Novel Theranostic Opportunities Offered by Characterization of Altered Membrane Lipid Metabolism in Breast Cancer Progression

Mika Hilvo[1], Carsten Denkert[2], Laura Lehtinen[5], Berit Müller[2], Scarlet Brockmöller[2], Tuulikki Seppänen-Laakso[1], Jan Budczies[2], Elmar Bucher[5], Laxman Yetukuri[1], Sandra Castillo[1], Emilia Berg[1], Heli Nygren[1], Marko Sysi-Aho[1], Julian L. Griffin[6], Oliver Fiehn[7], Sibylle Loibl[8], Christiane Richter-Ehrenstein[3], Cornelia Radke[4], Tuulia Hyötyläinen[1], Olli Kallioniemi[5], Kristiina Iljin[5], and Matej Orešič[1]

Abstract

Activation of lipid metabolism is an early event in carcinogenesis and a central hallmark of many cancers. However, the precise molecular composition of lipids in tumors remains generally poorly characterized. The aim of the present study was to analyze the global lipid profiles of breast cancer, integrate the results to protein expression, and validate the findings by functional experiments. Comprehensive lipidomics was conducted in 267 human breast tissues using ultraperformance liquid chromatography/ mass spectrometry. The products of *de novo* fatty acid synthesis incorporated into membrane phospholipids, such as palmitate-containing phosphatidylcholines, were increased in tumors as compared with normal breast tissues. These lipids were associated with cancer progression and patient survival, as their concentration was highest in estrogen receptor–negative and grade 3 tumors. *In silico* transcriptomics database was utilized in investigating the expression of lipid metabolism related genes in breast cancer, and on the basis of these results, the expression of specific proteins was studied by immunohistochemistry. Immunohistochemical analyses showed that several genes regulating lipid metabolism were highly expressed in clinical breast cancer samples and supported also the lipidomics results. Gene silencing experiments with seven genes [*ACACA* (acetyl-CoA carboxylase α), *ELOVL1* (elongation of very long chain fatty acid–like 1), *FASN* (fatty acid synthase), *INSIG1* (insulin-induced gene 1), *SCAP* (sterol regulatory element–binding protein cleavage–activating protein), *SCD* (stearoyl-CoA desaturase), and *THRSP* (thyroid hormone–responsive protein)] indicated that silencing of multiple lipid metabolism–regulating genes reduced the lipidomic profiles and viability of the breast cancer cells. Taken together, our results imply that phospholipids may have diagnostic potential as well as that modulation of their metabolism may provide therapeutic opportunities in breast cancer treatment. *Cancer Res; 71(9); 3236–45. ©2011 AACR.*

Authors' Affiliations: [1]Bio and Process Technology, VTT Technical Research Centre of Finland, Espoo, Finland; [2]Institute of Pathology, [3]Breast Cancer Center, Charité University Hospital; [4]Institute of Pathology, DRK Klinikum Berlin Köpenick, Berlin, Germany; [5]Bio and Process Technology, VTT Technical Research Centre of Finland, Turku, Finland; [6]Department of Biochemistry, University of Cambridge, Cambridge, United Kingdom; [7]Genome Center, University of California Davis, Davis, California; and [8]German Breast Group, GBG-Forschungs GmbH, Neu-Isenburg, Germany

Corresponding Author: Mika Hilvo, Bio and Process Technology, VTT Technical Research Centre of Finland, Tietotie 2, P.O. Box 1000, FI-02044 VTT, Espoo, Finland. Phone: 358-20-7226131; Fax: 358-20-7227071; E-mail: mika.hilvo@vtt.fi or Matej Orešič, Bio and Process Technology, VTT Technical Research Centre of Finland, Tietotie 2, P.O. Box 1000, Espoo, Finland 02044, Finland. Phone: 358-20-7224491; Fax: 358-20-7227071; E-mail: matej.oresic@vtt.fi

Introduction

An early and universal feature of tumors is the activation of lipid metabolism, and, in fact, the term "lipogenic phenotype" has been coined to describe the activation of the lipogenic enzymes in the malignant processes (1). Thus far, the lipid metabolism of cancer has been predominantly investigated at the level of genes and many of these have been shown to affect tumorigenesis. Recently, the field has gained additional interest, as accumulating evidence suggests that cancer and metabolic diseases are connected at the level of lipid metabolism (2). Human cells have 2 sources of fatty acids; diet or *de novo* synthesis (1). In healthy adults, the role of *de novo* fatty acid synthesis is usually minor, as the cells primarily rely on dietary fatty acids. Only a few healthy tissues, such as liver or adipose tissue, and specific physiologic processes, such as endometrial cell proliferation

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or milk production, use *de novo* synthesis to generate fatty acids (1, 3). However, increased lipogenesis is a hallmark of many cancers including prostate and breast cancer (4–6). The key enzymes of *de novo* fatty acid synthesis are fatty acid synthase (FASN) and acetyl-CoA carboxylase α (ACACA), which in co-operation catalyze the synthesis of palmitate, and whose upregulation is coordinated in cancer cells, occurring early in the tumor progression (7–9).

Although the earlier studies have implicated individual lipid metabolism genes in cancer, several questions remain unanswered. Given the importance of membrane lipid composition and fluidity to maintain the topology, mobility, or activity of membrane bound proteins and to ensure normal cellular physiology, cells have developed robust mechanisms to maintain the membrane lipid homeostasis (10). To understand lipid metabolism in cancer, it is thus important to measure its end products, the cellular lipids, at the molecular level. The so-called lipidomics approach, covering a global profile of structurally and functionally diverse lipids, may not only elucidate the lipid molecular composition of cancer cells but also deliver clues about the mechanisms behind the control of cellular lipid homeostasis (10–12). The current study applied the lipidomic approach to a series of breast cancer tissue samples, and the hypotheses derived from tumor tissues were followed-up with functional studies *in vitro*, revealing the key role of several lipid metabolism genes in the regulation of observed phospholipid remodeling in tumor progression.

