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Cardiolipin composition correlates with prostate cancer cell proliferation

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Abstract Prostate cancer (PC) is the second most diagnosed cancer in men. It has been recognized that diet can play a crucial role in PC genesis and progression. In this context, free fatty acids are considered as modulators of cell proliferation. Recently, a relationship between the composition of the mitochondrial phospholipid cardiolipin (CL) and cell proliferation has been discussed. The aim of this study was to analyse the interrelationship between CL composition and the proliferation of prostate cells by exposing PC-3 tumour cells to different fatty acids and by analysing the CL composition in prostate tissue from PC patients after prostatectomy. Among the applied fatty acids, palmitic acid was found to stimulate proliferation of PC-3 cells, whereas oleic acid (OA) had an inhibiting effect. The lipidomic analysis of CL revealed that fatty acids supplied to PC-3 cells were incorporated into CL molecules. Further, the CL content of palmitoleic acid (C16:1) exclusively correlated with the proliferation of PC-3 cells. The CL composition significantly differed between tumour and normal prostate tissue from PC patients. In five out of six patients, the CL content of palmitoleic acid was higher in tumour prostate tissue in comparison to normal prostate tissue. Our data illustrate that the composition of CL can be easily modified by the fatty acid environment of cells. OA was most effective in decreasing the amount of palmitoleic acid within the CL molecules and deceleration of PC-3 cell proliferation. In conclusion, a diet rich in OA might be beneficial in protecting from rapid proliferation of PC cells.

Keywords Prostate cancer · Free fatty acids · Cardiolipin · Lipidomic analysis · Tumour cell proliferation

Abbreviations

PC	Prostate cancer
CL	Cardiolipin
LA	Linoleic acid
SA	Stearic acid
AA	Arachidonic acid
DA	Docosahexaenoic acid
PA	Palmitic acid
OA	Oleic acid
ME	Methanol
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Introduction

PC is the second most commonly diagnosed cancer and the second most cause of cancer death of men in the USA [1]. Besides age and family history, there is increasing evidence that diet and life style play a crucial role in PC genesis [2]. In this context, it is of interest that there is evidence of increased incidence of PC in immigrant populations to the US and Europe compared to their countries of origin [3]. Several nutritional components have been evaluated with

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regard to their effect on PC. High fat diet, meat and milk intake have been recognized as risk factors for the development of PC, whereas polyphenols in green tea and tomatoes seem to be beneficial [4]. However, further studies are needed to shed more light on the mechanism of effects of particular nutritional constituents on PC initiation and progression [5].

It has been demonstrated that free fatty acids are able to alter the proliferation of tumour cells [6]. Stimulatory as well as inhibitory effects of free fatty acids on cell proliferation have been observed [7]. In their work, Hardy et al. also reported about fatty acid-induced changes in the mitochondrial phospholipid CL. However, the presented data do not allow conclusions about a causal link between CL and cell proliferation. Fatty acids are required for CL de novo synthesis. They are incorporated into the CL molecule as fatty acid residues. In this way, the supply of cells with specific fatty acids can affect the composition and distribution of molecular CL species. In a previous work, we have demonstrated an association between acyl-chain composition of CL and cell proliferation in lymphocytes from patients with acute and chronic leukaemia as well as in a Seax-tumour cell line [8]. This study revealed that fast growing lymphocytes are characterized by low numbers of double bonds within the CL-acyl chain residues, high content of C16:0 acyl chain residues and low amounts of C18:4 acyl chain residues in comparison to low proliferating lymphocytes.

The metabolism of high proliferating cells significantly differs from that of low or non-proliferating cells. To match the demands of cell growth, high proliferation cells depend on the establishment of an anabolic metabolism that is supported by glycolytic ATP production [9]. In contrast, low or non-proliferating cells receive their energy from oxidative phosphorylation. It has been shown that CL is associated with mitochondrial proteins, among them complexes of the mitochondrial respiratory chain [10]. Further, there is evidence that changes in composition and distribution of molecular CL species may be paralleled by impairment of oxidative phosphorylation [8, 11]. According to the hypothesis postulated by Warburg [12], of mitochondrial dysfunction in tumour cells, it is reasonable to assume that CL-mediated alterations of mitochondrial function may be essential for tumour genesis and progression.

The aim of this study was to analyse the composition and distribution of molecular CL species in biopsies from non-malignant and malignant tissue obtained from patients treated by radical prostatectomy. In addition, cells of a PC-3 tumour cell line were exposed to several fatty acids in order to study the interrelationship between cellular environment, composition and distribution of molecular CL species, as well as cell proliferation. For CL analysis, a LC–MS/MS-based lipidomic approach was applied.

Materials and methods

Cell culture

PC-3 cells were received from Cell Lines Service (Eppelheim, Germany) and stored at −196 °C prior to use. After thawing and washing, 5×10^6 cells were incubated in 30 ml of RPMI 1640 medium containing L-glutamin and 10 % fetal bovine serum (PAA Laboratory GmbH, Germany/The Netherlands) for seven days including two exchanges of media to allow initial cell growth. Subsequently, 2×10^5 cells were seeded in 2 ml RPMI medium with L-glutamin and 10 % FCS (fetal calf serum) in dishes of 35 mm diameter. The experiment was started by adding 50 µM fatty acids, 2 µM staurosporin or 20 µl methanol as vehicle control. Samples were withdrawn after 4 and 72 h for protein determination, MTT reduction analysis, lactate and cardiolipin analysis and estimation of caspase 3 activity. The cells were cultured in a gas atmosphere containing 5 % CO_2, 17 % O_2 and 78 % N_2 at 37 °C.

Determination of protein

The cells were dissolved in 1 % dodecylsulphate in the presence of 0.1 M NaOH. The amount of protein was determined by applying the method of Lowry et al. [13] using bovine serum albumin as the standard.

Determination of reduced MTT

Cells were incubated with 0.15 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in 2 ml medium for 30 min at 37 °C in a CO_2 incubator [14]. At the end of the incubation, the medium was removed and the formazan was extracted with 1 ml dimethylsulfoxide. The quantity of formazan was determined by measuring the absorption at 550 nm in the presence of 20 µl of 0.1 M NaOH to avoid pH-dependent changes in absorption.

