A specific lipid metabolic profile is associated with the epithelial mesenchymal transition program

Anna Maria Giudetti, Stefania De Domenico, Andrea Ragusa, Paola Lunetti, Antonio Gaballoe, Julien Franck, Pasquale Simeon, Giuseppe Nicolardi, Francesco De Nuccio, Angelo Santino, Loredana Capobianco, Paola Lanutti, Isabelle Fournier, Michel Salzet, Michele Maffia, Daniele Vergara.

1. Introduction

The epithelial-mesenchymal transition (EMT) program is defined as a cellular process whereby the transformation from epithelial to mesenchymal cells occurs through the coordinated activation of a series of molecular events [1,2]. These include alterations in the organization of cytoskeletal structure, cell-cell junction formation, and apical-basal polarity that fulfills the acquisition of a migratory phenotype, and the activation of a stemlike program [3]. These events occur through the down-regulation of epithelial markers that is orchestrated by a series of EMT-transcriptional factors (TFs) [4]. Overall, mesenchymal cells that undergo EMT exhibit profound genomic and proteomic differences compared to their epithelial counterpart [5,6], this includes also a metabolic reprogramming that occurs as a consequence of alterations in signaling pathways that support tumor metabolism [7-11]. It is well recognised, both in vitro that in vivo, the inverse correlation between oxidative phosphorylation (OXPHOS) and EMT [12] and the concomitant induction of glycolysis and lactate production [10,13]. It is still unclear to what extent metabolic changes are secondary to modifications of cellular pathways or if metabolic alterations themselves

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ABSTRACT

Several studies have identified a specific metabolic program that is associated with the process of epithelial-mesenchymal transition (EMT). Whereas much is known about the association between glucose metabolism and EMT, the contribution of lipid metabolism is not still completely understood. Here, we studied epithelial and mesenchymal breast cancer cells by proteomic and lipidomic approaches and identified significant differences that characterised these models concerning specific metabolic enzymes and metabolites including fatty acids and phospholipids. Higher levels of monounsaturated fatty acids together with increased expression of enzymes of de novo fatty acid synthesis is the distinct signature of epithelial with respect to mesenchymal cells that, on the contrary, show reduced lipogenesis, higher polyunsaturated fatty acids level and increased expression of genes involved in the triacylglycerol (TAG) synthesis and lipid droplets formation. In the mesenchymal model, the diacylglycerol acyltransferase (DGAT)-1 appears to be the major enzyme involved in TAG synthesis and inhibition of DGAT1, but not DGAT2, drastically reduces the incorporation of labeled palmitate into TAG. Moreover, knockdown of β-catenin demonstrated that this metabolic phenotype in under the control of a network of transcriptional factors and that β-catenin has a specific role in the regulation of lipid metabolism in mesenchymal cells.
may be the driver of the EMT program by altering the activity of EMT-TFs. Several experimental works support both hypotheses in different pathological conditions. For example, mutations in the genes encoding isocitrate dehydrogenase 1 and 2 (IDH1/2) caused an EMT phenotype, characterised by changes in EMT-related gene expression and cellular morphology, through the up-regulation of the transcription factor Zeb1 and down-regulation of the miR-200 family of microRNAs [9]. The loss of the mitochondrial citrate synthase enzyme induced a bioenergetics switch from mitochondrial respiration to glycolysis and promoted an EMT program through activation of Snail and Twist in Hela cervical carcinoma cells [11]. In basal-like breast cancers, loss of fructose-1,6-biphosphatase (FBP1) mediated by the EMT-TF SNAI is essential to induce glycolysis, inhibit OXPHOS and enhancing stem-cell like characteristics [10].

Overall, this links EMT to a specific metabolic signature. Understanding the molecular mechanisms behind this association may be useful to develop therapeutic strategies aimed at targeting specific enzymes.

Recent developments in the mass spectrometry analysis have provided valuable insights into the regulation of EMT [5]. In the present study, we applied a label-free shotgun proteomic approach to analyze the protein expression profile of breast tumor cell lines with epithelial and mesenchymal features. Our data identified a specific dataset of metabolic proteins, validated by qPCR, western blot and biochemical assays that differentially characterised the two models. More in detail, we demonstrated that the most aggressive mesenchymal cell lines displayed a lower de novo lipid synthesis compared to epithelial cells, with a lower incorporation of radiolabeled acetate into neutral lipids and phospholipids. On the contrary, mesenchymal cells significantly incorporated exogenous radiolabeled FAs into neutral lipids with a significant over-expression and increased activity of a set of enzymes involved in the metabolism of triacylglycerols (TAGs). Moreover, we demonstrated the association of this metabolic signature to a specific lipidomic profile assessed by MALDI-TOF/TOF mass spectrometry and NMR providing a link between molecular and metabolic alterations.

2. Materials and methods

2.1. Lentiviral particles, cell culture, data availability and reagents

Human tumor cells were purchased from the American Type Culture Collection (ATCC) or from Banca Biologica and Cell Factory (IRCCS Azienda Ospedaliera Universitaria San Martino-IST Istituto Nazionale per la ricerca sul cancro, Genova, Italy). Human cancer cell lines MCF-7, MDA-468, MDA-415, MDA-361, T47D, HBL-100, MDA-231, SKOV-3, and human normal fibroblast NHDF were cultured in DMEM medium (4500 mg/l or 1000 mg/l glucose, EuroClone) supplemented with 10% lipodermic pro-

2.2. In vivo tumor growth and bone metastasis

Experiments were performed using female Athymic nude mice (Harlan Laboratories, Italy) at 4 weeks of age and carried out in strict accordance with the European Committee Council 106 Directive (86/609/EC) and with the Italian animal welfare legislation (art 4 and 5 of D.L. 116/92). See also the Supplemental Materials and Methods.