Materials and Methods

Breast cancer samples

For lipidomic profiling, breast cancer tissue as well as adjacent normal tissue was dissected by a senior pathologist in the operating room and was immediately frozen in liquid nitrogen and stored at −80°C. The histopathologic quality control of the samples is explained in the Supplementary Materials and Methods. As control samples, paired normal breast tissue was used. It was obtained at the time of tumor surgery but did not contain tumor cells. From formalin-fixed, paraffin-embedded (FFPE) tissue of the same tumors, a tissue microarray (TMA) was constructed for immunohistochemical evaluation of protein markers. The investigations were carried out in the framework of the METAcancer FP7 project—ethic committee approval was obtained from the Institutional Review Board of the Charité hospital.

Lipidomic analyses of breast cancer tissue

The full details of the lipidomics methods are explained in the Supplementary Materials and Methods. In brief, the lipid extracts were analyzed on a Waters Q-Tof Premier mass spectrometer combined with an Acquity Ultra Performance LC (UPLC). The data were processed using MZmine 2 software (13) and the lipid identification was based on an internal spectral library and tandem mass spectrometry. The lipidomics results were normalized according to the protein content of the samples (mg protein/mg tissue) and the lipids were quantified using the internal standards.

In silico data mining and gene selection for functional studies

A list of 24 genes involved in lipid metabolism was collected and GeneSapiens database (14) was applied to bioinformatically explore the gene expression levels across 9,783 human tissue samples.

Immunohistochemistry

The antibodies and detailed procedures of immunohistochemical staining on TMAs are found in the Supplementary Materials and Methods. The stainings were evaluated by 2 pathologists (S.B. and B.M.). The intensity (negative, weak, moderate, and strong) and the percentage of positive tumor cells were evaluated, and from these parameters, an immunoreactive score (IRS) was calculated as described before (15). The following cutoff points were used in the analysis of the different markers: FASN and INSIG1 (insulin-induced gene 1), low = IRS 0–4 versus high = IRS 6–12; ACACA and SREBP1 (sterol regulatory element–binding protein 1), low = IRS 0–2 versus high = IRS 3–12.

Functional experiments in breast cancer cells

The details and materials of the cell line experiments can be found in the Supplementary Materials and Methods. The experiments were carried out in breast carcinoma cells ZR-75-1 and MDA-MB-468 and in nonmalignant MCF-10A cell line. After 72 hours of incubation, cells were assayed for viability and apoptosis. Scrambled siRNA and lipid were used as negative controls and siRNAs against KIF11 (kinesin family member 11) and PLK1 (polo-like kinase 1) as positive controls. On the basis of the cell viability results, 7 siRNAs (and 2 positive controls) were selected for lipidomics analysis. To validate the silencing of the target genes, quantitative real-time PCR was carried out from each sample prepared for lipidome analysis.

Statistical analyses of the data

All statistical analyses and visualizations were conducted using R software, version 2.9.1, and the details of all the analyses can be found in the Supplementary Materials and Methods. The differences between the tumor types were compared using the Wilcoxon rank-sum test. To account for the multiple testing issues, *q*-values indicating the false discovery rate (FDR) were calculated with the R package *q*-value. Median values were used for calculating fold changes and constructing heat maps. Predictive model for estrogen receptor (ER) status (Fig. 1C) was developed using the Kernel-based Orthogonal Projections to Latent Structures (K-OPLS) method, with the R package K-OPLS (16).