Determination of lactate concentration

An aliquot of 1 ml cell suspension of duplicate dishes of cell cultures was used for the determination of lactate content for each incubation condition. For the determination of lactate, a Cobas 6000 c 501 analyser(Roche Diagnostics GmbH, Mannheim, Germany) was used. The measurements were conducted according to the instructions of the manufactures. All chemicals of the assay were from Roche Diagnostics GmbH, Mannheim (Germany).

Cardiolipin analysis

Extraction of CL: 50 ng of tetra-myristoyl-CL [$(C14:0)_4$-CL; Avanti Polar Lipids Inc., Alabaster, AL] was added as internal standard to 10 µl of samples. For extraction of CL, 4.2 ml chloroform/methanol (2/1, v/v) containing 0.05 % BHT was added. The lipid and aqueous phases were separated by adding 800 µl of 0.01 M HCl, intensive shaking and subsequent centrifugation. After centrifugation, the lipid phase (lower phase) was collected and dried under nitrogen atmosphere and acidified. Ice-cold methanol (2 ml), chloroform (1 ml) and 1 ml of 0.1 M HCl were added. The solution was intensively mixed. After 5 min of incubation on ice, the samples were separated by the addition of chloroform (1 ml) and 0.1 M HCl (1 ml). The chloroform/methanol phase was recovered as CL-containing sample. Afterwards, the samples were dried under nitrogen and dissolved in 0.8 ml chloroform/methanol/water (50/45/5, v/v/v). After mixing and filtering of the mixture over 0.2 µm PTFE membranes, the samples were ready for analysis.

HPLC–MS/MS analysis: For measurement of molecular CL species, a TSQ Quantum Discovery Max (Thermo Fisher Scientific GmbH, Dreieich, Germany) was used in the negative ion electrospray ionization (ESI) mode. The HPLC system consisted of a Surveyor MS quaternary narrow bore pump with integrated vacuum degasser and a Surveyor auto sampler. Auto sampler tray temperature was held at 8 °C. In partial loop mode, a sample of 10 µl of lipid extract dissolved in chloroform/methanol/water (50/45/5, v/v/v) was injected and CL was separated by using a LiChroCart column (125 mm × 2 mm), LiChrospher Si60 (5 µm particle diameter; Merck, Darmstadt, Germany) and a linear gradient between solution A (chloroform) and solution B (methanol/water 9:1, v/v). All solutions contained 25 % aqueous ammonia (0.1 ml/l). The gradient was as follows: 0–0.2 min 92 % solution A, 8 % solution B; 0.2–4.5 min 92–30 % solution A, 8–70 % solution B; 4.5–6 min 30 % solution A, 70 % solution B; 6–6.5 min 30–92 % solution A, 70–8 % solution B; 6.5–11 min 92 % solution A, 8 % solution B. The flow rate was 300 µl/min. Total time of analyses was 11 min. The eluate between 0.3 and 6 min was introduced into the mass spectrometer. Nitrogen was used as the nebulizing gas and argon as collision gas at a pressure of 1.5 mTorr. The spray voltage was 3.5 kV, the ion source capillary temperature was set at 375 °C and the cone-voltage was 30 V. Daughter fragments from the doubly charged parent derived from $(C18:2)_4$-CL with m/z 723.6 ($(M-2H)^{2-}/2$) were obtained using a collision energy of 36 eV. This molecular CL species and the internal standard (m/z 619.6) were analysed by mass transfer reaction monitoring their doubly charged ions and their respective fatty acids linoleic acid m/z 279.2 and myristic acid m/z 227.2 using the selected reaction monitoring (SRM) mode. The same approach was used for parent and daughter fragments of other molecular species of CL. The quantity of these molecular species was related to the content of $(C18:2)_4$-CL. Oxidized CL [$(C18:2)_3$ monohydroxylinoleic acid-CL] was measured in the SRM mode as a transition from m/z 731.6 to m/z 279.2 (linoleic acid).

Prostate tissue samples

PC samples were analysed from patients treated by radical prostatectomy. At the time of surgical intervention, the age of the patients was between 63 and 75 years. PC stage was T2c, T3a and T3b, respectively, and the Gleason score ranged from 7 to 9. The concrete assignment of the stage and Gleason score of the patients is provided within the result section. Tumour tissue samples were taken from PC patients who underwent standard radical prostatectomy in the Department of Urology, University Medicine Greifswald. Tissue samples were immediately frozen in liquid nitrogen, and histological evaluation was done by experienced pathologists. Control sections of the PC tissue samples were cut into 4-µm-thick sections using a microtome and standard HE (hematoxylin/eosin) staining was performed. Areas of malignant cells were highlighted in a photography of the PC sample and subsequently used for harvesting malignant PC cells and adjacent non-malignant control cells for further analysis.

After histological evaluation, PC tissue samples were stored at −80 °C. This study was approved by the Ethics Committee of the University Medicine Greifswald (Registration No. BB 21/12) and all patients signed informed consent forms. All tissue samples and related clinical data were obtained from the Department of Urology, University Medicine Greifswald.

Statistical analysis

Data are presented as mean ± SEM. For statistical analysis, unpaired Student's t test was used. Differences were considered as significant with $p < 0.05$.

Results

Effect of fatty acids on growth of PC-3 prostate tumour cells

To get detailed information about PC-3 cell growth in the presence of fatty acids, we followed protein content over 72 h of cultivation in comparison to the vehicle control. As a control, the effect of the apoptosis inducer staurosporin

(Stau), known as caspase-3 activator, on protein content was tested reflecting loss of total protein by induction of apoptosis. In the slowly growing control cultures, the protein of the cultures increased from 1.03 ± 0.19 mg/ml after 4 h to 1.86 ± 0.11 mg/ml after 72 h of cultivation. The presence of the fatty acids SA (82 ± 5 % of control, $p = 0.049$), AA (86 ± 4 % of control, $p = 0.039$) and DA (81 ± 6 % of control, $p = 0.047$) as well as Stau(47 ± 12 % of control, $p = 0.003$) resulted in significant lower protein values after 72 h of cultivation (Fig. 1a). The protein content of a cell culture does not necessarily reflect the amount of viable cells, since dead cells also contribute to the protein content. Therefore, we determined in a second test the ability of the cells to reduce MTT. This parameter is a measure of the amount of vital cells. The corresponding data are presented in Fig. 1b. In this presentment, it turns out that only the presence of PA moderately elevated the ability for MTT reduction (106.01 ± 4.24 MTT reduction in % of control, $p = 0.17$), whereas linoleic acid (LA), arachidonic acid (AA) and OA decreased it. In the presence of these fatty acids, the amount of reduced MTT was about 20 % lower in comparison to the vehicle control after 72 h of cultivation.