2.3. Sample preparation, mass spectrometry analysis, and database searching

Whole protein extraction was carried out with the Illustra TriplePrep kit (GE Healthcare) and processed according to the filter-aided sample preparation (FASP II) protocol [16]. A generalized description of the experimental workflow is shown in Fig. 1A. To globally define proteomic differences between the two models, whole proteins were trypsin digested by FASP and peptides analyzed by a high-resolution mass spectrometer (Q-Exact) followed by data analysis in MaxQuant and Perseus. In detail, raw files obtained from nanolC-MS were processed using the MaxQuant proteomic software (version 1.5.2.8) [17], as described [14]. Q-Exact spectra were matched to peptide sequences in the human UniProt protein database (release November 2014, 88876 entries) using the Andromeda algorithm [18]. Label-free quantification of the proteins was conducted using the MaxLFQ algorithm [19]. Statistical analysis was performed with the software Perseus software (version 1.5.2.4). Functional annotation and characterization of the identified proteins were performed using STRING version 10.5 (https://string-db.org). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [20] partner repository with the dataset identifier PXD0011854.

2.4. RNA extraction and real-time PCR

Total RNA was extracted from cells grown in a T25 flask using the Illustra triplePrep kit (GE Healthcare) and analyzed as previously described [14]. See also the Supplemental Materials and Methods.

2.5. Western blot analysis

Western blot analysis was performed as previously described [14]. See also the Supplemental Materials and Methods.

2.6. Measurement of the mitochondrial citrate transporter (CIC) activity

Mitochondria were extracted from MCF-7 and MDA-231 human breast cancer cell lines (3 × 10^7 cells) by differential centrifugation. Mitochondria (10–15 mg proteins) were solubilized with a buffer containing 3% Triton X-100 (w/v), 20 mM Na_2SO_4, 1 mM EDTA, 10 mM Pipes, pH 7.0, at a final concentration of about 10 mg protein/ml. After incubation for 10 min at 2 °C, the mixture was centrifuged at 25,000 × g for 20 min at 2 °C thereby obtaining a supernatant, referred to as mitochondrial extract that was processed as described [21]. See also the Supplemental Materials and Methods.
2.7. Incorporation of labeled substrates into fatty acids, neutral lipids, and phospholipids and thin-layer chromatography analysis

The rate of [1-14C]acetate (16 mM, 0.96 mCi/mol) incorporation into fatty acids was measured essentially as described [14]. See also the Supplemental Materials and Methods.

2.8. Assay of enzymatic activities in digitonin-permeabilized cells

The activity of acetyl-CoA carboxylase (ACC) was determined as the incorporation of radiolabeled acetyl-CoA into fatty acids in a coupled assay with FASN reaction in digitonin-permeabilized cells as described [22]. Reactions were carried out at 37 °C for 4 min. To determine the
activity of FASN, malonyl-CoA was included in the digitonin-containing assay mixture, while ATP, butyryl-CoA, and FASN were omitted. The assay was allowed to proceed for 10 min [22]. To determine DGAT activity, cells were treated with DGAT1 or DGAT2 inhibitors (A922500 and PF-06424439, respectively) at the concentration of 40 μM for 4 h. DGAT activity was determined by permeabilizing plasma membranes with digitonin and using endogenous diacylglycerols as substrates, as described [22]. The assay medium contained the following components: 50 mM potassium phosphate, pH 6.5, 1.0 mM dithiothreitol, 50 mM NaF, bovine serum albumin (5 mg/ml) and 50 μM [1-14C]palmitoyl-CoA (240 Bq/mol) in a total volume of 0.5 ml. The incubation was terminated after 1 min by the addition of 2 ml of methanol-chloroform (2:1, v/v). After extraction of lipids, triacylglycerols were isolated by TLC on Silica G following the procedure above reported. The silica, containing the triacylglycerol fractions, was scraped from the plate and assayed for radioactivity.

2.9. Fatty acid beta-oxidation measurements

The rate of [14C]fatty acid oxidation was determined as the formation of CO2 as previously described [23]. See also the Supplemental Materials and Methods.

2.10. ATP-citrate lyase and glucose 6-P dehydrogenase assay

For ATP-citrate lyase (ACLY) activity assay, cells were scraped-off from the plate and homogenized in a buffer containing 0.25 M sucrose and 20 mM Tris, pH 8.0. After centrifugation at 15,000 × g for 30 min, the supernatant was used for the assay. ACLY activity was measured by following the decrease of NADH absorbance, at 340 nm, when the sample is added to a medium containing, in 1 ml of final volume, the following components: 0.2 M Tris buffer pH 8.4, 0.01 M MgCl2, 0.01 M 2-mercaptoethanol, 0.02 M potassium citrate, 0.2 mM CoA-SH, 0.2 mM NADH, 20 units/ml of malic dehydrogenase enzyme, and 0.01 M of ATP.

Glucose 6-P dehydrogenase (G6PD) activity assay was performed at 30 °C by measuring the increase in absorbance at 340 nm resulting from the reduction of NADP+ in a buffer containing: 55 mM Tris-HCl pH 7.9 with 3.3 mM MgCl2, 6 mM NADP+ and 0.1 M glucose-6-P. The reaction was started by sample addition.

2.11. In situ cellular MALDI TOF/TOF analysis of lipids

Lipids mass spectra were acquired in negative ion reflection mode (detection range: 500–2000 mass/charge, m/z), using a Bruker Daltonics UltraFlex Extreme MALDI-TOF/TOF mass spectrometer. Samples were analyzed using 9-aminoacridine (9-AA, SIGMA) as MALDI matrix. See also the Supplemental Materials and Methods.

