conventional anticancer agents can enhance their anticancer activities [18, 19]. Cytotoxic effect of Vit C was reported in a human breast cancer cell line [20]. Breast cancer cells maintain a higher intracellular hydrogen peroxide (H_2O_2) than normal cells suggesting that breast cancer cells are able to accumulate and tolerate H_2O_2 within a certain range [21]. However, mild elevation of H_2O_2 in cancer cells has been shown to arrest cell cycle and induce apoptosis of cancer cells [16, 22]. This indicates selective overload of H_2O_2 in cancer cells could be a therapeutic strategy. Several studies have confirmed that high-dose of Vit C has anticancer effects, exhibiting selective cytotoxicity to cancer cells [23-25] while in other studies, no such beneficial therapeutic effect was found. In a study, Vit C was reported to induce cytotoxicity in MCF-7 cells [26].

SST, a 14 amino acid peptide known to inhibit growth hormone. In the pancreas, SST is produced by the delta cells of Langerhans islets, where it serves to block the secretion of both insulin and glucagon from adjacent cells [27]. The relative concentrations of these hormones regulate the rates of absorption, utilization, and storage of glucose, amino acids, and fatty acids [28]. One recent study hypothesized that the enhanced insulin and glucagon secretion was a result of decreased SST secretion, showing that Free Fatty Acids (FFAs) are regulators not only of insulin, but also SST secretion [29]. The presence of SSTR receptors in MCF-7 and MDA-MB cell lines have been reported [30]. They are colocalized with Epithelial Growth Factor Receptor (EGFR) in these cell lines [31]. Antitumoral effects of SST and analogues, mediated *via* a family of five Somatostatin Receptors (SSTR) belonging to the G-Protein Coupled Receptors (GPCR), are reported in several *in vitro* and *in vivo* studies [32], and in mammary cancer models [33]. Expression of EGFR and EGF-stimulated proliferation have been reported to markedly decrease in MCF-7 cells expressing high levels of the SSTR2 [34].

We are interested in the follow-up of the effects of antitumoral drugs and antioxidants in cancer cell lines with an attention to membrane fatty acids, as the structural and functional hydrophobic moieties of the phospholipids. We previously published that co-administration of bleomycin with several antioxidants induced membrane fatty acid remodeling and changes of growth signaling pathways in testicular cancer germ cells, evidencing the importance of nutritionally important antioxidants [35]. Indeed, monounsaturated and polyunsaturated fatty acids (MUFA and PUFA) of cell membranes can be affected by oxidative stress in two ways: lipid peroxidation, with induction of membrane remodeling and decrease of PUFA moieties, and lipid isomerization with the formation of mono-trans isomers as marker of the thiyl radical generation in cells [36]. Natural compounds might inhibit growth of breast cancer cells affecting ROS levels [37].

There is no study demonstrating the effects of SST on membrane lipid profile in human breast cancer cells. Therefore, we investigated the effects of SST with or without Vit C on the membrane fatty acid profiles of two breast cancer cell lines (MCF-7 and MDA-MB-231), together with signaling pathways such as AKT1, p-AKT-1(Ser473), p-AKT-1 (Thr308), p-P44/42 MAPK (Thr202/Tyr204), S6-Ribozomal and p-S6-Ribozomal (Ser235/236) that furnish a combined scenario of molecular and biological consequences of SST and Vit C in breast cancer.

2. MATERIALS AND METHODS

2.1. Cell Lines

Both cell lines were supplied from the ATCC. The cells were cultured in DMEM (Invitrogen) or RPMI (Invitrogen) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS), 100 units/ml of penicillin and 100µg/ml of streptomycin. Cells were grown to confluence at 37°C in a humidified atmosphere containing 5% CO_2 in air and were passaged weekly using 0.25% trypsin. Cells were incubated with SST (Bachem) and/or Vitamin C (Merck) for 24h as indicated below. The standard protocols for cell culture were applied in the experiments and the cell groups were prepared under the same conditions.

2.2. Viability Assay

The viability of the cells was measured using the MTT (3-(4,5)dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The principle of this method is based on cellular reduction of MTT to a blue formazan product by mitochondrial dehydrogenases of viable cells. The intensity of the blue color formed by this procedure is a criterion for cell viability. Cell viability assay was performed by seeding $5x10^4$ cells/well in 96-well plates and incubating them for 1 day in DMEM or RPMI with 10% FBS. The following day, the cells were incubated with different concentrations of SST (10-320µM) and/or Vitamin C (500-10000µM) for 24h. The control cells were prepared in plates containing only medium. Following incubation, MTT was added into each well and incubated for 4h at 37°C. Viability analysis was performed in the breast cancer cell lines incubated with SST and/or Vitamin C for 24h in order to determine the EC_{50} doses for SST and Vit C. A microplate reader (BioTek) was used to measure the absorbances at 570nm. The average absorbance values of the cells incubated with different agents were compared with the average absorbance values of the control cells exposed to medium only to calculate the percentage of the viable cells. We determined EC_{50} of SST on MCF-7 cell viability as 38.58µM, for Vitamin C as 6506µM. For the MDA-MB-231 cells, EC_{50} concentration of SST was 96.75µM and that of Vitamin C was 591.2µM. We applied these doses in the incubations.

2.3. Phospholipid Extraction and Fatty Acid Analysis

Cells were detached using accutase, thoroughly washed with phosphate buffer, and pelleted by centrifugation at 14,000 ×g for 40min at 4°C after adding water. Phospholipids were isolated from the pellet and fatty acids were derivatized into their corresponding Fatty Acid Methyl Esters (FAME) to be recognized by Gas Chromatography (GC) in comparison with standard references as described before [35]. Fatty acid compositions are given as relative percentages of the total fatty acid content, which represents the major fatty acid components (>97%) present in cell membrane phospholipids.

2.4. Mitogen-Activated Protein Kinase (MAPK) Assay

Cells were lysed with Cell Lysis Buffer (Cell Signaling Technology), then the levels of AKT1, p-AKT-1(Ser473), p-AKT-1 (Thr308), p-P44/42 MAPK (Thr202/Tyr204), S6-Ribozomal and p-S6-Ribozomal (Ser235/236) in the cell lysates were determined using the PathScan MAPK Multi-Target Sandwich ELISA Kit (Cell Signaling Technology) according to the manufacturer's instructions.