The ability of cells to reduce MTT is a metabolic activity that may depend on the availability of substrates such as fatty acids. To estimate the cellular efficiency for MTT reduction after exposure of the cells with Stau of fatty acids, we calculated the ratio of MTT reduction and protein (specific MTT reduction). The result is depicted in Fig. 1c. In the presence of Stau (24.2 ± 3.7 % of control, $p = 0.001$), the specific MTT reduction was significantly decreased after 72 h of cultivation. Likewise, the fatty acids LA (89 ± 6 % of control, $p = 0.099$), AA (92 ± 4 % of control, $p = 0.084$) and OA (86 ± 6 % of control, $p = 0.23$) caused decrease in specific MTT reduction in tendency indicating at least partial impairment of the ability of the cells for MTT reduction. On the basis of these results, we consider MTT reduction (Fig. 1b) as a suitable parameter for the documentation of growth of metabolic vital PC-3 cells.

It is commonly assumed that tumour cells essentially differ from non-tumour cells in energy metabolism (Warburg hypothesis). They are characterized by a high glycolytic activity generating lactate as end product, even in the presence of oxygen. An applicative parameter for glycolytic activity is the concentration of lactate in the cell culture medium. Therefore, lactate concentration has been used as tumour marker in tissues. We determined lactate concentration in dependence on the presence of fatty acids in the culture medium after 72 h of cultivation. Figure 2 displays that the presence of PA in the culture medium was associated with about 25 % lower lactate concentration in comparison to the vehicle control. The other fatty acids caused in tendency lower lactate concentration in comparison to the vehicle control within 72 h of cultivation as well. In neither case, higher lactate concentrations than in controls were detected. These observations suggest that, in

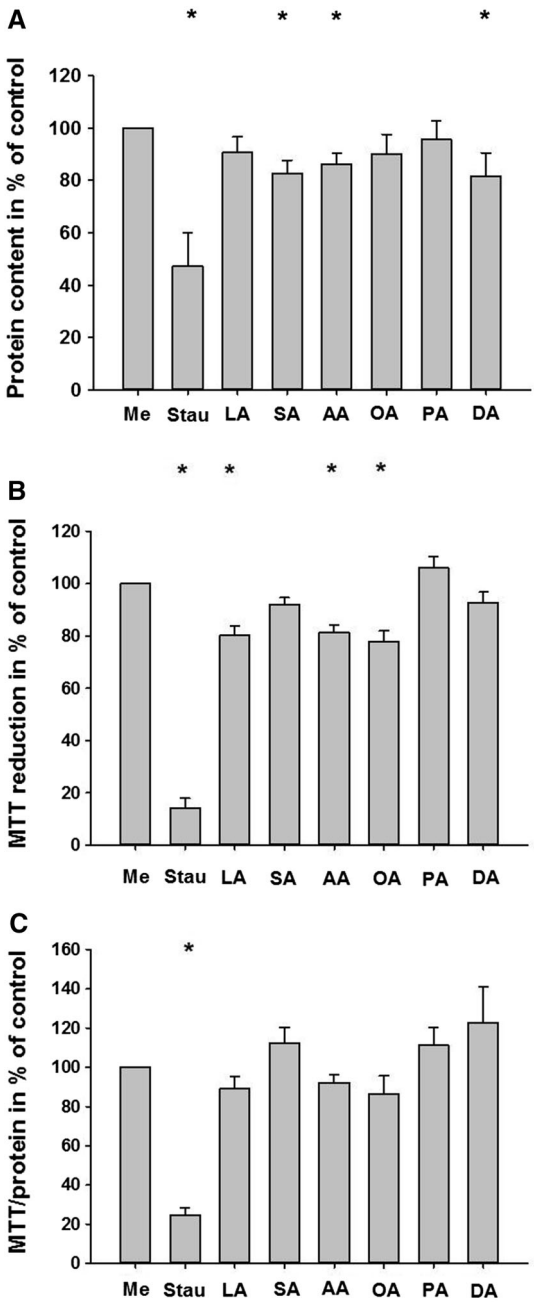

Fig. 1 Effect of fatty acids on proliferation of PC-3 cell cultures. Protein content and MTT reduction of PC-3 cells were analysed in dependence of external fatty acids (*LA* linoleic acid, *SA* stearic acid, *AA* arachidonic acid, *OA* oleic acid, *PA* palmitic acid, *DA* docosahexaenoic acid) and methanol (ME) as vehicle after 72 h of cultivation in the presence of the indicated fatty acids. Data of protein values (**a**), MTT reduction (**b**), and the ratio of both (**c**) are presented. Data of six cultures are presented as mean ± SEM. *Difference to methanol control was significant with $p < 0.05$

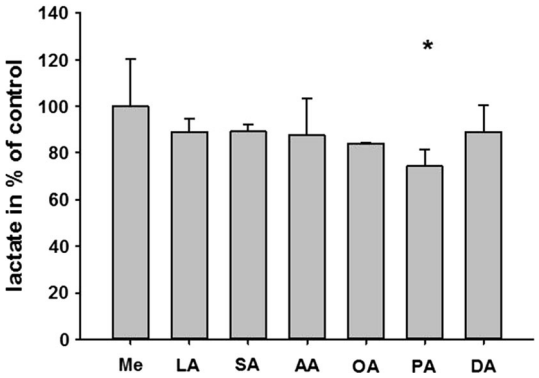

Fig. 2 Effect of fatty acids on lactate production of PC-3 cells. PC-3 cells were cultured in the presence of the indicated fatty acids (*LA* linoleic acid, *SA* stearic acid, *AA* arachidonic acid, *OA* oleic acid, *PA* palmitic acid, *DA* docosahexaenoic acid) and methanol (ME) as vehicle. After 72 h the accumulated lactate was determined. Data of six cultures are presented as mean ± SEM. *Difference to methanol control was significant with $p < 0.05$

contradiction to Warburǵs hypothesis, lactate concentration did not correlate with the proliferation rate of PC-3 cells under our experimental conditions.