Results

Lipidomic analysis of breast cancer tissue

Global lipidomic analysis using Ultra Performance Liquid Chromatography – Mass Spectrometry (UPLC-MS)
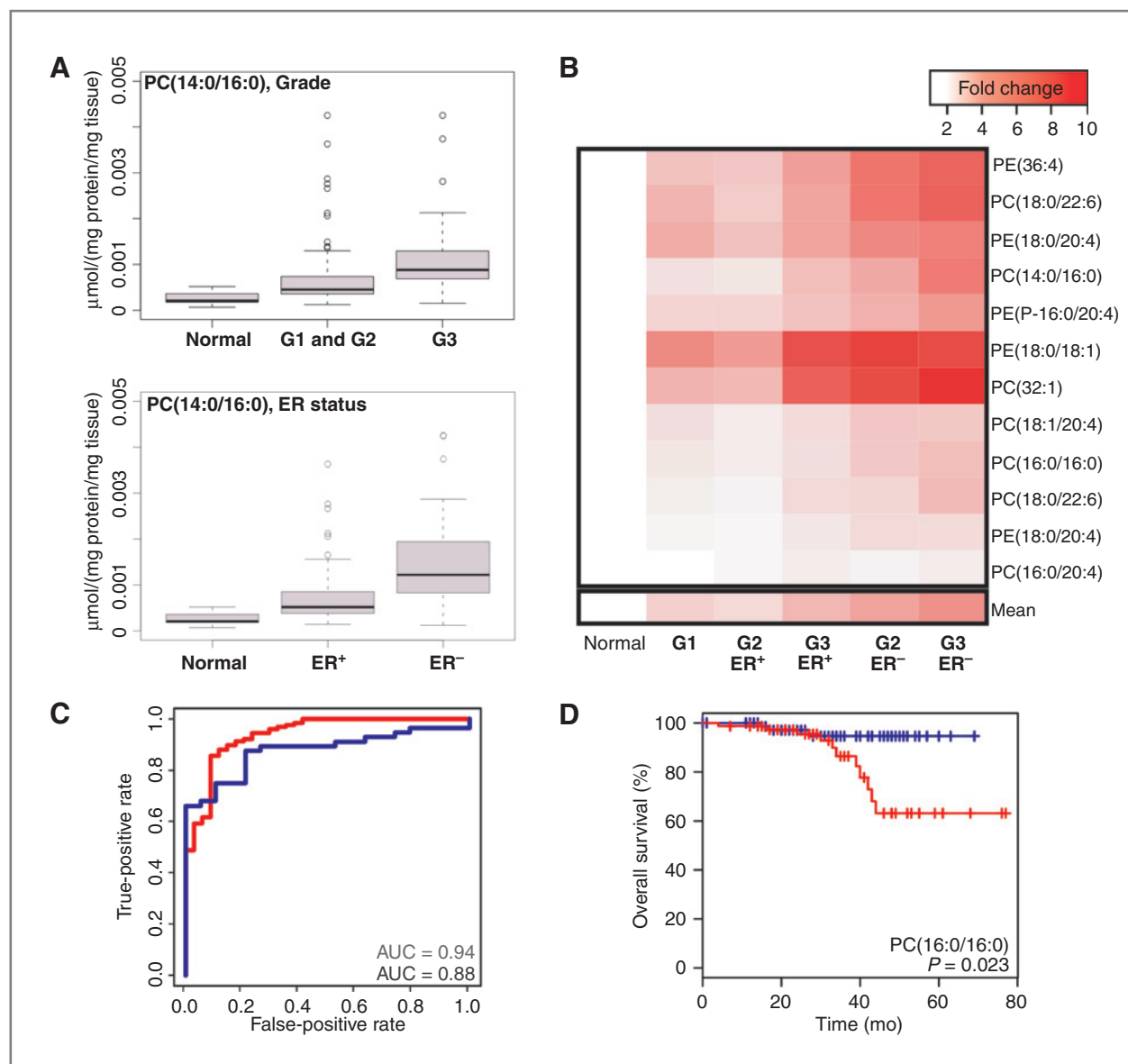
Figure 1. Lipidomic changes associate with estrogen receptor status and grade of the tumors as well as survival of the patients. A, box plots for PC(14:0/16:0) in normal tissues and in tumors of different grades and ER status. One outlier sample has been excluded from the figures. B, fold changes of the highest ranking differentially regulated phospholipids in tumors of different grade and ER status. The fold changes were calculated with respect to the healthy tissue samples. C, ROC curve for the model predicting the ER status [blue, validation set (AUC = 0.88); red, training set (AUC = 0.94)]. D, overall survival of the patients that were divided into 2 populations on the basis of the PC(16:0/16:0) levels (blue, patients with concentration below the median of all `samples; red, patients with concentration above the median of all samples).

methodology was carried out on 257 breast cancer samples as well as 10 samples from adjacent breast tissue. Before the start of the data acquisition, the samples were separated into 2 groups (Supplementary Table S2) using the Kullback–Leibler divergence to ensure a comparable distribution of clinico-pathologic variables. The initial results were derived from the training cohort of 183 samples, and the second cohort consisting of 84 samples was used to validate the results. Both positive (ESI$^+$) and negative (ESI$^-$) electrospray ionization modes were used in MS analyses. ESI$^+$ is more sensitive than

ESI$^-$ and shows neutral lipids, such as triacylglycerols (TG), but ESI$^-$ was relevant in this study due to sensitivity to detect the negatively charged signaling and membrane lipids including phosphatidylinositols (PI) and phosphatidylethanola-mines (PE). A total of 425 lipids were detected in ESI$^+$ mode and 126 lipids in ESI$^-$ mode. Supplementary Table S3 shows mass-to-charge (m/z) values, retention times, and abundance of the lipids in the training and validation cohorts. The overlapping lipids in these ion modes showed very similar results, and, unless otherwise stated, we report

Table 1. The most significantly changed lipids with respect to the ER status as well as tumor grade in both training and validation cohorts

Lipid	Training cohort						Validation cohort			
	ER$^-$ vs. ER$^+$		Grade 3 vs. 1 and 2		Tumor vs. normal		ER$^-$ vs. ER$^+$		Grade 3 vs. 1 and 2	
	q-value	Fc	q-value	Fc	q-value	Fc	q-value	Fc	q-value	Fc
PC(14:0/16:0)	<0.001	2.36	<0.001	1.94	<0.001	2.83	0.011	1.34	0.044	1.32
PC(16:0/16:0)	<0.001	1.84	<0.001	1.54	<0.001	2.20	0.012	1.31	0.167	1.24
PE(36:4)	<0.001	1.84	<0.001	1.44	<0.001	4.36	0.137	1.21	0.293	1.10
GlcCer[d18:0/24:0(2-OH)][a]	<0.001	−4.46	0.031	−1.36	0.014	1.26	<0.001	−5.94	0.001	−3.95
PC(32:1)	<0.001	2.10	<0.001	1.94	<0.001	5.10	0.168	1.21	0.180	1.27
GlcCer[d18:0/24:0(2-OH)][a]	<0.001	−3.40	0.035	−1.42	0.002	1.85	<0.001	−2.74	0.002	−2.41
PC(16:0/20:4)	<0.001	1.69	<0.001	1.66	<0.001	2.05	0.038	1.28	0.229	1.19
PE(18:0/20:4)[b]	<0.001	1.55	<0.001	1.61	<0.001	1.71	0.028	1.32	0.187	1.20
PC(18:0/22:6)[b]	<0.001	1.99	<0.001	1.76	0.003	2.05	0.073	1.62	0.239	1.35
PE(18:0/20:4)[b]	<0.001	1.51	<0.001	1.33	<0.001	4.44	0.055	1.30	0.239	1.12
PC(18:0/22:6)[b]	<0.001	1.69	<0.001	1.73	<0.001	4.25	0.070	1.18	0.166	1.15
PE(P-16:0/20:4)	<0.001	1.65	<0.001	1.43	<0.001	3.21	0.005	1.41	0.010	1.35
PI(18:0/20:4)	<0.001	1.62	<0.001	1.38	<0.001	1.73	0.002	1.31	0.180	1.14
PE(P-16:0/18:2)	<0.001	−1.84	0.102	−1.15	<0.001	2.48	0.020	−1.85	0.095	−1.34
SM(d18:1/24:0)	<0.001	1.65	<0.001	1.55	<0.001	2.67	0.025	1.19	0.090	1.19
PC(18:1/20:4)	<0.001	1.60	<0.001	1.48	<0.001	2.24	0.070	1.17	0.391	1.05
PC(18:0/20:4)	<0.001	1.31	<0.001	1.29	<0.001	2.10	0.127	1.13	0.468	−1.11
PE(18:0/20:3)	0.001	1.79	<0.001	1.58	<0.001	3.76	0.017	1.39	0.050	1.38
PE(P-38:4)	0.001	1.70	<0.001	1.58	0.001	1.52	0.003	1.56	0.060	1.31
PE(34:1)	0.001	1.54	<0.001	1.51	<0.001	4.43	0.426	1.05	0.336	1.02
PC(16:0/16:1)	0.001	1.34	0.002	1.21	0.002	1.87	0.312	−1.11	0.395	1.00
PE(18:1/18:0)	0.003	1.35	<0.001	1.36	<0.001	3.82	0.425	1.04	0.395	1.09