2.5. Epidermal Growth Factor Receptor (EGFR) Assay

Epidermal growth factor receptor levels were measured by sandwich ELISA kit (Biont), a sensitive colorimetric assay, in accordance with the procedure recommended by the manufacturer. The total protein content in the samples was determined by the

Table 1. Fatty acid methyl esters (FAME) obtained from membrane phospholipids of MCF-7 cells incubated with Somatostatin, Vitamin C, or SST+Vit C for 24h and from cells in the same conditions but not treated with these agents.

FAME[a]	Control[b] n=6	Somatostatin[b] n=6	Vitamin C[b] n=6	SST+Vit C[b] n=6
14:0	1.92±0.73	2.51±0.24	2.46 ± 0.53	2.58±0.24
16:0	26.23±4.26	23.35±2.37	24.06 ± 4.07	21.13±1.20**
16:1 6 c	1.25±0.45	1.52±0.02	1.31 ± 0.28	1.83±0.12**
16:1 9 c	3.81±1.18	4.76±0.32	6.29 ± 1.26**	6.61±0.32**
18:0	10.40±2.50	11.93±0.43	11.62 ± 1.86	11.96±0.51
18:1 9 trans	0.04±0.09	tr	tr	0.03±0.08
18:1 9 c	27.89±2.96	26.89±1.21	26.57 ± 1.57	25.64±0.55*
18:1 11 c	6.49±1.98	7.84±0.58	8.53 ± 0.85*	8.14±0.21**
18:2 trans	0.30±0.14	0.31±0.14	0.56 ± 0.47	0.41±0.38
18:2 ω6	5.70±3.39	3.44±0.06**	3.82 ± 0.40**	4.53±0.21**
18:3 ω6	0.11±0.05	0.11±0.06	0.39 ± 0.041**	0.37±0.03**
18:3 ω3	0.30±0.09	0.37±0.05	0.22 ± 0.06	0.25±0.04
20:0	0.41±0.22	0.37±0.14	0.37 ± 0.09	0.42±0.04
20:1 11c	0.91±0.35	0.95±0.07	1.13 ± 0.26	1.30±0.11
20:2w6	1.01±0.38	0.98±0.40	0.48 ± 0.06**	0.49±0.03*
DGLA w6	0.97±0.26	1.0±0.05	1.11 ± 0.13	1.47±0.11*
20:4 trans ω6	0.03±0.02	0.02±0.03	0.32 ± 0.28**	0.24±0.03**
20:4 ω6	5.48±1.01	5.96±0.28	5.26 ± 0.93	6.22±0.48
EPA	1.22±0.37	1.52±0.08	1.05 ± 0.19	1.12±0.08
22:0	0.74±0.87	0.69±0.50	0.45 ± 0.16	0.55±0.06
DPA	1.65±0.50	2.15±0.08**	1.75 ± 0.29	2.13±0.14**
DHA	2.47±0.51	2.81±0.08	2.25 ± 0.57	2.57±0.20
tot SFA	39.70±2.55	38.85±3.10	38.96 ± 4.05	36.65±1.42
tot MUFA	40.53±1.00	42.13±2.11	43.83 ± 2.98	43.55±0.69**
tot PUFA	19.77±3.18	19.02±1.00	17.22 ± 1.98	19.80±1.16
Tot ω6	12.63±3.45	10.81±0.82	10.84 ± 1.46	12.26±0.69#
Tot ω3	6.86±1.41	7.85±0.17*	6.38 ± 1.04	7.54±0.49#,&
tot trans	0.77±0.31	0.52±0.15	0.88 ± 0.73	0.68±0.38

The values are reported as the relative percentage (% rel.) of the total fatty acid peak areas detected in the GC analysis. The values are given as mean ± SD.

Bradford method [38] using Bovine Serum Albumin (BSA) as a standard. The total protein content was used to normalize the EGFR and MAPK values of each sample.

2.6. Statistical Analysis

Statistical analyses were conducted using t-test and performed with SPSS software. Statistical significance was based on 95% confidence limits ($p \leq 0.05$). Comparison of the non-parametric data among the groups was performed using the Mann-Whitney U test. Results were given as mean ± SD.

3. RESULTS

3.1. Membrane Fatty Acid Profile

Table **1** shows membrane fatty acid composition in the MCF-7 cells after incubations with SST, Vitamin C or SST+Vit C for 24h. Myristic acid (14:0) level was found similar in all groups, whereas palmitic acid (16:0) decreased significantly in the SST+Vit C group (Fig. **1**). The most relevant Monounsaturated Fatty Acid (MUFA), oleic acid (18:1, delta-9) decreased with SST+ Vit C compared to the control cells ($p<0.05$) (Fig. **1**), together with the other MUFA 6cis-16:1. (sapienic acid), 9cis-16:1 (palmitoleic acid) and 11cis-18:1 (cis-vaccenic acid) levels compared to the control cells ($p<0.05$). The level of omega-6 essential fatty acid, linoleic acid (18:2) significantly decreased in the cells incubated with SST, Vit C and SST+Vit C compared to the control group ($p<0.05$). Incubations with Vit C alone and SST+Vit C combination significantly elevated Polyunsaturated Fatty Acid (PUFA) family members; 18:3 ω6 (γ- linolenic acid) and Dihomo-γ-Linolenic Acid (DGLA) ω6 levels compared to the control cells. No increase in the arachidonic acid level (20:4 ω6) was found in all cell groups. Mono-trans arachidonic acid (20:4 t ω6) as a marker of endogenous lipid isomerization was significantly elevated in cells incubated with SST+Vit C and Vit C group compared to the control cells. The ω3 fatty acid, docosapentaenoic acid (DPA ω3) level was significantly higher in the cells incubated with SST and with SST + Vit C compared to the controls (Fig. **2**). It is worth underlining that with the single treatments of SST and Vit C a few changes were detected in the MCF-7 cell membranes compared to the lipidome changes detected in the cells with SST + Vit C incubation.