Cardiolipin and PC progression

To investigate the interrelationship between the composition of CL and the progression of PC cells, we additionally analysed the distribution of molecular CL species, total CL content, oxidized CL and the incorporation of fatty acids in the CL molecules in dependence of the presence of fatty acids in the cell culture medium. In Table 1, the distribution of molecular CL species is presented. These data clearly demonstrate that the presence of specific fatty acids in the incubation medium results in significant changes in distribution of molecular CL species in comparison to controls. In particular, it becomes evident that the supply of specific fatty acids caused their increased incorporation into CL molecules containing each four fatty acid residues. Thus, the presence of LA in the cell culture medium resulted in a strong elevation of the relative content of $(C18:2)_4$-CL from 2.7 ± 0.33 % of total CL (vehicle control) to 33.52 ± 2.74 % of total CL. Likewise, the other fatty acids caused increase in the amount of such molecular CL species that contain these particular fatty acids in their acyl chain residues. In conclusion, the presence of particular fatty acids in the culture medium can modify the distribution of molecular CL species.

The sum of the determined CL species (total amount of CL) was also affected by fatty acids in the cell culture medium. Figure 3 demonstrates that the presence of LA caused dramatic elevation of the CL content by a factor of about 5, whereas the presence of AA in the cell culture medium resulted in the reduction of the CL content to about 50 % of vehicle control (0.32 ± 0.04 nmol/mg protein versus 0.59 ± 0.08 nmol/mg protein).

To test the possibility that fatty acid-dependent changes in CL composition affect oxidation of CL, we determined the quantity of oxidized LA residues in $(C18:2)_4$-CL as marker of CL oxidation in dependence of the presence of fatty acids in the cell culture medium. The corresponding data are depicted in Fig. 4. Significant decrease in oxidized $(C18:2)_4$-CL was only observed in the presence of AA, OA and PA. The other fatty acids had no effect on this parameter in comparison to the vehicle control. Based on these data, we conclude that the presence of fatty acids in the culture medium causes changes in CL composition, total CL content and CL oxidation. However, changes in CL content and CL oxidation did not correlate with MTT reduction and concomitantly not with the proliferation of PC-3 cells.

The next analysis focused on the question whether the composition of CL is causally linked to proliferation of PC-3 cells. Therefore, we analysed the relationship between the amount of fatty acids that were incorporated in the CL molecules and MTT reduction after cultivation in the presence of different fatty acids. As shown in Fig. 1b, fatty acids are able to modulate proliferation within a window between 77 and 106 % with respect to the control culture. The content of all incorporated fatty acids was separately correlated with MTT reduction. Among all investigated incorporated fatty acids, only the content of palmitoleic acid in CL displayed a clear correlation with MTT reduction (correlation coefficient: $r^2 = 0.67$, Fig. 5).

On the basis of these results with the PC cell line PC-3, we analysed CL composition in tissues of PC patients after total prostate resection. In particular, CL composition of non-malignant tissue was compared with CL composition of tumour tissue of each PC patient. The data are summarized in Table 2. It is evident that the CL composition of these tissue samples basically differed from that found in PC-3 cells. This primarily concerns the content of $(C18:2)_4$-CL that was higher in the tissues of tumour patients. Most of interest was the direct comparison between non-malignant and malignant tissues in each individual. The direct comparison of the distribution of molecular CL species between non-malignant and malignant prostate tissue revealed significant differences in 4 out of 6 patients. Most impressive was the observation of lower contents of $(C18:2)_4$-CL in tumour tissues. Further evident differences were found in the content of up to 13 additional molecular CL species (for example, see data of patient 5 in Table 2). However, direction of changes in CL composition between normal and tumour tissue was not equal in all cases (compare values of patient 5 with patient 6).

Table 1 Effect of fatty acids on the composition of molecular CL species in PC-3 cells

Supplement Molecular CL-species	Me Content in % of total	LA	SA	AA	OA	PA	DA
$(C18:2)_4$-CL	2.70 ± 0.33	33.52 ± 2.74*	2.56 ± 0.69	4.72 ± 0.54*	1.63 ± 0.49*	2.49 ± 0.28	3.64 ± 0.51*
$(C18:2)_3$C18:1-CL	3.33 ± 0.43	20.00 ± 3.70*	3.80 ± 0.85	4.00 ± 0.56	1.04 ± 0.22*	3.30 ± 0.56	4.25 ± 0.65
$(C18:2)_2/(C18:1)_2$-CL	7.89 ± 0.83	8.59 ± 0.35	10.91 ± 1.39	5.87 ± 0.41*	4.63 ± 0.33*	6.48 ± 0.56	8.31 ± 1.05
C18:2/C18:1/$(C16:0)_2$-CL	1.44 ± 0.39	1.18 ± 0.36	1.16 ± 0.43	1.48 ± 0.76	0.73 ± 0.51*	1.63 ± 0.26	1.61 ± 0.69
$(C18:1)_2/(C16:0)_2$-CL	23.49 ± 2.59	1.25 ± 0.39*	14.48 ± 1.89*	23.33 ± 2.20	19.88 ± 4.45	29.88 ± 2.87	16.52 ± 2.60*
$(C18:2)_3$/C16:1-CL	1.12 ± 0.23	4.13 ± 0.35*	1.07 ± 0.38	1.62 ± 0.30	0.48 ± 0.31*	1.22 ± 0.20	1.54 ± 0.39
$(C18:2)_2$/C18:1/C16:1-CL o. $(C18:2)_3$/C16:0-CL	4.15 ± 0.19	4.73 ± 0.58	3.79 ± 0.42	3.02 ± 0.47*	1.87 ± 0.49*	4.87 ± 0.48	4.23 ± 0.42
$(C18:1)_2$/C18:2/C16:1-CL o. $(C18:2)_2$/C18:1:C16:0-CL	9.22 ± 0.76	1.66 ± 0.76*	7.99 ± 1.18	4.75 ± 0.93*	3.33 ± 0.84*	11.33 ± 1.34	7.48 ± 1.12
$(C18:1)_2$/C18:2/C16:0-CL	4.31 ± 0.14	1.71 ± 0.52*	4.43 ± 0.29	2.91 ± 0.48*	1.57 ± 0.18*	4.70 ± 0.39	4.09 ± 0.37
$(C18:1)_3$/C16:0-CL	18.89 ± 2.24	0.30 ± 0.06*	16.80 ± 3.85	12.36 ± 3.78	42.55 ± 2.87*	14.83 ± 3.03	13.12 ± 2.95
$(C18:1)_2/(C18:3)_2$-CL	2.44 ± 0.20	0.55 ± 0.04*	1.73 ± 0.26	4.68 ± 1.28*	0.67 ± 0.17*	2.40 ± 0.27	6.09 ± 1.17*
$(C18:1)_3$/C18:2-CL	8.09 ± 1.22	2.01 ± 0.11*	12.11 ± 2.61	5.85 ± 1.41	12.83 ± 4.03*	5.70 ± 0.87	7.37 ± 1.37
$(C18:2)_3$/C20:4-CL	2.69 ± 2.13	3.72 ± 0.69	3.58 ± 3.50	6.15 ± 4.28*	2.52 ± 2.77	2.25 ± 1.77	5.20 ± 2.99
$(C18:2)_3$/C20:3-CL o. $(C18:2)_2$/C18:1/C20:4-CL	2.63 ± 0.58	5.86 ± 0.83*	3.93 ± 2.57	4.84 ± 2.09	1.17 ± 0.88*	1.92 ± 0.60	5.04 ± 0.98*
$(C18:2)_3$/C20:2-CL o. $(C18:2)_2$/C18:1/C20:3-CL	3.10 ± 0.35	7.01 ± 1.03*	4.51 ± 1.24	7.67 ± 1.05*	1.78 ± 0.51*	2.38 ± 0.33*	4.52 ± 0.41
$(C18:2)_2$C18:1/C20:2-CL	2.57 ± 0.27	2.75 ± 0.45	4.64 ± 0.80*	4.01 ± 0.61*	1.41 ± 0.27*	2.24 ± 0.25	3.30 ± 0.22*
$(C18:2)_3$/C20:0 o. $(C18:1)_2$C18:2/C20:2-CL	1.58 ± 0.65	1.02 ± 0.26	2.36 ± 0.53	2.70 ± 1.66*	1.84 ± 1.04	1.42 ± 0.76	2.04 ± 0.95
C22:6/$(C18:2)_3$-CL	0.27 ± 0.07	0.01 ± 0.00*	0.10 ± 0.07*	0.02 ± 0.02*	0.08 ± 0.06*	0.67 ± 0.35	1.36 ± 0.31*
C22:6/$(C18:2)_2$/C18:1-CL	0.07 ± 0.03	0.00 ± 0.00	0.05 ± 0.06	0.02 ± 0.02	0.02 ± 0.01*	0.28 ± 0.16*	0.28 ± 0.07*