2.12. NMR analysis

Dried raw organic lipid extracts of MCF-7, and MDA-231 cell lines (1 × 10⁶ cells/well, 6 replicates each) were dissolved in 600 μl mixture of CDCl3/CD3OD (2:1, v/v) containing 0.03% (v/v) tetramethylsilane (TMS), transferred into 5-mm NMR tubes and analyzed. The 1H NMR spectra were recorded at 298 K on a 500 MHz Varian INOVA spectrometer (Varian Inc., CA) by using the zsped function. See also the Supplemental Materials and Methods.

Spectra were imported into NMRProcFlow online software [24] and divided into 0.04 ppm-wide segments for a spectral window that ranged from 0.60 to 6.05 ppm. The segments in the intervals 2.36–2.43 ppm and 4.15–4.35 ppm were removed because of the overlapping methanol and water signals, respectively. The obtained 131 buckets were normalized with a constant sum method and imported into SIMCA 14.1 software (MKS Umetrics) to perform, after Pareto scaling, Principal component analysis (PCA), and orthogonal partial least-squares discriminant analysis (OPLS-DA).

2.13. Gas chromatography-mass spectrometry analysis

To analyze fatty acids, cellular total lipids were extracted by the Bligh and Dyer procedure. Methyl esters were prepared by transesterification with toluene and methanolic boron trifluoride (17% BF3) at 65 °C for 20 min. GC-MS analyses were performed using an AGILENT 5977E gas chromatograph. See also the Supplemental Materials and Methods.

2.14. Statistical analysis and multivariate analysis

All data represent the average of at least three independent experiments, unless otherwise specified. Normally distributed data was compared with a two-tailed Student’s t-test using GraphPad PRISM software (version 6.0). The graphs represent mean ± S.D. Differences were considered significant when P was < 0.05. Multivariate statistical analyses were performed using MetaboAnalyst or SIMCA.

3. Results

3.1. Proteomic characterization of breast cancer models

To get insight into the biological processes associated with EMT, we conducted a mass spectrometry analysis of proteins isolated from the low metastatic breast cancer cell line MCF-7 and the highly metastatic MDA-231 triple negative breast cancer (TNBC) model. These two cell lines also display a different expression of epithelial and mesenchymal markers, respectively (Supplementary Fig. 1). As indicated in Fig. 1, 397 proteins were identified as differentially expressed between MCF-7 and MDA-231 cells. In particular, the heat-map generated by Perseus, segregated samples into two separated branches characterised by two clusters of up- and down-regulated proteins (Fig. 1B). Cluster 1 represents proteins up-regulated in MDA-231 cells (156 proteins) (Supplementary Table 3), while cluster 2 proteins up-regulated in MCF-7 (239 proteins) (Supplementary Table 4). This separation clearly indicated the profound proteomic differences between the two cell models. A protein-protein interaction (PPI) network and a gene ontology analysis of our dataset were generated by STRING (Fig. 1C). Identified proteins were classified in terms of KEGG pathways with a great predominance of proteins involved in the regulation of metabolic processes in MCF-7 compared to MDA-231 (59 members, false discovery rate 4.99e-10). Overall, this analysis identified cellular pathways that are enriched for proteins associated with the two models.

3.2. Mesenchymal breast cancer models have reduced expression and activity of enzymes involved in the fatty acid synthesis

The dysregulation of metabolism is an established feature of EMT. Our dataset of identified proteins contains a large group of proteins associated with different metabolic-related processes. This may reflect a specific metabolic demand required to support the growth of the two models. For this purpose, we examined the viability of MCF-7 and MDA-231 cells when cultured in the absence of glucose or lipids. As shown in Supplementary Fig. 2, the viability of MDA-231 was significantly affected in these culture conditions. On the contrary, MCF-7 showed a greater metabolic plasticity that made these cells more adapt to adjust their metabolism in response to the different environmental stimuli. This is accomplished by a differential expression of enzymes involved in lipids and glucose metabolism. If we consider glucose metabolism, data reported on Supplementary Fig. 3 show proteins identified by MS/MS and those validated by western blot. In MDA-231, we observed a higher expression of glucose transporter 1 (GLUT1), lactate
dehydrogenase (LDH)-A, LDH-B, and hexokinase type II (HXKII) suggesting a higher anaerobic glycolytic profile of this cell model compared to MCF-7. Moreover, MDA-231 cells showed reduced levels of G6PD as demonstrated by MS/MS, western blot, qPCR, and enzymatic activity (Supplementary Fig. 3B). Overall, this characterizes the two models respect to a metabolic dataset of glycolytic enzymes. We then validated this signature with microarray data retrieved from the GEO dataset GSE41313 [15] and confirmed that HKI, HKII, FBP1, G6PD, aldose reductase (AR), LDH-B, monocarboxylate transporter 4 (MCT4), and basigin (BSG) represent differentially expressed genes in luminal and basal-like models of breast cancer (Supplementary Fig. 4).

If alterations in enzymes related to glucose metabolism seem to make functional the dependence of MDA-231 from glucose, the down-regulation of FASN may be coherent with the observed dependence of these cells from lipids. We zoomed in on genes of fatty acids synthesis pathway and we observed that mRNA levels of CIC, ACLY, ACACA (also referred here as ACC), ACACB, and FASN were all significantly down-regulated in MDA-231 compared to MCF-7 cells supporting the significant metabolic differences between the two cell models (Fig. 2A). Moreover, a different expression level of the transcriptional factors Myc, Carbohydrate-Responsive Element-Binding Protein (ChREBP) and Sterol Regulatory Element-Binding Protein-1c (SREBP-1c), was also measured between the two cell models, demonstrating that the differential expression of the key lipogenic enzymes was mostly due to a different transcriptional regulation (Fig. 2A) with a consequent effect at protein level (Fig. 2B) and enzymatic activity (Fig. 2C). Finally, GEO analysis confirmed that FASN down-regulation is a characteristic of basal breast cancer compared to luminal models (Supplementary