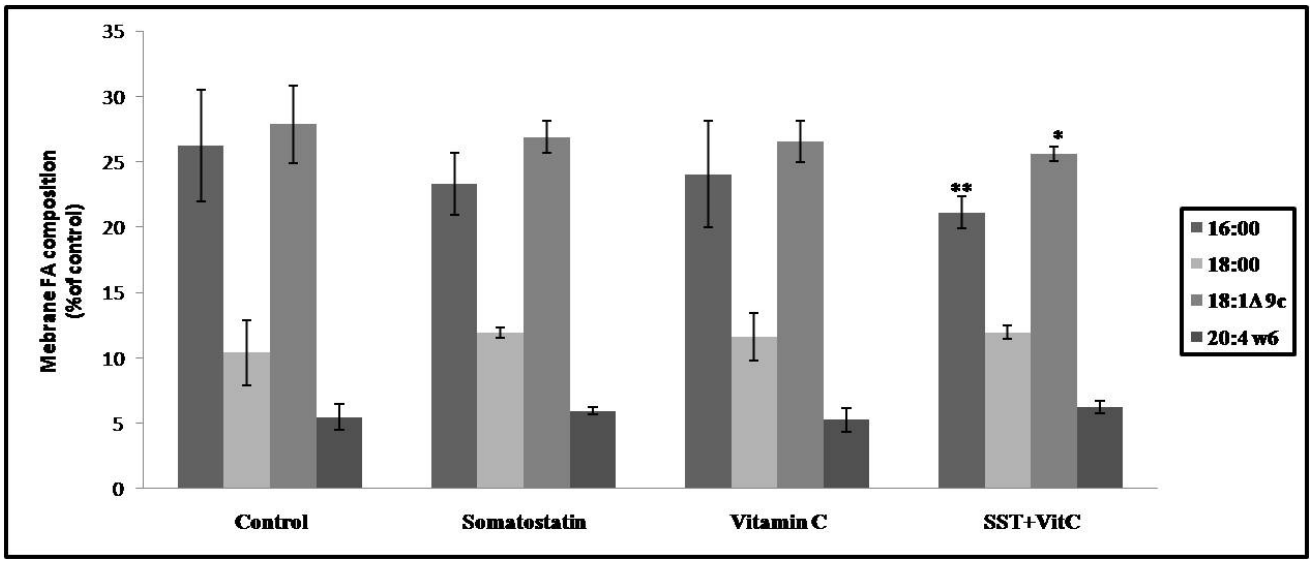

Fig. (1). Main membrane fatty acid residues of the MCF-7 cells following incubations with Somatostatin (SST), Vitamin C (Vit C), or SST+Vit C for 24h (n=6 in each experiment). Values are mean±SD. See Table **1** for values and complete analysis. *,**Values significantly different from the control $p < 0.05$, $p < 0.01$. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

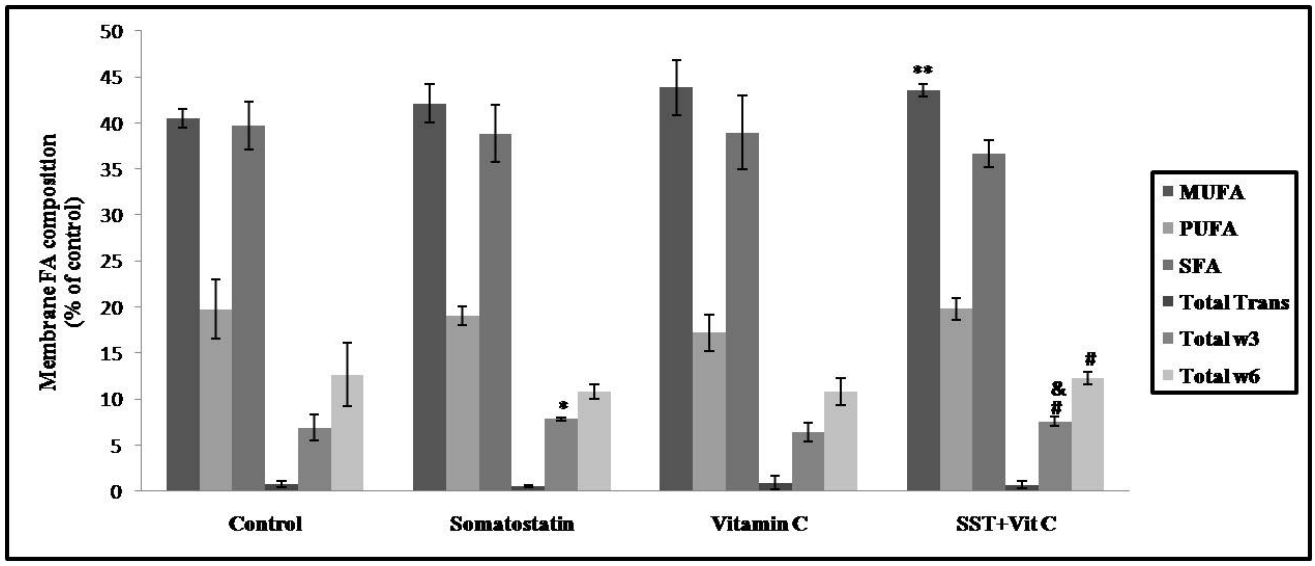

Fig. (2). MUFA, PUFA, SFA, total ω3, total ω6 and total trans fatty acid moieties in the MCF-7 cell membranes following incubations with Somatostatin (SST), Vitamin C (Vit C) or SST+Vit C for 24h (n=6 in each experiment). Values are mean±SD. See Table **1** for values and complete analysis. *,**Values significantly different from the control $p < 0.05$, $p < 0.01$. $^{\&}$Values significantly different from the Vit C $p < 0.05$. $^{\#}$Values significantly different from the SST $p < 0.05$. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Table **2**, and Figs. **3** and **4** depict membrane fatty acid composition in the MDA-MB231 cells incubated with SST, Vit C or SST+Vit C for 24h. SST incubation did not cause membrane FA changes significantly except long chain 20:0 and 20:4t, whereas Vit C decreased significantly ω3 DHA and increased total SFA, total trans isomer, and 18:2 t, compared to untreated cells. Incubation with SST+Vit C decreased significantly PUFA ω3 family (DPA and DHA) and decreased PUFA ω6 (18:2), in contrast increasing significantly SFA especially stearic acid (Fig. **3**). 20:4 ω6 fatty acid level in the MDA-MB 231 cells incubated with SST+Vit C was significantly lower compared to the controls, and lower than the cells incubated with SST alone. In the SST + Vit C incubated cells, the 18:2 t isomer remained significantly higher than the controls. SST+Vit C incubation caused an increase in the SFA levels compared to the cells incubated with SST. Fig. (**4**) depicts the membrane lipidome fatty acid monitoring and the levels of SFA, MUFA, PUFA, total trans, total ω3 and total ω6 under experimental conditions.