PC-3 cells were exposed to the indicated fatty acids. After 72 h of cultivation, molecular CL species were analysed by applying mass spectroscopy. Data of six cultures are presented as mean ± SEM

* Difference to methanol control was significant with $p < 0.05$

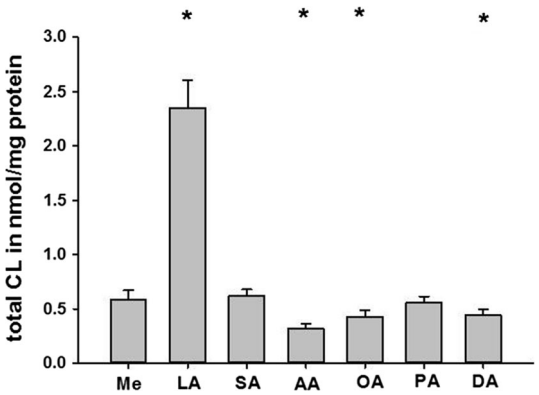

Fig. 3 Effect of fatty acids on the content of CL in PC-3 cells. The content of CL in PC-3 cells was determined that were exposed to the indicated fatty acids (*LA* linoleic acid, *SA* stearic acid, *AA* arachidonic acid, *OA* oleic acid, *PA* palmitic acid, *DA* docosahexaenoic acid) and methanol (ME) as vehicle after 72 h of cultivation. Data of six cultures are presented as mean ± SEM. *Difference to methanol control was significant with $p < 0.05$

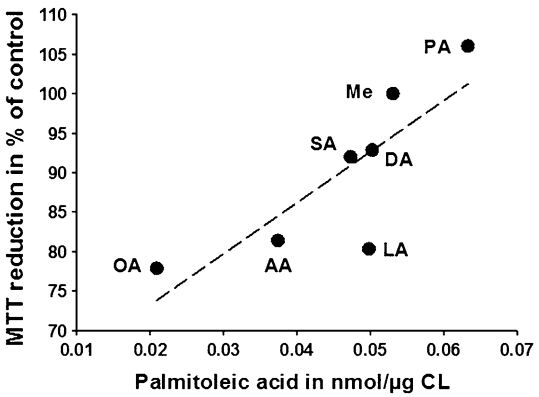

Fig. 5 Relationship between proliferation of PC-3 cells and the content of palmitoleic acid within CL. After treating PC-3 cells with the indicated fatty acids for 72 h of cultivation, MTT reduction and the content of palmitoleic acid within CL were determined. Data of six cultures are presented as mean ± SEM. *Difference to methanol control was significant with $p < 0.05$

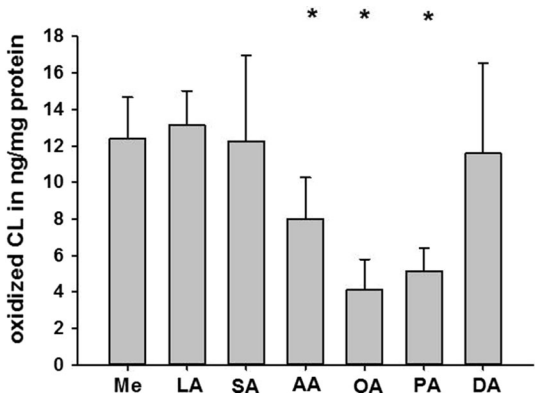

Fig. 4 Effect of fatty acids on CL oxidation in PC-3 cells. The amount of $(C18:2)_3$ monohydroxylinoleic acid-CL in PC-3 cells was determined after cultivation in the presence of the indicated fatty acids (*LA* linoleic acid, *SA* stearic acid, *AA* arachidonic acid, *OA* oleic acid, *PA* palmitic acid, *DA* docosahexaenoic acid) and methanol (ME) as vehicle for 72 h. Data of six cultures are presented as mean ± SEM. *Difference to methanol control was significant with $p < 0.05$

The lipidomic analysis of CL composition in PC-3 cells revealed the exceptional relevance of palmitoleic acid incorporated in CL molecules with respect to cell proliferation. Therefore, we determined the content of incorporated palmitoleic acid in CL molecules in tissues of PC patients. In Fig. 6, the differences in palmitoleic acid content between tumour tissue and benign tissues of the patients are presented. In the tissues of five patients out of six, the palmitoleic content of CL was in malignant tissue higher in comparison to non-malignant controls. The difference was the highest in patients 1 and 5. In line with the extraordinary CL composition of the tumour tissue of patient 6 is the finding of smaller palmitoleic acid content in CL in the tumour tissue of this patient.