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**Fig. 2.** Expression and activity of proteins related to lipid metabolism in breast cancer models. A) mRNA levels, obtained by qPCR analysis, for citrate carrier (CIC), ATP-citrate lyase (ACLY), acetyl-CoA carboxylase α and β (ACACA and ACACB), fatty acid synthase (FASN), MYC, Carbohydrate-Responsive Element-Binding Protein (ChREBP), and Sterol Regulatory Element-Binding Protein-1c (SREBP-1c) in MCF-7 and MDA-231 cells. B) Western blot analysis of CIC, ACC, ACLY, FASN protein expression in MCF-7 and MDA-231 cell lines. α-Tubulin (α-Tub) was used as loading control. C) Enzymatic activity of ACLY, ACC, FASN and CIC. CIC activity was measured in proteoliposomes reconstituted with the mitochondrial extract from MCF-7 and MDA-231 cells. FASN and ACC activities were measured in digitonin-permeabilized MCF-7 and MDA-231 cells and ACLY activity by a spectrophotometric assay as reported in the Materials and methods section. Values are means ± S.D. of four independent experiments. P value * < 0.05, ** < 0.01, *** < 0.001.
Fig. 5A). We provided further evidence in *vitro* by culturing MCF-7 and MDA-231 cells as mammospheres (Supplementary Fig. 5B) and in *vivo* using xenograft models (Supplementary Fig. 5C), by comparing subcutaneous tumors obtained from MCF-7 and subcutaneous tumors and bone metastasis obtained from MDA-231 cells (Supplementary Fig. 4C). In both conditions, we observed a higher expression of proteins related to fatty acid metabolism in the epithelial MCF-7 model.

The differential expression of lipogenic enzymes may affect the rate of *de novo* fatty acid synthesis with important cellular consequences including the activation of compensatory metabolic pathways to sustain lipid demand. We determined how the reduced expression of lipogenic enzymes might affect the incorporation of labeled acetate into fatty acids, and we profiled this association in a panel of normal and cancer cells with a different expression of FASN and EMT markers (Fig. 3A). As shown in Fig. 3B, high [1-14C]acetate incorporation into fatty acids was observed in cell models with a higher FASN level, demonstrating a correlation between FASN expression and fatty acid precursor utilization. This association was further verified in MCF-7 cells stable silenced for FASN (MCF-7 shFASN) (Supplementary Fig. 6). Overall, this may push mesenchymal models towards the activation of specific metabolic strategies to overcome their low FASN expression.

### 3.3. Mesenchymal models preferentially incorporated exogenous labeled fatty acids into triacylglycerols

Mammalian FASN catalyzes the incorporation of acetyl-CoA units into palmitoyl-CoA. Thus, new synthesized cellular fatty acids, eventually elongated and desaturated, are mainly incorporated into complex lipids. We determined if decreased FASN activity could be correlated to a different incorporation of radiolabeled acetate into phospholipids and neutral lipids. After 1 h of cell incubation with [1,14C]acetate a general decrease of labeled precursor incorporation into both neutral lipids and phospholipids was observed in MDA-231 with respect to MCF-7 (Fig. 4A).

Analysis of the radioactivity incorporated in polar lipid classes indicates that 15.41 ± 3.43% and 16.92 ± 2.68% of the radioactivity was associated with phosphatidylethanolamine (PE), 61.84 ± 6.96% and 65 ± 6.62% with phosphatidylcholine (PC), 22.75 ± 2.84% and 21.25 ± 3.31% with phosphatidylinositol/phosphatidylserine (PI/PS) in MDA-231 and MCF-7, respectively. Thus, although we measured lower total acetate incorporation into phospholipid species in MDA-MB-231 with respect to MCF-7 cells, the efficiency in the synthesis of each phospholipid species was not different between the two cell lines. Regarding the acetate incorporation into neutral lipids, the behavior was different. In particular, although the amount of labeled substrate incorporated into neutral lipids in MDA-231 was about half of that measured in MCF-7 cells, MDA-231 had a greater incorporation of acetate into triglycerides (TAG) and ester of cholesterol (about 23% CE and 33% TAG in MDA-231 and 4% CE and 14% TAG in MCF-7) and a lower acetate incorporation into diacylglycerol and cholesterol (DAG + CHOL) with respect to MCF-7 cells. This data allowed us to hypothesize a greater efficiency of TAG synthesis of MDA-231 with respect to MCF-7 cells.

Considering that acetate is the precursor of fatty acids, the lower efficiency of acetate incorporation into neutral and polar lipid of MDA-231 cells could be related to the lower lipogenic efficiency of these cells with respect to MCF-7.