NOTE: Comparison between tumor versus normal tissue is also included for the training cohort.

Abbreviation: Fc, fold change.

[a]These represent different adducts of the lipid.

[b]The lipids have different retention times, and, thus, have likely fatty acid chains in different sn-1 and sn-2 positions [e.g., PE(18:0/20:4) vs. PE(20:4/18:0)].

here only the ESI$^-$ data (ESI$^+$ data shown in Supplementary Table S4). The lipids were identified by tandem mass spectrometry, and those for which the exact structure of the 2 acyl chains could not be elucidated, the total number of carbon atoms and double bonds [e.g., PC(32:1)] is shown.

Tumor grade and ER status associate with altered membrane lipid composition

The most striking difference in the lipid profiles were observed between the breast tumor and normal tissue samples. The FDR q-values were significant for 70% of the lipids even with a very stringent cutoff value ($q < 0.001$). The membrane phospholipids including phosphatidylcholines (PC), PEs, and PIs, as well as sphingomyelins (SM) and ceramides (Cer), were the most increased lipids in tumors (Table 1 and all lipids listed in Supplementary Table S5). TGs were mainly unaltered, although some were downregulated in cancer compared with normal breast (Supplementary Table S4).

In tumors, the most prominent difference associated with the altered phospholipid metabolism was related to ER status (Table 1 and Supplementary Table S5). Many phospholipids (PC, PE, and PI) and SMs were upregulated in ER$^-$ tumors, whereas TGs were not different when comparing ER$^+$ and ER$^-$ breast cancer samples (Supplementary Table S4). Some glycosylated ceramides were highly downregulated in ER$^-$ tumors (Table 1 and Supplementary Table S5).

Another important parameter associating with the tissue phospholipid profiles was tumor grade. Because no large differences were observed between grade 1 and 2 tumors (data not shown), we first combined these into 1 group and compared this group to grade 3 tumors. Interestingly, the most significantly changed lipids were the same that were also affected by the ER status. In fact, 17 of the 22 most altered lipids were found in both ranked lists [grade and ER status; Table 1, complete data shown in Supplementary Tables S5 (ESI$^-$) and S4 (ESI$^+$)]. Overall, the phospholipid concentrations

were increased in grade 3 tumors, whereas TGs remained unchanged. For this reason, we also investigated whether the overall increase in phospholipids alone could explain the results or whether the specific lipids associate with more aggressive tumors. The phospholipids in each sample were normalized by the total phospholipid content (of identified lipids) in the sample. Statistical analyses confirmed that, indeed, specific lipids are associated with ER status and tumor grade, and the most significant was PC(14:0/C16:0), as shown in Supplementary Table S6. The effect of both ER status and grade for PC(14:0/16:0) without total phospholipid normalization is shown in Figure 1A.

It is known that the majority of the ER$^-$ tumors are of grade 3 (17) and this was also the case in our patient population, as only 7% of the grade 1 and 2 tumors were ER$^-$, whereas 44% of the grade 3 tumors were ER$^-$. Thus, either grade or ER status alone could explain our results. We therefore analyzed the ER status only within grade 3 tumors and the grade only within ER$^+$ tumors and confirmed that both ER status and grade independently affected the same lipids (Fig. 1B and Supplementary Table S7), with the highest levels found in ER$^-$ grade 3 tumors (the only exception being the glycosylated ceramide, which was only slightly affected by tumor grade but highly downregulated in ER$^-$ tumors).

Interestingly, the most significantly increased lipids (Table 1) in grade 3 tumors were PC(14:0/16:0) and PC(16:0/16:0), that is, phospholipids incorporating *de novo* synthesized fatty acid chains. Palmitate (C16:0) is the major FASN product, but usually smaller amounts of myristic (C14:0) and stearic (C18:0) acids are also produced (18, 19). Indeed, a common characteristic for the altered lipids was the incorporation of at least 1 saturated fatty acyl side chain, either palmitate or stearic acid, the latter one being also the first product of the elongases. The results indicate that the *de novo* synthesized fatty acids incorporated into the membrane phospholipids are increased in tumors as compared with healthy tissue, and this process further increases during cancer progression, that is, in ER$^-$ and grade 3 tumors. Of the unsaturated fatty acids, arachidonic acid (C20:4) was the most commonly identified in the phospholipids.