Discussion

This study aimed to investigate the interrelationship between proliferation of PC cells and CL composition. In this context, free fatty acids were used to actively alter CL composition. Several aspects of the interplay between CL composition and cell proliferation can be investigated by using PC cell lines. In this study, the well-established PC model cells PC-3 were applied as an androgen-independent and androgen receptor-negative PC cell type reflecting an advanced stage of PC progression [15].Even though PC is a fairly slow growing tumour entity, however, PC-3 cells have been demonstrated as comparatively fast growing cells within the panel of PC cell lines [16].

Cell proliferation is commonly documented by measuring DNA-synthesis rate, cell number, protein and/or MTT reduction. In contrast to the other parameters, MTT reduction additionally covers metabolic activity [17]. In this study, we applied protein content and MTT reduction to record the development of the cell cultures. Both parameters reflected the growth of PC-3 cell cultures within 72 h as well as the influence of external free fatty acids on this process. However, the effect of fatty acids on protein values and MTT reduction quantitatively differed. Likewise, the ratio of MTT reduction and protein content differed among the incubation conditions. We take this as an indication for the mixture of metabolic active (alive) and metabolic inactive (dead) cells within the cultures. Since we were interested in studying the development of metabolically active tumour cells, we focused on MTT reduction as marker for cell proliferation. On the basis of our experiments, we recognized LA, AA and OA as proliferation inhibiting fatty acids and PA as proliferation

Table 2 Composition of molecular CL species in normal and tumour tissue of patients with prostate carcinoma

Patient	1		2		3		4		5		6	
Stage	T 3a		T 3a		T 2c		T 2c		T 3b		T 2c	
Tissue	Control	Tumour	Control	Tumour	Control	Tumour	Control	Tumour	Control	Tumour	Control	Tumour
Molecular CL-species	Content in % of total											
$(C18:2)_4$-CL	33.33	27.50	38.44	26.82	35.95	36.98	36.52	38.22	41.90	21.51	40.33	28.92
$(C18:2)_3$/C18:1-CL	23.35	25.10	23.98	23.97	22.78	21.56	26.18	25.66	22.85	21.87	25.77	26.86
$(C18:2)_2$/$(C18:1)_2$-CL	10.93	13.66	9.64	12.10	9.15	7.98	10.88	9.69	7.38	15.87	9.12	12.31
C18:2/C18:1/$(C16:0)_2$-CL	0.51	0.16	0.22	0.28	0.39	0.47	0.11	0.16	0.25	0.17	0.11	0.43
$(C18:1)_2$/$(C16:0)_2$-CL	4.94	2.62	1.95	3.77	4.26	4.20	1.73	1.95	1.81	4.10	1.03	2.19
$(C18:2)_3$/C16:1-CL	2.33	2.93	1.57	1.46	1.99	2.28	2.40	3.16	1.99	2.26	2.20	1.63
$(C18:2)_2$/C18:1/C16:1-CL o. $(C18:2)_3$/C16:0-CL	1.68	2.74	1.02	1.38	1.24	1.48	1.53	2.01	1.26	3.37	1.50	1.46
$(C18:1)_2$/C18:2/C16:1-CL o. $(C18:2)_2$/C18:1/C16:0-CL	0.75	1.32	0.56	0.82	0.49	0.54	0.61	0.77	0.60	2.73	0.63	0.94
$(C18:1)_2$/C18:2/C16:0-CL	0.40	0.58	0.38	0.57	0.32	0.28	0.30	0.34	0.32	1.01	0.29	0.53
$(C18:1)_3$/C16:0-CL	0.41	0.63	0.41	0.54	0.30	0.26	0.33	0.29	0.49	1.85	0.29	0.41
$(C18:1)_2$/$(C18:3)_2$-CL	0.13	0.16	0.12	0.12	0.12	0.10	0.08	0.12	0.10	0.21	0.17	0.27
$(C18:1)_3$/C18:2-CL	2.37	3.14	1.89	2.71	1.70	1.55	2.09	1.74	1.37	5.89	1.63	2.39
$(C18:2)_3$/C20:4-CL	2.74	1.54	2.39	1.47	3.04	3.49	2.09	2.21	3.50	1.42	2.24	2.16
$(C18:2)_3$/C20:3-CL o. $(C18:2)_2$/C18:1/C20:4-CL	7.81	5.90	6.74	7.35	8.77	9.46	6.45	6.60	7.92	5.82	6.60	6.84
$(C18:2)_3$/C20:2-CL o. $(C18:2)_2$/C18:1/C20:3-CL	5.18	6.59	6.00	8.83	5.67	5.93	5.10	4.50	5.46	6.45	4.86	7.06
$(C18:2)_2$/C18:1/C20:2-CL	2.37	4.08	3.16	5.46	2.28	2.08	2.66	1.91	2.09	4.12	2.36	4.36
$(C18:2)_3$/C20:0-CL o. $(C18:1)_2$/C18:2/C20:2-CL	0.67	1.28	0.92	1.68	0.66	0.58	0.79	0.46	0.49	1.18	0.59	1.08
C22:6/$(C18:2)_3$-CL	0.08	0.07	0.38	0.43	0.41	0.45	0.05	0.07	0.10	0.04	0.15	0.05
C22:6/$(C18:2)_2$/C18:1-CL	0.03	0.02	0.12	0.14	0.38	0.23	0.02	0.03	0.02	0.01	0.03	0.01

After prostate resection, normal and tumour tissue was evaluated by pathologists including staging of the tumour. Specimens of both tissue types of each patient were used for the lipidomic analysis of molecular CL species. The data of six patients are presented