Thus, we followed the fate of exogenous added labeled fatty acids into complex lipids by incubating cells with different 14C-labeled fatty acids, such as palmitate (16:0), oleate (18:1), and linoleate (18:2). As shown in Fig. 4B, the total amount of radioactivity into phospholipids was higher in MCF-7 but to a lesser extent with respect to what observed using acetate, whereas radiolabeled fatty acids were extensively incorporated into the TAG fraction of MDA-231 with respect to MCF-7 cells. These findings suggest that a coordinated molecular program may be responsible for the different incorporation rate of fatty acids into TAG in MDA-231. By MS/MS we found an increased expression in MDA-231 cells of proteins involved in the fatty acid trafficking and lipid droplets formation including fatty acid-binding protein 5 (FABP5), caveolin1 (CAV1), and perilipin-3 (PLIN3) (Supplementary Table 3). These proteins mediate the fatty acid trafficking and are associated with lipid droplets (Fig. 5A). Transcripts of these proteins, analyzed by qPCR, were modulated in the two breast models as well as those of other enzymes with a central role in triacylglycerol metabolism (Fig. 5B). Among these, the lipid transporter CD36, seipin, DGAT1 and DGAT2, that mediates the final step of triacylglycerol synthesis, and the monoacylglycerol lipase (MGAL) enzyme involved in the utilization of cellular fat stores. We reported higher levels of DGAT1 and MGAL in MDA-231 cells with respect to MCF-7 (Fig. 5B) but no a significant change in DGAT2 expression supporting a specific role for DGAT1 isof orm in MDA-231 lipid metabolism. To confirm this data, enzymatic activity and western blot analysis of DGAT were performed in MCF-7 and MDA-231 cells. As shown in Fig. 5C, total DGAT activity was significantly higher in MDA-231 as compared to MCF-7 cells. The protein level of DGAT1 was also increased in MDA-231 whereas the expression of DGAT2 was significantly reduced compared to MCF-7 (Fig. 5D). This led us to confirm that the increased DGAT activity measured in MDA-231 cells was mainly due to DGAT1 isof orm. In order to further confirm the contribution of both isof orms to the DGAT activity, we measured the enzymatic activity in presence or absence of specific DGAT1 or DGAT2 inhibitors (Fig. 5E). In MDA-231, a significant reduction of enzymatic activity was observed only in presence of DGAT1 inhibitor, confirming the major role of this enzyme in MDA-231 triacylglycerol synthesis.

Moreover, this result was also supported by TLC analysis that highlighted a higher TAG level in MDA-231 with respect to MCF-7 cells (Fig. 5F). Finally, as fatty acids present into TAG may be used to generate lipid mediators [25] or for energetic purposes, we measured fatty acid oxidation efficiency in MDA-231 and MCF-7 cell lines. As shown in Fig. 5G, MDA-231 cells showed a higher rate of [14C]CO2 production,
Fig. 4. Incorporation of radiolabeled substrates into neutral and phospholipids in MCF-7 and MDA-231 cells. A) Incorporation of [1-14C]acetate into polar and neutral lipids. B) Incorporation of [1-14C]oleate, [1-14C]palmitate and [1-14C]linoleate, into lipid fractions. Values are means ± SD of four independent experiments. Phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), cholesterol ester (CE), triacylglycerol (TAG), diacylglycerol (DAG), cholesterol (CHOL), free fatty acids (FFA).
the end product of labeled palmitate oxidation, with respect to MCF-7 cells. This is also consistent with the increased expression, in MDA-231 cells, of acyl-CoA synthetases (ACSL)-5, ACSL-4, and ACSL-3 enzymes that activate long-chain and very-long-chain fatty acids to form acyl-CoA substrates for both lipid synthesis and β-oxidation (Supplementary Fig. 7).

Finally, our experiments demonstrated that, in the mesenchymal model, the backbone for TAG synthesis was probably furnished by glycolytic glycerol-P as demonstrated by the higher incorporation of labeled glucose into total lipids of mesenchymal vs epithelial cells (Supplementary Fig. 8).

In order to evaluate the role of DGAT1 in the control of lipid metabolism in MDA-231 cells, we followed the rate of fatty acid synthesis from labeled acetate, using a concentration of DGAT1 inhibitor capable to induce a reduction of 70% of the enzymatic activity (see Fig. 5E). In this experimental condition, MDA-231 cells signiﬁcantly decrease the incorporation of acetate into fatty acids by elongation, and hence makes the analysis representative of the incorporation of acetate into fatty acids by de novo synthesis. Thus, we speculated that the reduction of incorporation of acetate into fatty acids observed after DGAT1 inhibition might occur through the regulation of de novo synthesis by a similar mechanism. To validate this, we investigated the expression of FASN and ACC by western blot. As shown in Supplementary Fig. 9A, total levels of FASN and ACC were not affected. By contrary, we observed an up-regulation of the phosphorylation of ACC at Ser79 associated with an up-regulation of its upstream regulator phospho-AMP-dependent kinase (AMPK). Overall, the cellular effects of DGAT1 inhibition are associated with a condition of metabolic stress with a subsequent increase of the phosphorylation of AMPK and its downstream target ACC. This effect was not observed when MDA-231 was treated with the inhibitor of DAGT2, suggesting the critical role of DAGT1 in supporting MDA-231 metabolism (Supplementary Fig. 9A).

We next asked if DGAT1 inhibition has a consequence on the high proliferative and migratory cellular features of MDA-231. Data show that DGAT1 inhibition affects cell proliferation and migration mediated by epidermal growth factor (EGF) in vitro (Supplementary Fig. 9B and C), and reduces the expression of the EMT markers cyclin D1 and Zeb1 (Supplementary Fig. 9D).

3.4. Epithelial and mesenchymal models have a unique lipidomic profile

If MCF-7 and MDA-231 adopt specific strategies to support their metabolic demand this may reflect in a different lipidomic profile. Comprehensive fatty acids profiling of MCF-7 and MDA-231 performed by GC–MS indicated that MDA-231 had a higher percentage of saturated and fatty acids and polyunsaturated fatty acids (PUFA) compared to MCF-7 cells which instead present a higher percentage of mono-unsaturated fatty acids (MUFA) (Fig. 6). These data are consistent with the higher expression in MCF-7 cells of the stearoyl-CoA desaturase-1 (SCD1) enzyme that we identiﬁed by MS/MS/MS analysis and validated by western blot (Supplementary Table 4 and Supplementary Fig. 10).

The data obtained by GC–MS were conﬁrmed and complemented by NMR analysis conducted on the total lipids extracted from both cell lines. A representative 1H NMR spectrum is shown in Supplementary Fig. 11A. Resonance assignment was performed according to the
literature [26–28] and two-dimensional analyses, allowing identifying a total of 25 lipid signals corresponding to several species, such as cholesterol, saturated and unsaturated fatty acids, phosphatides, and triglycerides, as reported in Supplementary Table 5.