We also tested if the results could be validated in the validation cohort. This series did not contain normal tissue samples, and therefore normal breast to tumor comparison could not be conducted. The results for the ER status were highly reproducible in this independent sample series (Table 1). For grade, *q*-values were not as strong as the ones obtained for ER status, but the results showed similar trends with the training set. We also applied predictive regression models for tumor grade and ER status using the training set and tested the models in the validation set. For the ER status, a good model could be developed with area under curve (AUC) values of the receiver operating characteristic (ROC) curves of 0.94 and 0.88 in the training and validation sets, respectively (Fig. 1C), whereas for grade, no strong diagnostic model was found.

Progesterone receptor (PR) and HER2 status did not cause as dramatic alterations in the lipid profiles as the ER status and grade. However, similar to ER status, also PR negativity

was associated with downregulation of glycosylated ceramides, and some phospholipids [e.g., PC(32:1) and PC(32:2)] were increased in HER2$^+$ tumors both in the training (Supplementary Tables S4 and S5) and validation sets (data not shown).

Tumor membrane lipid composition is associated with patient survival

Because many lipids were associated with tumor parameters predicting worse prognosis such as grade 3 and ER$^-$ status (17), we also studied associations of lipidomic profiles with the survival of the patients. For these analyses, both ESI$^-$ and ESI$^+$ data were used, and the patients were divided into 2 populations for each lipid, grouped according to concentration above or below the median value across all samples. Several lipids were found statistically significant ($P < 0.05$). Interestingly, increased concentrations of PC(30:0), PC(16:0/16:0) = PC(32:0), PC(32:1), and PC(32:2) were all associated with poorer overall survival [Kaplan–Meier curve for PC(16:0/16:0) is shown in Figure 1D]. All of these lipids were increased in grade 3 tumors, which may explain their association to poorer survival.

Expression of selected lipid metabolism–related proteins associates with altered lipid profiles

To link the observed lipidomic changes to expression of lipid-related genes, we carried out a comprehensive bioinformatic analysis of the mRNA expression profiles for multiple genes across data sets of 9,783 tissue samples representing 43 healthy and 68 malignant tissue types in the GeneSapiens database (14). On the basis of the expression and function of the genes, we selected several genes (Fig. 2) for follow-up studies by immunohistochemistry in clinical tumors and functional gene silencing studies in breast cancer cells. The selected genes function in many aspects of lipid metabolism, as illustrated in Figure 2. The expression of these genes in the database is shown in Supplementary Figure S1. In summary, ACACA, SCD (stearoyl-CoA desaturase), SREBP1, and THRSP (thyroid hormone–responsive protein) were highly expressed in clinical breast cancer samples.

To integrate lipidomics results with gene expression, immunohistochemical stainings were carried out in the same samples for which lipidomic profiles were determined. Following the initial screening to determine which of the above selected gene products were significantly altered in tumors, we finally studied 4 proteins, ACACA, FASN, INSIG1, and SREBP1 (Fig. 3A). On the basis of the staining intensity and percentage of positive tumor cells, the samples were divided for each protein into 2 categories (low and high). While ER and HER2 status did not associate with changes in protein expression, differences were found for the tumor grade (Supplementary Table S8), in particular for ACACA, as the group containing low levels of this protein had 24% of grade 3 tumors, whereas the high level group had 51% of grade 3 tumors. Also INSIG1 and SREBP1 showed differences (high expression found in low grade tumors), although the results were not statistically significant. Interestingly, although lipidomics showed similar results in ER$^-$ and grade 3 tumors, this
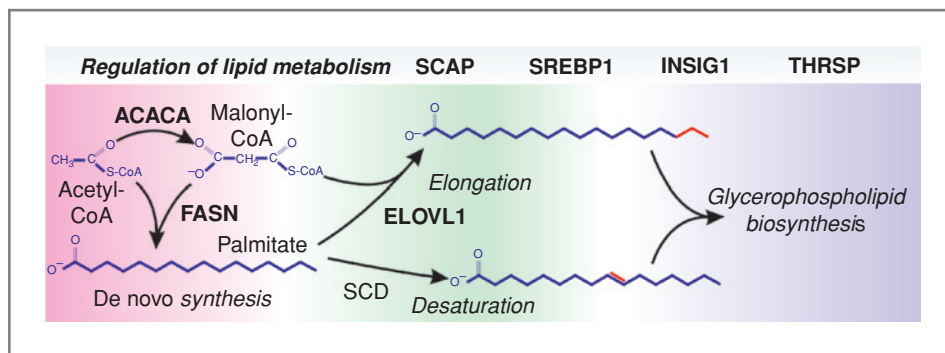
Figure 2. Schematic figure showing the genes and proteins investigated in the present study. ACACA produces malonyl-CoA, which is used by FASN to produce palmitate *de novo*. The *de novo* synthesized fatty acids can be elongated by elongases, such as ELOVL1, which is elongating saturated lipids, and/or desaturated by desaturases, such as SCD. The lipid metabolism is regulated by several genes, such as *SREBP1* and its regulator SCAP. In addition, INSIG1 and THRSP participate in lipid metabolism regulation.

phenomenon was not evident in the immunohistochemistry results for these 4 proteins.