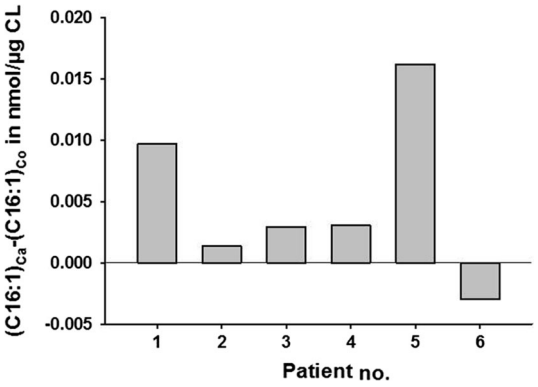

Fig. 6 CL content of palmitoleic acid in tumour tissues of patients with prostate carcinoma. The content of palmitoleic acid within CL in tumour (Ca) and normal tissues (Co) of patients with prostate cancer was determined after evaluation of the biopsies by pathologists. Data of six individuals are presented

stimulating fatty acid in PC-3 cells. The influence of free fatty acids on proliferation has been recognized for several tumour entities and model systems [17–19]. In particular, in the case of PC, dietary intake of LA promotes PC progression in humans [20], whereas other polyunsaturated fatty acids such as DHA and eicosapentaenoic acid (EPA) decrease prostate cancer development [21, 22]. One can speculate that the difference to our results may be due to the diversity of PC cells in patients and cell lines such as PC-3 cells. This observation reveals that the interrelationship between PC cell type, specific fatty acids and tumour cell proliferation still remains unclear.

There is evidence that lipid metabolism plays an essential role in tumour progression [23]. High lipid synthesis is required for the establishment of new cell membranes. In order to shift the metabolic status from catabolism to anabolism, oxidative phosphorylation needs to be adapted to anabolic conditions. In this context, Otto Warburg hypothesized that tumour cells are characterized by anaerobic glycolysis and lactate production [9]. In our experiments, PC-3 cells produced lactate under all conditions. Although OA clearly reduced PC-3 cell proliferation, the reduction in lactate production did not reach the level of significance. Most surprisingly, the treatment of the cells with PA stimulated cell proliferation but significantly reduced lactate production. Thus, our results suggest that in the presence of PA, PC-3 cells use oxidative phosphorylation to meet the energy demand for the stimulation of proliferation in contradiction to Warburgs hypothesis. The deviation of tumour cell metabolism from Warburgs hypothesis also has been reported by other groups [24, 25].

The phospholipid CL has been shown to be essential for proper oxidative phosphorylation [26]. Additionally, it has been recognized that the composition of molecular CL species and distribution of fatty acid residues are of significant impact for physiological function of CL [27]. Therefore, it is reasonable to assume that fatty acid-depending changes in proliferation of PC-3 cells may be linked to changes in CL composition as the consequence of different incorporations of fatty acids into CL molecules. In fact, we have detected impressive changes in CL composition of PC-3 cells after treatment with different fatty acids. Those changes were related to CL content as well as to distribution of molecular CL species. Thereby, the administration of particular fatty acids not only caused incorporation of this species but also induced changes in the CL content of other fatty acids. This observation points towards a complex relationship between cellular lipid metabolism and CL synthesis.

Administration of free fatty acids to tumour cells causes extensive effects on lipid metabolism. Numerous changes in the composition of triacylglycerides and phospholipids including CL are initiated by exposing cells to fatty acids. In order to designate changes in lipid composition of a particular lipid to cellular function such as cell proliferation requires specific concepts. A straight forward strategy is the genetic manipulation of the synthesis of the particular lipid. Alternatively, one can surge for explicit correlations between lipid composition and cellular function. Here, we correlated the amount of specific fatty acids that were incorporated into CL to the proliferation of PC-3 cells. Among the fatty acids that were detected within CL, only the amount of palmitoleic acid strongly correlated with proliferation. This observation is a strong indication for a causal relationship between CL composition and cell proliferation. In contrast, CL content did not correlate with proliferation. This is impressive documented by the finding that administration of PC-3 cells with LA caused about fivefold increase in CL content but decrease in proliferation, whereas the administration with AA was accompanied by decrease in both CL content and proliferation. It has been shown that drugs targeting mitochondrial function possess anti-cancer potential [28]. It is further known that lowering the CL content below a threshold level leads to the impairment of mitochondrial function [29].

It had been manifold demonstrated that oxidative stress can induce apoptosis in several cell types [30]. Oxidative conditions may be accompanied by increased oxidation of CL. However, the content of oxidized CL did not correlate with the proliferation of PC-3 cells. Thus, fatty acid-dependent induction of oxidative stress seems not to be the main factor for stimulation of PC-3 cell proliferation under our experimental conditions. A reason for that may be that tumour cells can tolerate relative high concentrations of reactive oxygen species [31].

Based on the results we obtained in the PC-3 in vitro model, it is conclusive that CL could play a role in the proliferation of human PC cells under in vivo conditions. In

our study, the 6 patients who were included in the analysis received total prostate resection. Because of the high variety in age, tumour stage as well as the heterogeneity in the composition of tissue samples, the few numbers of patients in this preliminary study led us to dissociate from statistical analysis. Instead of that we present, the data of each individual separately. Although we did not found clinical parameters (e.g. patients' age, body mass index, Gleason score, prostate mass) that correlated to the CL pattern, the PC tissue analysis underscored the findings of our cell culture experiments with respect to the correlation of CL composition and cell proliferation. We detected differences in nearly all of the analysed molecular CL species between cancer and normal tissue at least in 4 out of 6 patients. These differences in CL composition between normal and cancer tissue indicate significant distinctions in lipid metabolism, specifically in CL metabolism. It should be noted that the differences in CL composition between normal and tumour tissue were specific for each patient. Reasons for this observation could be the constitution of the tissue samples due to anatomical conditions and other individual factors such as stage of the tumour. The impact of individual factors on the CL composition of tissue samples is also reflected by the scattering of the data in normal tissue samples between the patients. The finding that the content of palmitoleic acid within the CL molecules of PC tissue was in 5 patients higher than in CL molecules of normal tissues points towards higher proliferation rates within the tumour tissues. This conclusion results from the data we generated with PC-3 cell cultures.

Conclusions

Our data on a PC-3 cell line illustrate that the composition of CL can be easily modified by changing the fatty acid environment of cells and that fatty acids in the culture medium can modulate cell proliferation. Among the fatty acids investigated, OA was most effective in deceleration of PC-3 cell proliferation. Under all conditions, the incorporation of palmitoleic acid into CL correlated with the proliferation rate of the cells. Preliminary data of prostate tissues of PC patients have demonstrated that the composition of CL differed between normal and tumour tissue and that the content of palmitoleic acid was increased in tumour tissues in 5 out of 6 PC patients. In conclusion, a diet rich in OA might be beneficial in protecting from rapid proliferation of PC cells.