A comparative evaluation of the membrane lipidome changes in both cells demonstrated that the fatty acid families involved in MDA-MB231 cells were different than the MCF-7 cells, not causing increase in MUFA and omega-3 fatty acids at the expenses of decrease in SFA, but in contrast diminished omega-3 fatty acids and increased SFA families.

3.2. MAPK Pathway Member Levels

Absorbances of AKT1, p-AKT-1(Ser473), p-AKT-1 (Thr308), p-P44/42 MAPK (Thr202/Tyr204), S6-Ribozomal and p-S6-Ribozomal (Ser235/236) in the MCF-7 cell lysates are shown in Fig. (**5**). MCF-7 cells were incubated with SST, Vit C or SST + Vit C for 24h, leading to the inactivation of S6-Ribozomal, p-S6-Ribozomal

Table 2. Fatty acid methyl esters (FAME) obtained from membrane phospholipids of MDA-MB231cells incubated with Somatostatin, Vitamin C, or SST+Vit C for 24h and from cells in the same conditions but not treated with these agents.

FAME[a]	Control[b] n=6	Somatostatin[b] n=6	Vitamin C[b] n=6	SST+Vit C[b] n=6
14:0	1.18 ± 0.43	1.23 ± 0.12	1.13 ± 0.28	1.53 ± 0.34
16:0	17.93 ± 5.46	18.84 ± 3.09	18.78 ± 4.02	23.72 ± 6.94
16:1 6 c	1.93 ± 0.50	2.03 ± 0.38	1.93 ± 0.16	1.81 ± 0.31
16:1 9 c	1.74 ± 0.52	1.75 ± 0.39	1.61 ± 0.12	1.54 ± 0.28
18:0	18.99 ± 1.31	19.04 ± 1.40	20.06 ± 0.72	21.78 ± 3.74*
18:1 9 trans	0.09 ± 0.10	0.14 ± 0.14	0.12 ± 0.09	0.10 ± 0.07
18:1 9 c	22.27 ± 0.96	22.26 ± 0.56	21.88 ± 1.71	19.09 ± 4.01
18:1 11 c	6.28 ± 0.24	6.42 ± 0.08	6.56 ± 0.65	5.82 ± 1.23
18:2 trans	0.24 ± 0.21	0.35±0.20	0.72 ± 0.09**	0.61 ± 0.21*
18:2 ω6	4.75 ± 0.87	4.61 ± 0.36	5.05 ± 0.45	3.87 ± 1.93*
18:3 ω6	0.30 ± 0.13	0.26 ± 0.10	0.42 ± 0.06	1.07 ± 1.65
18:3 ω3	0.37 ± 0.15	0.31 ± 0.10	0.32 ± 0.08	0.40 ± 0.13
20:0	0.62 ± 0.17	0.41 ± 0.22*	0.61 ± 0.12	0.51 ± 0.14
20:1 11c	0.59 ± 0.15	0.54 ± 0.12	0.70 ± 0.08	0.63 ± 0.20
20:2w6	0.73 ± 0.18	0.64 ± 0.24	0.77 ± 0.04	0.70 ± 0.19
DGLA w6	1.92 ± 0.47	1.88 ± 0.22	2.05 ± 0.20	1.69 ± 0.61
20:4 trans ω6	0.08 ± 0.13	0.32 ± 0.08*	0.30 ± 0.06*	0.32 ± 0.61*
20:4 ω6	8.50 ± 1.68	8.40 ± 0.96	7.63 ± 0.63	6.75 ± 1.46
EPA	1.80 ± 0.62	1.75 ± 0.44	1.59 ± 0.23	1.46 ± 0.26
22:0	0.58 ± 0.49	0.53 ± 0.12	0.63 ± 0.32	0.48 ± 0.17
DPA	4.29 ± 0.88	3.29 ± 0.80	3.49 ± 0.27	2.94 ± 0.56*, #, &
DHA	4.51±0.72	4.16 ± 0.66	3.85 ± 0.36*	3.32 ± 0.67*,#
tot SFA	39.29 ± 5.42	40.06 ± 2.11	41.02 ± 4.64#	48.02 ± 10.52
tot MUFA	33.06 ± 0.90	33.29 ± 0.67	32.66 ± 2.47	28.88 ± 5.95
tot PUFA	27.65 ± 4.80	26.65 ± 2.73	26.18 ± 2.05	22.80 ± 4.68
Tot ω6	14.62 ± 2.46	14.42 ± 1.39	14.88 ± 1.03	12.99 ± 2.67
Tot ω3	12.93 ± 2.56	12.02 ± 1.69	11.29 ± 1.04	9.81 ± 2.04*,#
tot trans	0.60 ± 0.36	0.80 ± 0.26	1.02 ± 0.13**	1.04 ± 0.13**

The values are reported as the relative percentage (% rel.) of the total fatty acid peak areas detected in the GC analysis. The values are given as mean ± SD.