Each lipid species usually gave more NMR proton signals and the average peak area was considered, as in the case of fatty acids. On the other hand, when proton signals overlapped with peaks of other metabolites or with solvent peaks, only the integral area of clean signals was considered for the quantitative analysis, as in the case of cholesterol. Unfortunately, all the signals from glycerol derivatives overlapped with other peaks and it was not possible to quantify them. After normalization of the total integral area, the NMR analysis allowed to determine the relative differences in concentration of various lipid species in the two cell lines, which were reported as the times-fold the corresponding signal was over- or under-expressed in MDA-231 relatively to MCF-7 cells.

Fig. 6. Fatty acid profile of MCF-7 and MDA-231 cells by GC-MS. Total lipids extracted by the two cell lines were transesterified with methanolic boron trifluoride and analyzed by GC-MS following the procedure reported in the Materials and methods section. The different saturated fatty acid, monounsaturated fatty acid (MUFA), and polyunsaturated fatty acid (PUFA) composition of MCF-7 and MDA-231 cells is reported. Values are means ± S.D. of three independent experiments. P value * < 0.05, ** < 0.01, *** < 0.001.

According to the NMR analysis, cholesterol and PUFA were the only...
species over-expressed in the MDA-231 cell line, all the other lipid being more abundant in the epithelial model. The total amount of fatty acids was 11% higher in the MCF-7 cells, as showed by the methylene hydrogen protons in α and β to the carbonyl at 1.62 and 2.35 ppm, respectively. The terminal methyl group at 0.89 ppm gave a more significant 15% increase although this value was probably spiked by the cholesterol signals in the same spectral region. Differently from PUFA, MUFA were under-expressed in MDA-231 cells by over 20%, which was averaged from the allylic and olefinic peaks at 2.02 and 5.35 ppm, respectively. The signal at 1.32 ppm relative to all allylic protons gave a smaller 10% decrease probably because of the error caused by the partial overlap with the methylene proton signal of the acyl chain. The peak at 3.10 ppm showed a 22% decrease of phosphatidylethanolamine with respect to the MCF-7 cell line, while the total choline-containing phospholipids were estimated to be 8% lower by integrating the resonance about 3.2 ppm, characteristic of the four methyl groups bound to the ammonium ion. Finally, the glycerol methine peak at 5.23 ppm showed a 25% higher concentration of total phospholipids in MCF-7.
A multivariate statistical analysis was then performed to investigate the greatest variations in the NMR data by PCA. A quite good separation along PC2 was observed between MCF-7 and MDA-231 cellular lipid extracts, although the latter were quite dispersed on both axes (Supplementary Fig. 11). Nevertheless, the two cell lines showed a quite different lipid profile, as observed by the corresponding loadings, which respected the observations previously reported. On the other hand, good very clustering was observed when supervised OPLS-DA was performed on the samples representing the two cell lines. Clear separation between MCF-7 and MDA-231 cells was observed along the main component (Fig. 7A) with an impressive cumulative R²X of 0.94. The analysis also yielded a very good predictive model with a Q²(cum) of 0.95, obtained by cross-validating the data. The most significant differences detected by the OPLS-DA were in total fatty acid concentration, as indicated by the buckets corresponding the terminal methyl and various methylene groups in the loadings column plot (Fig. 7B). The allylic proton signals of PUFA and MUFA were also considered significantly different by the obtained model and, to a lesser extent, the choline derivatives. According to the supervised analysis, the other lipid signals were not statistically significant in differentiating between the two groups, although quantitative differences could be measured in the NMR spectra.

The profound differences in the fatty acid composition of these cells may affect their phospholipid composition. By MALDI-TOF/TOF, we analyzed the phospholipid species present in MDA-231 and MCF-7 cells. Negative ion mode spectra obtained by in situ analysis of MCF-7 and MDA-231 using 9-AA as the matrix is shown in Fig. 7C. PLS-DA analysis showed a clear separation between the two groups, suggesting that significant differences occur in the phospholipid profile of MCF-7 and MDA-231 (Fig. 7D). According to VIP, the most significant discriminant lipid species were phosphoinositols species PI(38:4), PI(38:3), PI(34:1), PI(40:5), PI(40:4), PI(38:2), PI(38:5), PI(40:6), and phosphatidylethanolamine PE(36:1) (Fig. 7E). The changing trend of these lipid species is shown in Fig. 7E, and their fragmentation spectra, as determined by MS/MS, are reported in Supplementary Table 6. Moreover, we also observed changes in the saturation of the FA chain constituents with an increased abundance of PUFA-containing phospholipids in MDA-231 cells as compared to MCF-7. In particular, MDA-231 showed a significant increase in the arachidonic-containing PI(38:4) at m/z 885. By comparing the lipidomic profile of MDA-231 to that of MDA-415, MDA-361, and T47D we demonstrated that the phospholipid fingerprint of MDA-231 characterizes this cell line with respect to a panel of epithelial models. Consistently with what was observed in MCF-7 and MDA-231, PLS-DA 3D score plot showed a clear separation between MDA-231 and the other epithelial models (Supplementary Fig. 12A) and confirmed the higher abundance of PI(38:4) in MDA-231 (Supplementary Fig. 12B).