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Compliance with ethical standards

Conflict of interest There is no conflict of interest.

References

1. Siegel R, Naishadham D, Jemal A (2013) Cancer statistics. Cancer J Clin 63:11–30
2. Chan JM, Van Blarigan EL, Kenfield SA (2014) What should we tell prostate cancer patients about (secondary) prevention? Curr Opin Urol 24:318–323
3. Shimizu H, Ross RK, Bernstein L, Yantani R, Henderson BE, Mack TM (1991) Cancers of the prostate and breast among Japanese and white immigrants in Los Angeles Country. Br J Cancer 63:963–966
4. Zhu Y, Wang HK, Qu YY, Ye DW (2015) Prostate cancer in East Asia: evolving trend over the last decade. Asian J Androl 17:48–57
5. Mandair D, Rossi RE, Pericleous M, Whyand T, Caplin ME (2014) Prostate cancer and the influence of dietary factors and supplements: a systematic review. NutrMetab (Lond) 11:30
6. Hardy S, El-Assaad W, Przybytkowski E et al (2003) Saturated fatty acid-induced apoptosis in MDA-MB-231 breast cancer cells. A role for cardiolipin. J Biol Chem 278:31861–31870
7. Murray M, Hraiki A, Bebawy M, Pazderka C, Rawling T (2015) Anti-tumor activities of lipids and lipid analogues and their development as potential anticancer drugs. Pharmacol Ther 150:109–128
8. Schild L, Lendeckel U, Gardemann A, Wiswedel I, Schmidt CA, Wolke C, Walther R, Grabarczyk P, Busemann C (2012) Composition of molecular cardiolipin species correlates with proliferation of lymphocytes. Exp Biol Med (Maywood) 237:372–379
9. Ponisovskiy MR (2011) Warburg effect mechanism as the target for theoretical substantiation of a new potential cancer treatment. Crit Rev Eukaryot Gene Expr 21:13–28
10. Paradies G, Petrosillo G, Paradies V, Ruggiero FM (2009) Role of cardiolipin peroxidation and Ca^{2+} in mitochondrial dysfunction and disease. Cell Calcium 45:643–650
11. Claypool SM, Koehler CM (2012) The complexity of cardiolipin in health and disease. Trends Biochem Sci 37:32–41
12. Warburg O (1956) On the origin of cancer cells. Science 123:309–314
13. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275
14. Musser DA, Oseroff AR (1994) The use of tetrazolium salts to determinate sites of damage to the mitochondrial electron transport chain in intact cells following in vivo photodynamic therapy with photophrine H. Photochem Photobiol 59:621–626
15. Stope MB, Sauermann A, Rönnau C, Zimmermann U, Walther R, Burchardt M (2012) Androgen receptor and heat shock proteins in progression of prostate cancer cells. Int J Clin Pharmacol Ther 50:65–67
16. RomijnVerkoelen JC, RomijnVerkoelen CF, Schroeder FH (1988) Application of the MTT assay to human prostate cancer cell lines in vitro: establishment of test conditions and assessment of hormone-stimulated growth and drug-induced cytostatic and cytotoxic effects. Prostate 12:99–110

17. Kwan HY, Chao X, Su T, Fu X, Liu B, Tse AK, Fong WF, Yu Z (2014) Dietary lipids and adipocytes: potential therapeutic targets in cancers. J Nutr Biochem S0955–2863(14):00239–00243. doi:10.1016/jjnutbio.2014.11.001
18. Oh YS (2015) Mechanistic insights into pancreatic beta-cell mass regulation by glucose and free fatty acids. Anat Cell Biol 48:16–24
19. de Jong AJ, Kloppenburg M, Toes RE, Ioan-Facsinay A (2014) Fattyacids, lipid mediators, and T-cell function. Front Immunol 13(5):483
20. Jackson MD, Walker SP, Simpson-Smith CM, Lindsay CM, Smith G, Mc Fartane-Anderson N, Bennett FI, Coard KC, Aiken WD, Tulloch T, Paul TJ, Wan RL (2012) Association of whole-blood fatta acids and dietary intakes with prostate cancer in Jamaica. Cancer Causes Control 23:23–33
21. Moreel X, Allaire J, Leer C, Caron A, Labonte ME, Lamarche B, Julien P, Desmeules P, Têtu B, Fradet V (2014) Prostatic and dietary omega-3-fatty acids and prostate cancer progression during active surveillance. Cancer Prev Res (Phila) 7:766–776
22. Eser PO, VandenHeuvel JP, Jl Araujo, Thompson JT (2013) Marine- and plant-derived omega-3 fatty acids differentially regulate prostate cancer cell proliferation. Mol Clin Oncol 1:444–452
23. Hopperton KE, Duncan RE, Bazinet RP, Archer MC (2014) Fatty acid synthase plays a role in cancer metabolism beyond providing fatty acids for phospholipid synthesis or sustaining elevations in glycolytic activity. Exp Cell Res 320:302–310
24. Schulze A, Harris AL (2012) How cancer metabolism is tuned for proliferation and vulnerable to disruption. Nature 49:364–373
25. Moreno-Sanchez R, Rodriguez-Enriquez S, Marin-Hernandez A, Saavedra E (2007) Energy metabolism in tumor cells. FEBS J274:1393–1418
26. Raja V, Greenberg ML (2014) The functions of cardiolipin in cellular metabolism-potential modifiers of the Barth syndrome phenotype. Chem Phys Lipids 179:49–56
27. Schug ZT, Frezza C, Galbraith LC, Gottlieb E (2012) The music of lipids: how lipid composition orchestrates cellular behaviour. Acta Oncol 51:301–310
28. Ralph SJ, Low P, Dong I, Lawen A, Neuzil J (2006) Mitocans: mitochondrial targeted anti-cancer drugs as improved therapies and related patent documents. Recent Pat Anticancer Drug Discov 1:327–346
29. Ren M, Phoon CK, Schlame M (2014) Metabolism and function of mitochondrialcardiolipin. Prog Lipid Res 55:1–16
30. Sinha F, Das J, Pal PB, Sil PC (2013) Oxidative stress: the mitochondria-dependent and mitochondria-independent pathways of apoptosis. Arch Toxicol 87:1157–1180
31. Manda G, Isvoranu G, Comanescu MV, Manea A, DebelecButuner B, Korkmaz KS (2015) The redox biology network in cancer pathophysiology and therapeutics. Redox Biol 25:347–357