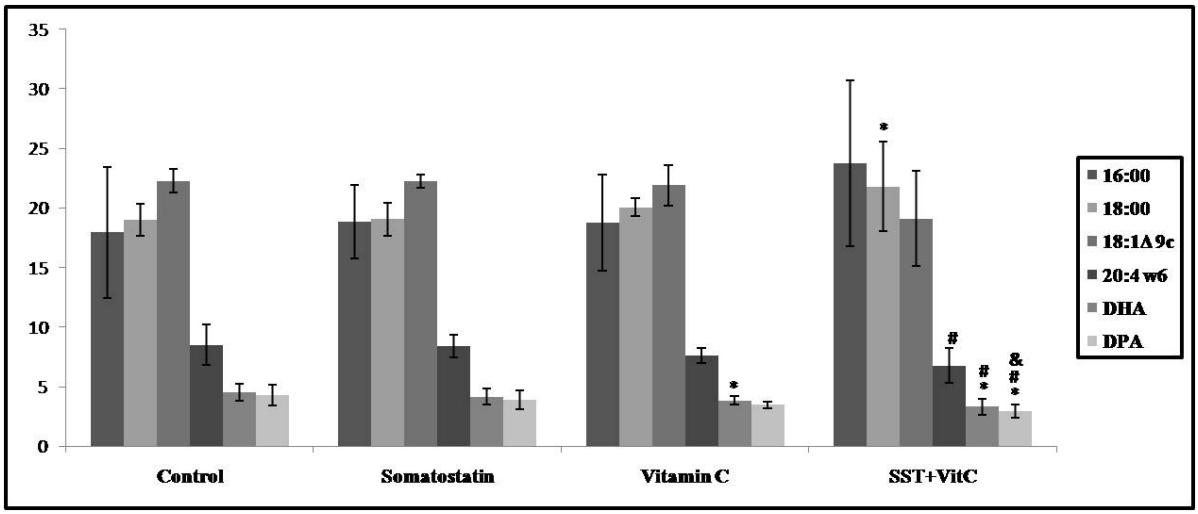

Fig. (3). Main membrane fatty acid residues of the MDA-MB231 cells following incubations with Somatostatin (SST), Vitamin C (Vit C), or SST+Vit C for 24h (n=6 in each experiment). Values are mean±SD. See Table **2** for values and complete analysis *Values significantly different from the control $p<0.05$. #Values significantly different from the SST $p<0.05$. &Values different from Vit C $p<0.05$. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

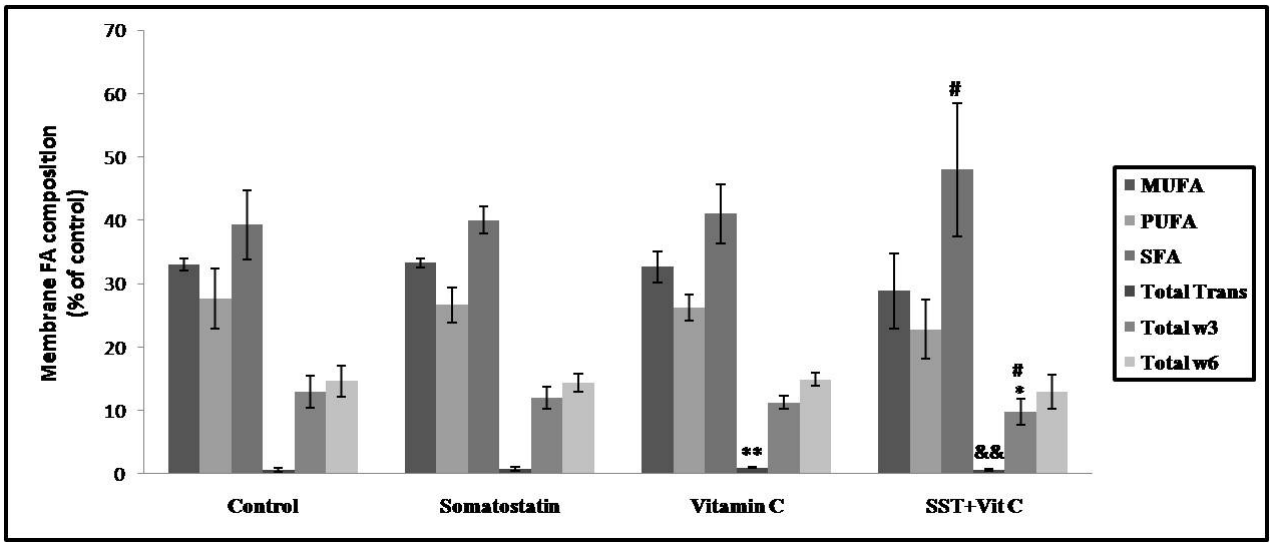

Fig. (4). MUFA, PUFA, SFA, total ω3, total ω6 and total trans fatty acid moieties in the MDA-MB231 cell membranes following incubations with Somatostatin (SST), Vitamin C (Vit C) or SST+Vit C for 24h (n=6 in each experiment). Values are mean±SD. See Table **2** for values and complete analysis.[*,**]Values significantly different from the control p<0.05, p<0.01. [&,&&]Values significantly different from the Vit C p<0.05, p<0.01. [#]Values significantly different from the SST p <0.05. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

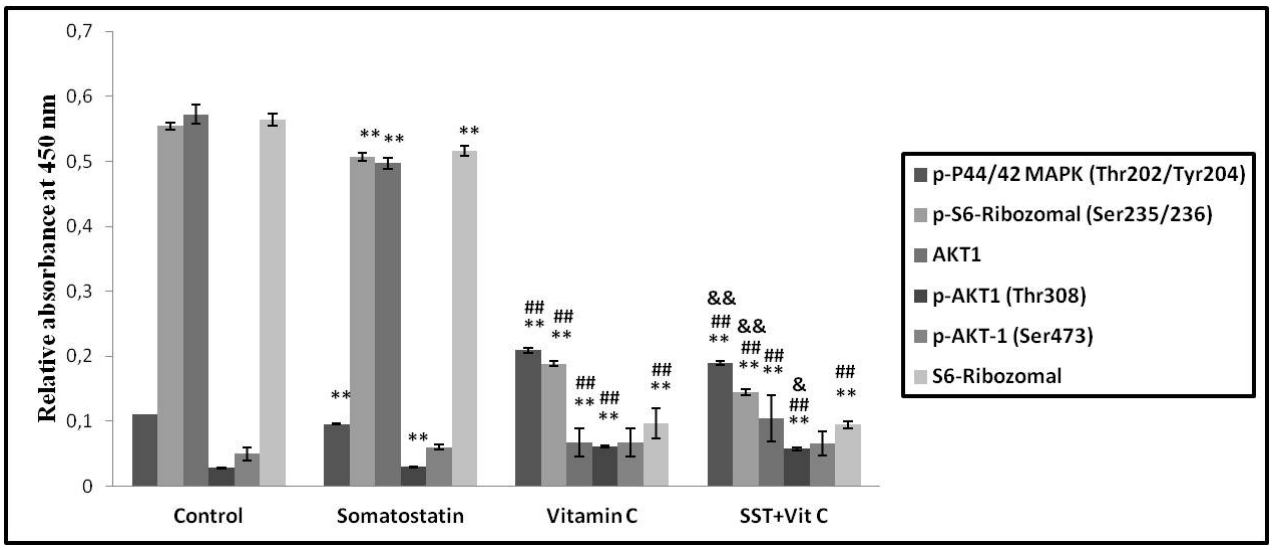

Fig. (5). Absorbances of the MAPK pathway members; AKT1, p-AKT-1(Ser473), p-AKT-1 (Thr308), p-P44/42 MAPK (Thr202/Tyr204), S6-Ribozomal and p-S6-Ribozomal (Ser235/236) measured in the MCF-7 cells after incubations with Somatostatin (SST), Vitamin C (Vit C) and SST + Vit C for 24 h (n=6).[**]Values significantly different from the control p <0.01. [&,&&]Values significantly different from the Vit C p <0.05, p < 0.01. [##]Values significantly different from the SST p <0.01. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