To correlate the gene expression with lipidomics results, we investigated the levels of the phospholipids from Table 1 in the groups with either low or high expression of the proteins. Unsupervised hierarchical clustering revealed 2 major clusters (Fig. 3B). High expression of FASN and ACACA together with low expression of SREBP1 formed a cluster in which the levels of the phospholipids were considerably higher as compared with the other cluster. This finding is in accordance to the expectations, as FASN and ACACA are known to increase fatty acid *de novo* synthesis. In addition, the association of the low SREBP1 expressing group with high phospholipid levels was anticipated on the basis of the results showing that this group had more grade 3 tumors than the high expressing group.
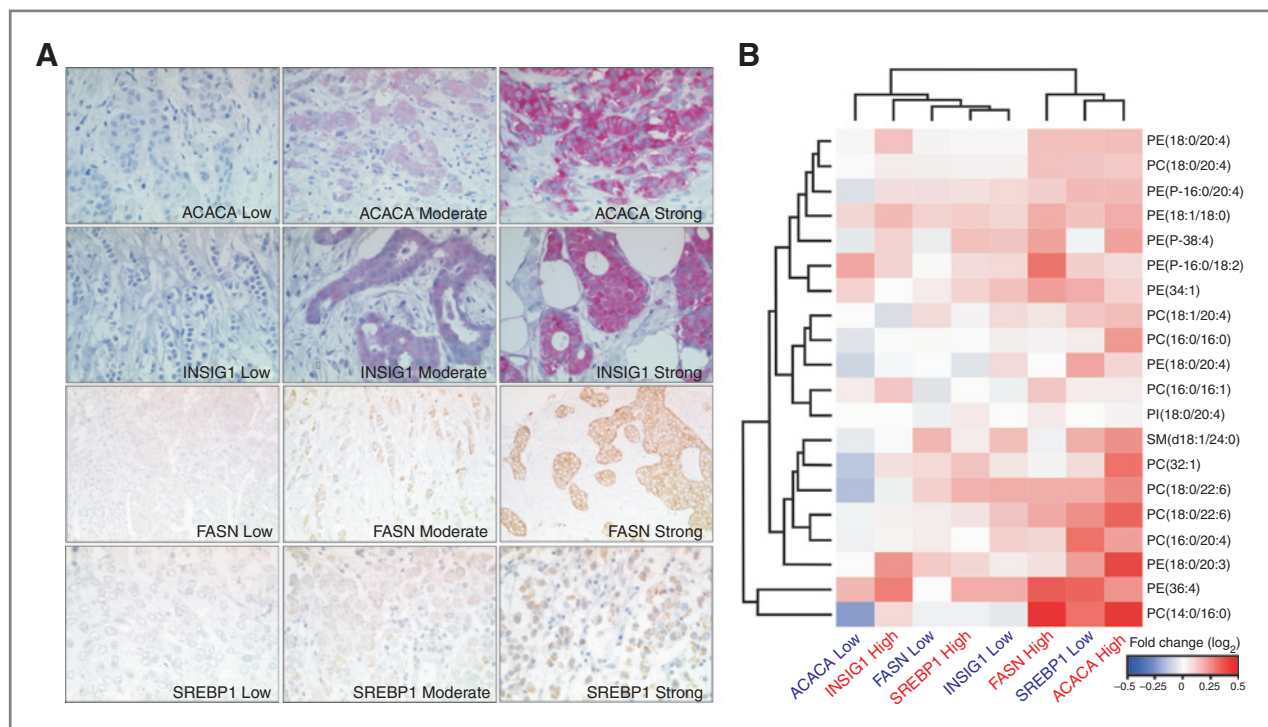
Figure 3. Immunohistochemical analyses integrated with lipidomics results. A, representative figures showing low, moderate, or strong staining for the investigated proteins (ACACA, FASN, INSIG1, and SREBP1) in breast cancer tissues. B, heat map showing the level of highest ranking differentially regulated phospholipids from Table 1 in tumors with either low or high expression of the selected lipid metabolism proteins. The low and high expression were defined on the basis of the IRS, which takes into account both the intensity and area of the staining, and the median of samples within each group was compared with the median of all samples by $\log_2$ fold change.
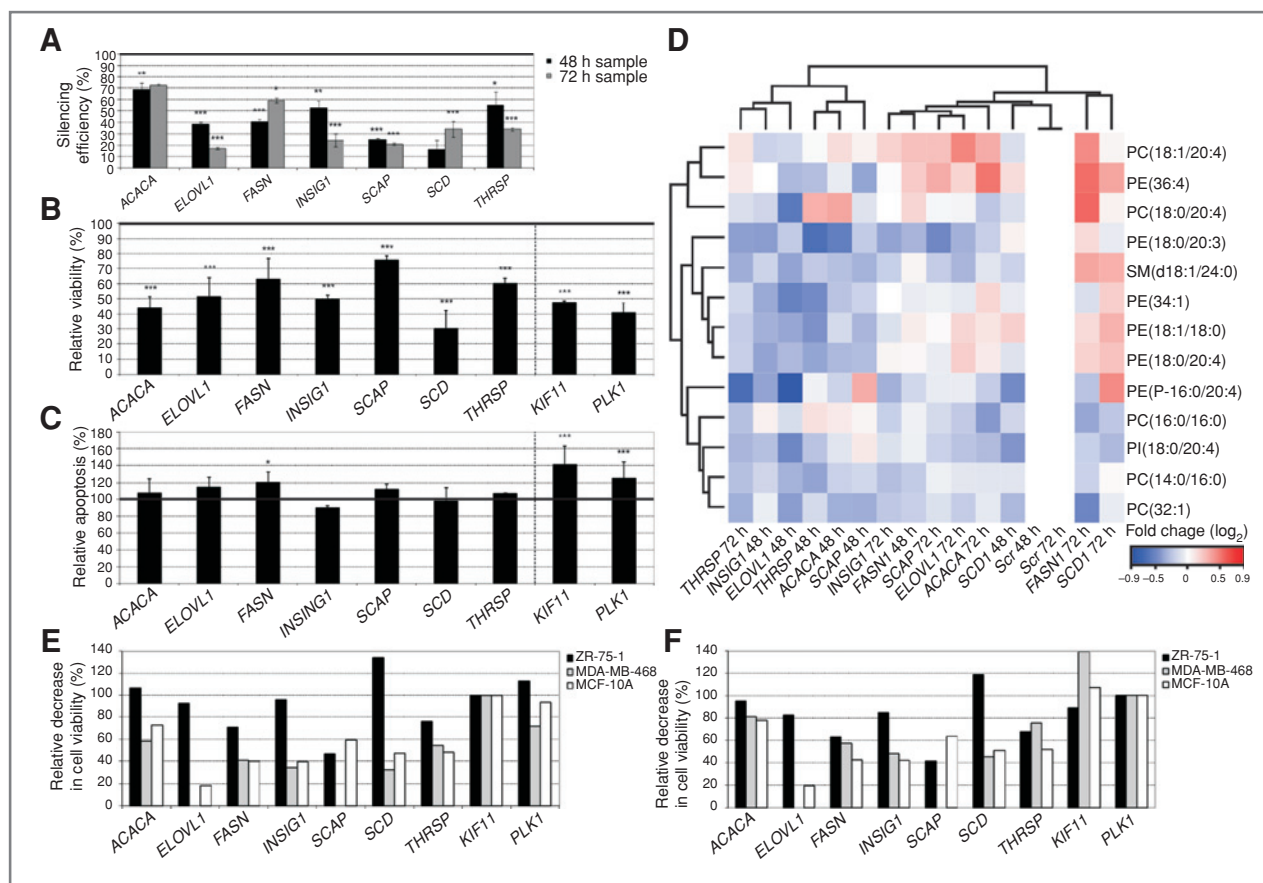
Figure 4. Knockdown of lipid metabolism–related genes affects the viability and lipidomic profiles of breast cancer cells. A, the efficacy of target gene silencing by siRNAs in ZR-75-1 cells. The effect of gene silencing to (B) cellular viability and (C) apoptosis in ZR-75-1 cells. A to C, the changes are relative to the scrambled siRNA control sample results (set as 100%), and the significance levels are as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Scr, scrambled siRNA. D, the effect of gene silencing on highest ranking differentially regulated phospholipids from Table 1 in ZR-75-1 cells: the $\log_2$ fold change was calculated by comparing the mean of 2 replicates relative to the appropriate (48 or 72 hours) scrambled siRNA samples. E and F, relative decrease in cell viability in response to gene silencing in 2 malignant (ZR-75-1 and MDA-MB-468) and 1 nonmalignant (MCF-10A) cell line. The results are normalized with respect to transfection efficacy by dividing the results with control gene results (KIF11 in E and PLK1 in F).