3.5. β-Catenin regulates MDA-231 lipid metabolism

We previously demonstrated that β-catenin is involved in the regulation of fatty acid metabolism in MCF-7 [14]. Here, we investigated the role of β-catenin in the regulation of genes involved in lipid metabolism in MDA-231. To do this, we used a short-hairpin RNA (shRNA) vector targeting β-catenin and verified by qPCR its down-regulation. Significant lower levels were observed in MCF-231 shβ-caten cells compared to control cells (scramble) (Fig. 8). By qPCR, we screened shβ-caten and scramble cells for the expression of EMT markers and metabolic enzymes. As shown in Fig. 8, β-catenin regulates the expression of both groups of genes. Of note, while the expression of the main analyzed genes was positively correlated with β-catenin, DGAT2, and MGL expression showed an inverse relationship. In particular mRNA and protein level, as well as the enzymatic activity of DGAT2 was increased in shβ-caten with respect to scramble cells (Fig. 8C). To note that, the down-regulation of β-catenin, upregulating the DGAT2 expression determined an increased TAG level, as revealed by TLC analysis, in shβ-caten with respect to scramble MDA-231 cells.

Differences between scramble and shβ-caten MDA-231 cells were also highlighted by NMR analysis of lipid extracts (Supplementary Table 7). The main changes observed in shβ-caten MDA-231 with respect to scramble cells were as follow: increased amount of cholesterol (6%), total PUFA (10–15%) and MUFA these last reaching values similar to those found in MCF-7 cells. In addition, the level of choline and phosphatidylethanolamine derivatives, as well as that of total phospholipids increased in shβ-caten MDA-231 with respect to scramble cells.

Multivariate PCA was not able to separate the two groups (Supplementary Fig. 13A–B), while they clustered quite well with the supervised discriminant analysis (Fig. 8D–E). Investigation of the loadings plot showed that discrimination was mainly accomplished through the signals just over 2.0 ppm, corresponding to the allylic protons of PUFA and MUFA, and the bin at 3.2 ppm, corresponding to the choline derivatives.

4. Discussion

In the present work, we used multiple approaches, including shotgun proteomic analysis, GC–MS, and lipidomics, to characterize MCF-7 and MDA-231 cells. Overall, these data provided a comprehensive characterization of these two models and allowed to capture specific changes in several cellular pathways. One of the most prominent network of modified proteins consisted of metabolic enzymes, and we decided to investigate these differences more deeply not only as metabolism is a hallmark of cancer but also as there is a growing interest towards the specific metabolic alterations that characterised cancer subtypes. For example, a large proteomic analysis provided important information about the metabolism of estrogen-receptor positive tumors compared to healthy tissues and lymph node metastasis [29]. Here, we focus on defining the metabolic differences between cancer models with different EMT features. However, in addition to this focus on metabolism, a large number of differentially expressed proteins were listed in Supplementary Tables 3 and 4 that may represent the basis for future investigations on other pathways and networks.

Differently expressed proteins identified through LC–MS/MS include enzymes involved in canonical metabolic pathways such as glycolysis and lipid metabolism. In particular, we observed an up-regulation of GLUT1, HKII and LDH-A, LDH-B, MCT4 and BSG in MDA-231 compared to MCF-7 (Supplementary Fig. 3). A high expression of LDH-B has been already observed in various types of cancer, including aggressive breast tumors, and associated with a poor survival rate [30]. Triple-negative cells may release high lactic acid amount that promotes metastatic invasion and malignant aggressive transformation [31]. On the contrary, MCF-7 showed an increased glucose branching to pentose phosphate pathway (PPP) as demonstrated by the higher expression of G6PD, the key enzyme of this metabolic pathway (Supplementary Fig. 3). Another feature of MCF-7 cells is the increased lipogenic activity with respect to MDA-231 cells as highlighted by the higher level of FASN and other lipogenic enzymes whose expression is controlled transcriptionally (Fig. 2). In contrast, MCF-7 cells exhibit a fall of the de novo lipid synthesis from acetate and a dependency on exogenous FA for TAG synthesis. This is in accord with the increased level of mRNAs and proteins involved in FA activation (Supplementary Fig. 7), FA binding, and TAG synthesis observed in the mesenchymal model (Fig. 5), and correlates with the higher basal levels of lipid droplets described in triple negative cells compared to estrogen-receptor positive cell lines [32].

These results have a substantial effect on the utilization of metabolic substrates. The incorporation of labeled acetate into lipid fractions was significantly lower in MDA-231, while labeled fatty acids incorporate efficiently into TAG. Our data also demonstrated that, when labeled glucose is utilized as lipid synthesis precursor, mesenchymal cells incorporate this substrate into total lipids in a greater amount with 354
Fig. 8. Metabolic alterations in β-catenin knockdown MDA-231 cells. A) β-catenin levels were determined by qPCR in MDA-231 scramble and shβcat cells. Expression levels determined by qPCR of EMT markers and EMT-transcriptional factors (TFs), and of enzymes of glucose, fatty acids and TAG metabolism in MDA-231 scramble and shβcat cells. P value * < 0.05, ** < 0.01, *** < 0.001. B) DGAT2 protein expression and activity in MDA-231 scramble and shβcat cells. α-Tubulin was used as loading control. P value ** < 0.01. C) Neutral lipid separated by one-dimensional thin-layer chromatography. Values are means ± S.D. of three independent experiments. P value * < 0.05, *** < 0.001. D) OPLS-DA scatter score plot of MDA-231 scramble and shβcat cells cellular lipid extracts. E) OPLS-DA loadings column plot of MDA-231 scramble and shβcat cells cellular lipid extracts.
respect to the epithelial model (Supplementary Fig. 7). Considering that glucose participates in the lipid synthesis or by de novo pathway or by furnishing glycerol-phosphate for complex lipid synthesis, we can assert that the mesenchymal model utilizes glucose in the form of glycerol-phosphate for TAG synthesis.