(Ser235/236) and AKT1 and activation of P-AKT1 (Thr308). p-P44/42 MAPK (Thr202/Tyr204) pathway's level was significantly decreased in the SST group compared to the control group. In contrast to SST, Vit C increased the activation of p-P44/42 MAPK (Thr202/Tyr204). However, when combined incubation with SST + Vit C decreased significantly p-P44/42 MAPK (Thr202/Tyr204), S6-Ribozomal and p-AKT-1(Ser473) levels compared to the cells incubated with Vitamin C alone. The S6-Ribozomal and AKT1 levels were diminished by SST and Vit C, whereas the AKT1 level showed a little recovery with SST + Vit C treatment. Vit C significantly enhanced p-AKT-1 (Thr308), p-P44/42 MAPK (Thr202/Tyr204) and p-S6-Ribozomal (Ser235/236) levels that were not affected by the combined SST + Vit C.

As shown in Fig. (**6**), 24h incubation of MDA-MB231 cells with SST, there was no significant difference in the p-S6-Ribozomal (Ser235/236) and p-AKT-1(Ser473) pathways in comparison to the control group. Vit C and SST+Vit C led to an increase in p-S6-Ribozomal (Ser235/236), p-AKT-1(Ser473) and S6-Ribozomal levels. p-P44/42 MAPK (Thr202/Tyr204) level significantly decreased in the breast cancer cells incubated with SST, Vit C and SST + Vit C. AKT1 level increased significantly by SST treatment, whereas decreased by Vit C and SST + Vit C combination.

3.3. EGFR Levels

As shown in Fig. (**7**), 24h incubation of MCF-7 cells with SST diminished the level of EGFR compared to the control cells. In contrast, Vit C and combination of SST with Vit C led to an increase in EGFR levels. Fig. (**8**) depicts the level of EGFR in MDA-MB231 cells. The EGFR level was not significantly changed

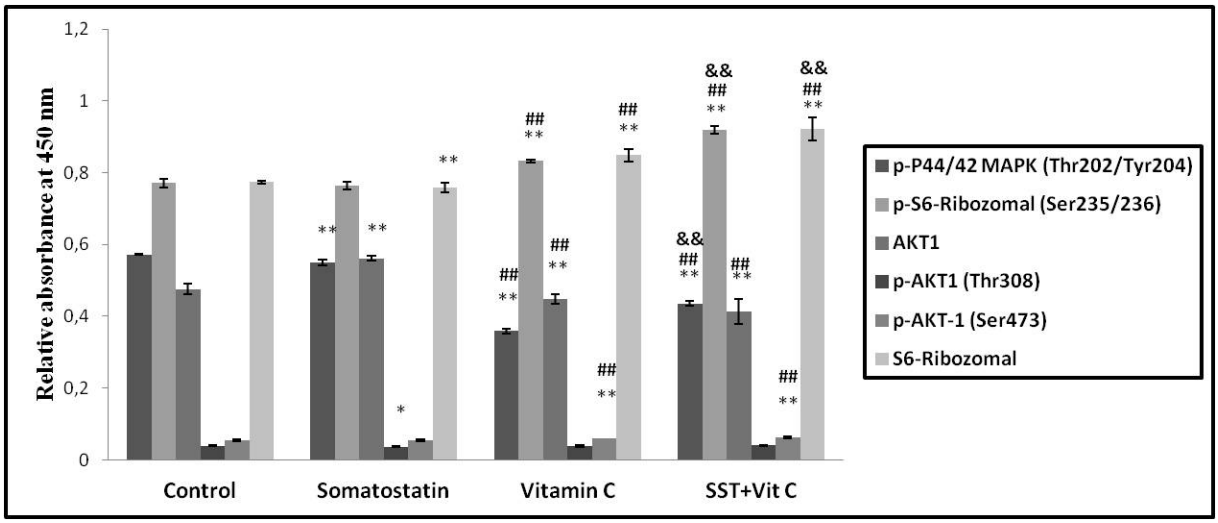

Fig. (6). Absorbances of the MAPK pathway members; AKT1, p-AKT-1(Ser473), p-AKT-1 (Thr308), p-P44/42 MAPK (Thr202/Tyr204), S6-Ribozomal and p-S6-Ribozomal (Ser235/236) measured in the MDA-MB231 cells after incubations with Somatostatin (SST), Vitamin C (Vit C) and SST + Vit C for 24 h (n=6). *,**Values significantly different from the control p <0.05, p < 0.01. $^{\&\&}$Values significantly different from the Vit C p <0.01. $^{\#\#}$Values significantly different from the SST p <0.01. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

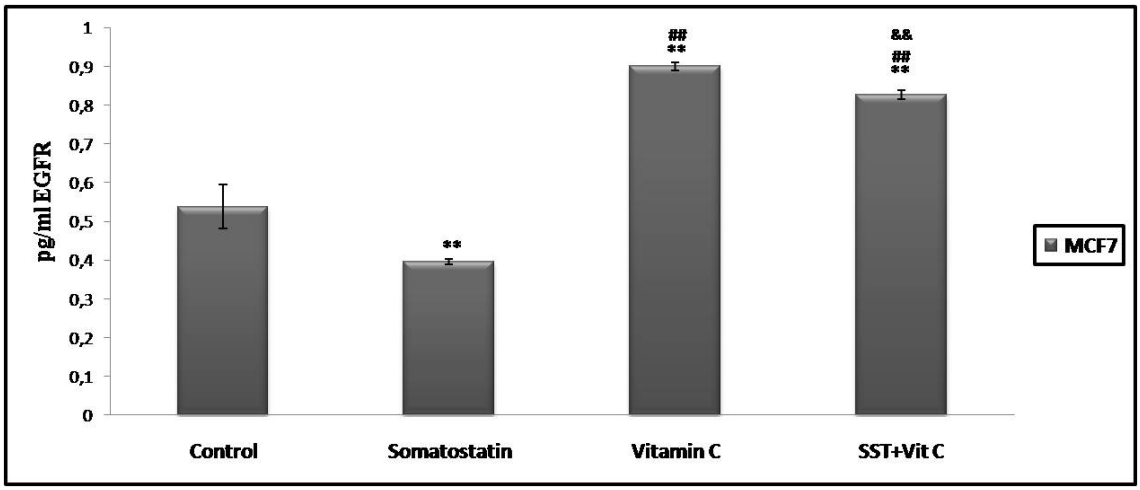

Fig. (7). EGFR levels in MCF-7 cells after incubation for 24h with SST, Vit C and SST + Vit C (n=6). **Values significantly different from the control p <0.01. $^{\&\&}$Values significantly different from the Vit C p <0.01. $^{\#\#}$Values significantly different from the SST p <0.01. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