Gene silencing reveals that *de novo* fatty acid synthesis and phospholipid remodeling pathways are required for breast cancer cell growth

To establish a direct role of selected genes in regulating the observed phospholipid remodeling, we conducted gene silencing studies of 7 genes (Fig. 2) in ZR-75-1 and MDA-MB-468 breast cancer cells. We selected these cell lines because ZR-75-1 showed strong expression of the investigated genes, whereas the gene expression in MDA-MB-468 was more close to nonmalignant MCF-10A breast epithelial cells (Supplementary Fig. S2). Seventy-two hours after transfection, cell viability and induction of apoptosis were measured as endpoints. The results are shown in Supplementary Table S9 and for 1 selected siRNA per gene in ZR-75-1 cells in Figure 4A–D. The efficacy of target gene silencing by the selected siRNAs was confirmed by quantitative real-time PCR (Fig. 4A). The results indicated that ELOVL1 (elongation of very long chain fatty acid–like 1), FASN, SCD, and SCAP (sterol regulatory element–binding protein cleavage–acti-

vating protein) siRNAs silenced their target genes efficiently (>50% of the scrambled control), whereas for ACACA, THRSP, and INSIG1 silencing was only partial. All the genes were shown to reduce cell viability significantly (Fig. 4B) and the phenomenon was observed with multiple siRNAs per gene. SCD silencing had the strongest effects, resulting in the reduction of cell growth to 30% of the levels seen with scrambled control siRNAs. ACACA (44%), INSIG1 (50%), and ELOVL1 (52%) silencing also had a strong impact on cell survival, and THRSP1 and FASN siRNA transfections reduced cell viability to 60% and 63% and SCAP siRNA to 76% of the scrambled control. Importantly, ACACA showed a strong effect, as already a partial silencing led to significant decrease in cell viability. An increase (20% relative to the control) in apoptosis was seen by FASN knockdown (Fig. 4C). Silencing of 4 of the 7 genes (*ACACA*, *FASN*, *INSIG1*, and *THRSP*) reduced cell viability also in MDA-MB-486 cells and apoptosis was induced in response to FASN, INSIG1, SCAP, SCD, and THRSP silencing, supporting the critical role

of these genes for breast cancer cell survival (Supplementary Table S9).

To study whether the observed changes in cell viability were specific for malignant cells, silencing experiments were also carried out in nonmalignant MCF-10A breast epithelial cells. The results showed that these genes are also important for viability in these cells (Fig. 4E and F). However, the transfection efficacy of MCF-10A cells was extremely good, and if the cell viability results are compared with the transfection efficacies in the respective cell lines, the silencing of all these genes, apart from SCAP, had a stronger impact in cancer cells compared with the nonmalignant ones (Fig. 4E and 4F). A stronger effect was observed in ZR-75-1 cells than in the MDA-MB-468 cells, which is understandable based on the high expression of lipogenic genes in ZR-75-1 cells (Supplementary Fig. S2). Notably, apart from INSIG1, the gene expression was not statistically different between the MDA-MB-468 and MCF-10A cells, yet the relative effect of silencing was stronger in cancer cells.