The differences so far reported between MDA-231 and MCF-7 cells place the mesenchymal model in a condition of greater dependence on energetic substrates. In fact, MDA-231 showed a reduced viability when maintained under conditions of metabolic stress (Supplementary Fig. 2), while MCF-7 demonstrated a major plasticity of MCF-7 cells to changes in the environment as also confirmed by their greater ability to convert acetate, a “non-conventional” substrate, into fatty acids with respect to MDA-231. These data indicate that both glucose and exogenous fatty acids may be essential for MDA-231 metabolism. While the dependence from glucose well correlates with the higher expression of GLUT1, it remains unclear whether the dependence from exogenous fatty acids has some effects on lipid transporters. In this context, our MS/MS and qPCR data revealed a reduced expression of CD36 in MDA-231 cells. This correlates with the lower levels of EMT markers observed in CD36+ cells compared to the CD36− counterparts in primary lesions and lymph node metastases [33], and with the possible involvement of other transporters and enzymes [34]. Moreover, our results indicate that MDA-231 cells had a higher β-oxidation rate with respect to MCF-7 cells (Fig. 5G) thus further emphasizing the essentially of fatty acids in MDA-231 metabolism. This observation raises the question of whether targeting fatty acid β-oxidation for cancer therapy may be effective in aggressive breast cancer cell lines. Knock-down or chemical inhibition of fatty acid oxidation rate-limiting enzymes carnitine palmitoyltransferase-IA (CPT1) and CPT2 significantly decrease tumor growth and distant metastasis by regulating the activation of Src pathway in TNBC models [35].

Metabolic profiles determined by GC-MS, NMR, and MALDI-MS/MS follow the distinct molecular program providing a link into how genomic/proteomic changes are associated with molecular alterations. Significantly increased levels of MUFA were observed in MCF-7 cells that on the contrary showed a lower level of PUFA with respect to MDA-231 cells. The enzyme responsible for the conversion of saturated fatty acids to MUFA, mainly as palmitoleyl- and oleyl-CoA, is the stearoyl-CoA desaturase (SCD1) whose expression was down-regulated in MDA-231 (Supplementary Fig. 10). The under-utilization of saturated fatty acids in MDA-231 could justify the higher percentage of these fatty acids with respect to MCF-7. Moreover, the presence of a higher percentage of PUFA in MDA-231 is compatible with the overexpression of genes involved in the long-chain fatty acid desaturation enzymes that characterize the mesenchymal model (Supplementary Fig. 10).

Interestingly, the different fatty acid pattern of the two cell models was mirrored in a different phospholipid fatty acid profile with higher levels of PI (38:4) in MDA 231 cells with respect to MCF-7 cells. This was also confirmed on the basis of multivariate analysis of the lipidomic profile of a panel of breast cancer models (Supplementary Fig. 12) and consistent with previous studies that described differential levels of phospholipid species in breast models [36]. Thus, shifting lipid metabolism towards lipid uptake more than de novo lipogenesis may significantly affect phospholipids composition, and TAG profile that becomes dominated by arachidonic acid as previously demonstrated [37]. This means that PI is an important source of PUFA in MDA-231 cells and a potential substrate for the activity of phospholipase A2 that results in over-expressed in mesenchymal cells (Supplementary Table 4).

DGAT enzymes are involved in the synthesis of TG from FA and diacylglycerols. We found that epithelial and mesenchymal cells use different DGAT isoforms to sustain their triacylglycerol metabolism. In particular, MDA-231 cells not only present higher expression of DGAT1 isoform but also a higher DGAT total activity and cellular TAG level. It is reported that DGAT1 is involved in the synthesis of TAG from exogenous fatty acids whereas DGAT2 mainly utilizes those produced by the de novo pathway [38]. This result is in line with the observed reduction of de novo fatty acid synthesis, the increased incorporation of exogenous labeled fatty acids, and with the inhibition of TAG synthesis demonstrated after DGAT1 inhibition by other authors [39]. Our study demonstrates that DGAT1 inhibition decrease acetate incorporation into fatty acids and we hypothesize that this is mediated by activation of AMPK and subsequent ACC phosphorylation and inhibition. Experimental data also showed that DGAT1 inhibition reduces MDA-231 proliferation, migration, and EMT markers expression including Zeb1. It will be interesting to study whether the molecular mechanisms by which DGAT1 inhibition affect Zeb1 expression.

It must be considered that MDA-231 cells also show a lipolytic tendency as highlighted by their higher expression of MAGL, an enzyme involved in TAG hydrolysis. DGAT1 may have a role in limiting lipidotoxicity under basal or lipolysis-stimulating conditions by a rapid re-esterification of hydrolysed fatty acids [40]. Additionally, DGAT1 can be selectively targeted resulting in the inhibition of growth, cell cycle block and cell death of tumor models [41].

We demonstrated that the ability to regulate TAG synthesis and breakdown is a characteristic of MDA-231 and other mesenchymal models [28]. Less is known about the transcriptional regulation of this process. Here, we demonstrated the β-catenin is critical in the control of several metabolic enzymes and that is directly correlated with DGAT1 and inversely correlated with DGAT2 expression. This finding is somewhat surprising in light of our previous work showing that knockdown of β-catenin in MCF-7 does not modify DGAT2 levels [14]. The basis for this remains unclear although the specific EMT-TFs background may be considered, supporting a specific role for the mesenchymal TFs in the regulation of lipid metabolism. This is a realistic hypothesis considering the key role of Zeb1 as a central transcriptional component of fat cell differentiation [42], that should be validated in future studies. Moreover, accumulation of lipid droplets was demonstrated to occur in neuroblastoma cells after targeting of Myc [43], suggesting that also Myc may be an important mediator of the metabolic phenotype observed in MDA-231 spheroid cell.

Overall, our analysis highlighted significant metabolic differences and identified a group of enzymes that is under the transcriptional control of β-catenin. Based on our present results, we can speculate that targeting TAG metabolism could be a feasible therapeutic strategy for breast cancer with down-regulated FASN.

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Conflict of interest

The authors declare that they have no conflict of interest.

Transparency document

The Transparency document associated with this article can be found, in online version.

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