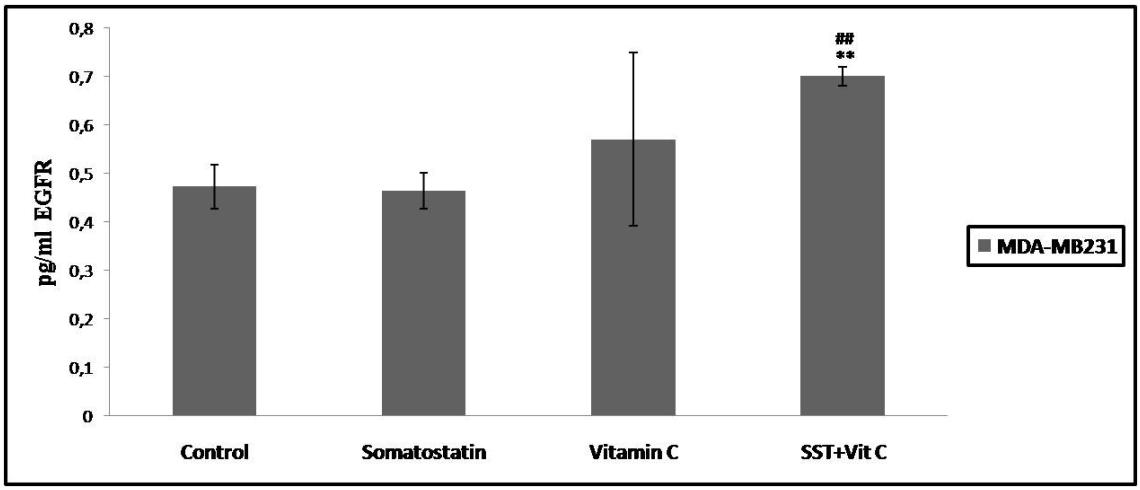

Fig. (8). EGFR levels in MDA-MB231 cells after incubation for 24h with SST, Vit C and SST + Vit C (n=6). **Values significantly different from the control p <0.01. $^{\&\&}$Values significantly different from the Vit C p <0.01. $^{\#\#}$Values significantly different from the SST p <0.01. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

compared to controls except in the SST+Vit C cells, where EGFR levels were significantly elevated compared to the controls.

4. DISCUSSION

We used MCF-7 and MDA-MB231 breast cancer cell lines, in order to investigate membrane fatty acid changes and signaling pathways related to incubation with SST and Vit C, alone and in combination. The role of cell membrane fatty acids and phospholipids in cancer growth is well known, starting from the simple evidence that cells cannot live without membranes. The need for fatty acids for cell growth is represented by an increased de novo lipogenesis with formation of palmitic acid and subsequent elongation/desaturation steps, forming all the other components of the SFA and MUFA families [39, 40]. Therefore, analysing the membrane lipidome of cultured cancer cells, important information can be obtained for treatments as well as for nutritional elements, having capacity to influence fatty acid formation and incorporation in cell membranes [41]. In membrane lipidome, detection/presence of trans fatty acids can be informative of free radical stress, that is generated also during chemotherapies. In our previous study, we reported the role played by antioxidant treatments in NTera-2 human testicular germ cancer cells treated with bleomycin and several antioxidants [35]. Our results shown in Tables **1** and **2** indicate an initial difference between MCF-7 and MDA-MB231 cell lines in terms of FFAs that was not known previously. In both cells, the relative percentages of SFA found in membrane phospholipids are similar, whereas the MUFA/PUFA ratio is different: MCF-7 membranes contain a 2:1 ratio and MDA-MB231 membranes contain a 1.2/1 ratio. The higher percentage of PUFA in MDA-MB231 cells indicates a different membrane arrangement, and it is noticeable that, within PUFA, the omega-6/omega-3 ratio is 1.2/1 whereas in MCF-7 cells it is 1.8/1. This is an important difference since the PUFA moieties of phospholipids are involved in cell signaling and their role for cancer risk and progression is highly debated [42]. The higher percentage of arachidonic acid in MDA-MB231 cells than in MCF-7 (8.50±1.68% *vs*. 5.48±1.01%) cells predisposes the cell signaling toward more inflammatory outcomes. It is then important to see the effects of SST and Vit C treatments on the PUFA moieties, that resulted to be different either for the cell type and for the fatty acids involved: 1) as reported in Table **1** and Figs. **1** and **2**, in MCF-7 cells, the levels of total PUFA ω6 and ω3 under our experimental conditions changed significantly in the combined SST + Vit C incubation compared to the control cells, and ω3 PUFA level significantly increased. The increase in ω3 PUFA was found also in the SST group compared to the controls; whereas Vit C alone did not cause any change in membrane fatty acid families. This is an important element that suggests to deepen SST interaction with membrane lipids; 2) as shown in Table **2** and Figs. **3** and **4** in MDA-MB231 cells treated with SST+Vit C the effect on ω3 PUFA was the opposite of MCF-7, since this FA family diminished, involving DPA and DHA, and the ω6/ω3 ratio changed from in favor of the first one. Such changes were not observed in the separated SST and Vit C experiments. This result suggests that depending on the cell type, the SST interaction can give different membrane reorganization and this can greatly influence the biological and pharmacological outcomes; 3) besides PUFA moieties, the other FA families involved in lipidome changes were not the same for MCF-7 and MDA-MD231 cells: in the former, the MUFA family significantly increased with the diminution of SFA, in the latter the significant increase of the SFA family and trans fatty acid levels was evidenced (Tables **1** and **2**).