To compare the molecular lipid changes in in vitro functional assays with those of tumor tissues, the lipid profiles were measured from the siRNA-treated ZR-75-1 breast cancer cells at 48 and 72 hours after transfection and compared with the respective control samples. Figure 4D shows the levels of those phospholipids that were found to be most strongly associated with grade and ER status (Table 1) and detectable in cultured cells. Mirroring the results obtained from tumor tissue, the most significantly changed lipids were PC(14:0/16:0) and PC(32:1), whose concentrations were decreased in response to silencing of nearly all proteins modulating lipid synthesis. These are the same lipids showing the strongest associations also with ER status, grade, and survival of the patients. The most dramatic decrease in PC(16:0/16:0) levels, containing direct products of the ACACA and FASN enzymes, was obtained in response to silencing of these genes at 72 hours, as expected.

For many samples, there was a difference in lipidomic profiles between the 2 time points. Usually, the decreased lipid concentrations were more evident at 48-hour time point (e.g., in the case of ACACA). Interestingly, for FASN in the 72-hour samples, the lipids containing de novo synthesized fatty acids were further decreased, whereas the lipids containing fatty acids, such as arachidonic acid, were increased compared with the 48-hour samples. This implies that the cells can develop alternative routes to provide FASN with malonyl-CoA instead of ACACA, but FASN cannot be substituted, and the cells start to build phospholipids from fatty acids that are not synthesized by the cells.

Discussion

We investigated systematically the lipidomic changes in both clinical breast cancer tissues, and in cultured breast cancer cells in response to silencing of the key metabolic enzymes by RNA interference. One of the consistently implicated lipids was PC(14:0/16:0) which was the most significantly changed lipid with respect to both tumor ER status and grade. Silencing of lipid metabolism key regulators

downregulated PC(14:0/16:0) and PC(32:1) levels the most, and in integrated lipidomics and immunohistochemistry results PC(14:0/16:0) showed the strongest association with FASN and ACACA upregulation. Interestingly, PC(16:0/16:0), containing only palmitate fatty acid chains, also showed significant changes but they were less pronounced as compared with PC(14:0/16:0). Thus, the results show that the increased de novo fatty acid synthesis is important for tumorigenesis and also suggest that the function of FASN may be modulated in cancer cells so that the proportions of myristic (C14:0) versus palmitic acid (C16:0) produced by the enzyme change during malignant processes. The overall amount of saturated fatty acids known to make the cell membrane less fluid (20) increases in the most aggressive tumors, and further studies are needed to investigate cell membrane physiology in tumor growth and anticancer therapy. In fact, it has been suggested that altered membrane fluidity may affect the sensitivity of tumor cells to chemotherapy (21).

In the study, we identified metabolic enzymes that have not been studied intensively in cancer before. ELOVL1 is an enzyme elongating saturated and monounsaturated fatty acids (22), which were detected at high levels in the phospholipids of cancer samples. Although ELOVL1 mRNA was not overexpressed in breast cancer compared with normal breast, the gene silencing results indicated that ELOVL1 is vital for breast cancer cells. SCD is a desaturase converting palmitic (C16:0) and stearic (C18:0) acids to palmitoleic (C16:1) and oleic (C18:1) acids, respectively. It has been reported to be downregulated in prostate cancer but shown to have an important role in the tumorigenesis of hepatocellular carcinomas and lung adenocarcinomas (23–25). Fatty acids catalyzed and produced by SCD were increased in the lipids associated with tumor grade or ER status, and SCD downregulation reduced cell proliferation. Recent results show a role for SCD in de novo fatty acid synthesis (26) and reduction in, for example, PC(16:0/16:0) was seen also in our gene silencing results.

Differentiation and ER status influenced the lipid profiles dramatically, with the highest levels of membrane phospholipids found in the most aggressive tumors. Indeed, it has been previously shown that the overexpression of FASN is most intense in carcinomas with higher risk of both disease recurrence and death, and steroid hormones, growth factors (EGFR, ERBB2), and the phosphatidylinositol-3 kinase (PI3K)-Akt signaling pathway regulate FASN expression (1, 27–29). There are also several publications showing the interconnection of FASN activity and HER2 signaling pathway (27, 30, 31). In the lipidomic and immunohistochemical analyses, we did not find major associations to the HER2 status, and thus our results challenge the earlier conceptions that HER2 signaling would have a major effect on the lipid metabolism (27).

Immunohistochemistry combined with lipidomics showed that low SREBP1 expression clustered with high FASN and ACACA levels, which was unexpected as the activity of SREBP1 is known to upregulate these enzymes (32). Moreover, INSIG1 complexes with SREBP1 and SCAP in the endoplasmic reticulum thus preventing their function in the regulation of transcription (33). Thus, one would expect to observe inverse lipidomic changes with INSIG1 and SREBP1 expression, or

when INSIG1 and SCAP are silenced. However, the overall changes observed in the protein expression and lipidomic profiles in response to gene silencing were not in accordance with these expectations. Thus, our results suggest that the regulation of lipid metabolism in breast cancer may differ from that of normal physiology.

Although attempts to investigate lipids during carcinogenesis were reported decades ago (34), the present study is the first one to characterize the molecular lipid changes in breast cancer. The lipid metabolism is an attractive target for cancer therapy, as it differs between tumors and normal tissues, with the most dramatic changes observed in the most aggressive tumors. Our results show that only comprehensive understanding, both at the gene expression and metabolite levels, will provide the information on the most crucial components of the lipid metabolism in tumor cells that could be exploited as therapeutic targets.

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Novel Theranostic Opportunities Offered by Characterization of Altered Membrane Lipid Metabolism in Breast Cancer Progression

Mika Hilvo, Carsten Denkert, Laura Lehtinen, et al.

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