The differences shown by the two cell lines are worthy of more comments on the fatty acid involvement, especially related to the MDA-MB231 cells, which are recognized to be resistant cell type. In this case, membrane lipidome changes point to the increase of SFA, which is an important biological effect. In fact, as mentioned in the introduction, it is well known that not only lipogenesis, but also desaturase enzymatic steps are necessary for cancer growth. These two metabolic pathways must be carefully evaluated for the tuning of antineoplastic activity: the increase of SFA detected with Vit C treatment in MDA-MB231 cells occurs without increase of MUFA. These changes point to two opposite outcomes for cancer cells: from one side to the diminution of the fluidity balance, that is associated with the suppression of cancer proliferation [40] and it is also known that SFA induce apoptosis in MDA-MB231 cells [43]. On the other side, SFA increase is interpreted as less reactivity of the membrane bilayer to free radical-mediated oxidation, thus diminishing the effects of chemotherapeutics [44]. In addition to these two aspects, we can contribute to this debate with our approach of fatty acid-based membrane lipidomics and fatty acid diversity. As a matter of fact, in MDA-MB231 cell the SFA-MUFA changes observed with the Vit C treatment are accompanied by the depletion of DHA and increase of trans isomers, which point to an overall effect on the fluidity loss, that can be detrimental for cell survival. In the treatment with Vit C combined with SST, other changes added to the previous ones, such as the significant diminution of linoleic acid, DPA and total ω3, all PUFA moieties again point to the fluidity diminution. Using PUFA and MUFA moieties of membrane lipids, we could also examine the effects of the two treatments concerning free radical stress, which is another crucial point to address together with the role of antioxidants. It is known that the natural unsaturated lipid cis geometry can be altered by the thiyl radical-catalysed cis-trans isomerization process, giving rise to trans lipids which are toxic for eukaryotic cells [36]. In particular, the biomarker role of trans fatty acid isomers in chemotherapy was advanced in bleomycin-treated cell lines [35]. The role of antioxidants as preventive effects for lipid isomerization was discussed and also considered to develop dietary strategies [45]. The formation of thiyl radicals can be induced by the increased oxidative state of cancer cells and for the drug mechanism, therefore it was interesting to evaluate the results with Vit C, a well known antioxidant molecule, and SST, a peptide containing sulfur moieties in the form of a disulfide bond. In both cell lines, trans isomers were detected in controls: in MCF-7 we found the trans isomer of linoleic acid (18:2 t, 0.30 ± 0.14) and trans isomers of arachidonic acid (20:4 t ω6, 0.03 ± 0.02), and in MDA-MB231 we found the trans isomer of linoleic acid (18:2 t, 0.24 ± 0.21) and trans isomers of arachidonic acid (20:4 t ω6, 0.08 ± 0.13), whereas for the trans isomer of oleic acid (18:1 9t), the levels were under the detection limits. In MCF-7 cells, with Vit C treatment, as well as in the treatment with SST+Vit C, 20:4 t became significantly elevated. In the MDA-MB231 cells 18:2 t was significantly increased with Vit C treatment as well as in the treatment with SST+Vit C, whereas 20:4 t was significantly elevated in both single treatments. We do not deepen the evaluation of the type of isomer involved in the transformation. The overall increase of trans isomers can be explained by the fact that the EC_{50} concentration of Vit C was used, a high dosage that produces pro-oxidant effects known in the Vit C-induced apoptosis [46]. It is important to investigate whether a high concentration of Vit C associated autoxidation is critical for its anticancer effects, particularly in breast cancer cells which maintain a higher intracellular H_2O_2 than normal cells suggesting breast cancer cells are able to accumulate and tolerate H_2O_2 within a certain range [21]. The results obtained following EGFR and MAPKs pathways are shown in Figs. **5-8** and the cascades of signals do not behave always similarly. SST is known to interact with SSTR in MCF-7 and MDA-MB231 cells inducing apoptosis with the status of MAPK signaling modulated in a cell-specific manner [47]. As far as the EGFR level is concerned (Figs. **7** and **8**), SST was the only treatment efficient to reduce the EGFR level in MCF-7 cells, whereas Vit C and combined SST+Vit C experiments increased it in both MCF-7 and MDA-MB231. In our experiments, the survival MAPK pathways was diminished in MDA-MB231 breast cancer cell lines incubated with STT, Vit C or with their combination by significantly diminishing p-P44/42 MAPK(ERK) level compared to the control group

in cells (Fig. **6**). The ERK was also affected in the same way by all treatments in MCF-7 cells. The rest of the signaling pathway was affected distinctively by the conditions used: p-AKT1 (Thr308) level was significantly increased in MCF-7 cells incubated with SST, Vit C and SST+Vit C, whereas p-AKT1 (Ser473) seemed to be more sensitive (increased) to the treatments in the MDA-MB231 cells. The phosphorylation of AKT is an important link in the cell apoptosis process. Phosphorylated AKT (p-AKT) proteins cause cell death and regulate various signaling pathways [48]. It is worth underlining here that in our study decreased cancer cell proliferation due to incubation with STT and SST+Vit C was evidenced by MTT viability assay and these results were further supported by downregulation of MAPK signaling pathway proteins such as ERK.

Having shown that in our experiments both the level of activation of proliferative cascades and fatty acid quality display specific modulation by each treatment, we can suggest the interplay between membrane composition and signaling as a crucial signature for the cell fate.

CONCLUSION

In conclusion, our results regarding two different breast cancer cell lines highlight the relevance of the membrane fatty acid monitoring to evidence basic differences in the membrane asset of SFA, MUFA and PUFA levels as well as of trans fatty acid isomers, which could account for metabolic differences relevant to cancer aggressivity. The lipid remodelling under incubation with STT and/or Vit C evidenced the specific lipid response to the conditions, consequent or synergic with the inactivation of the signaling pathways involved in the proliferation of cancer cells. These results can inspire further research and improve a better understanding of the complex scenario of cancer biology.

AUTHOR CONTRIBUTIONS

C.F. and T.O. formulated the idea and prepared the manuscript; C.C., C.F., T.O. designed the methodology and the research plan; A.H., E. K., F.H., G.M. performed experiments and organized the data and prepared all graphics; E.K., A.S., T.O., C. F., GDB supervised the data acquisition; G.D.B., C.C., T.O., C.F. discussed the results. All authors participated in the data discussion and ameliorated the draft. C.F. and T. O. acquired financial support.

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest regarding the study.

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