Aconitase 2 inhibits the proliferation of MCF-7 cells promoting mitochondrial oxidative metabolism and ROS/FoxO1-mediated autophagic response

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BACKGROUND: Deregulation of the tricarboxylic acid cycle (TCA) due to mutations in specific enzymes or defective aerobic metabolism is associated with tumour growth. Aconitase 2 (ACO2) participates in the TCA cycle by converting citrate to isocitrate, but no evident demonstrations of its involvement in cancer metabolism have been provided so far.

METHODS: Biochemical assays coupled with molecular biology, in silico, and cellular tools were applied to circumstantiate the impact of ACO2 in the breast cancer cell line MCF-7 metabolism. Fluorescence lifetime imaging microscopy (FLIM) of NADH was used to corroborate the changes in bioenergetics.

RESULTS: We showed that ACO2 levels are decreased in breast cancer cell lines and human tumour biopsies. We generated ACO2-overexpressing MCF-7 cells and employed comparative analyses to identify metabolic adaptations. We found that increased ACO2 expression impairs cell proliferation and commits cells to redirect pyruvate to mitochondria, which weakens Warburg-like bioenergetic features. We also demonstrated that the enhancement of oxidative metabolism was supported by mitochondrial biogenesis and FoxO1-mediated autophagy/mitophagy that sustains the increased ROS burst.

CONCLUSIONS: This work identifies ACO2 as a relevant gene in cancer metabolic rewiring of MCF-7 cells, promoting a different utilisation of pyruvate and revealing the potential metabolic vulnerability of ACO2-associated malignancies.

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interfering with tumour metabolic phenotype is a novel therapeutic strategy that can be exploited to preferentially kill cancer cells.\textsuperscript{13}

Considering the advantages, in terms of energetic and anabolic precursors, that a cancer cell can acquire by deregulating the TCA cycle, in this paper, we have assessed whether the modulation of aconitase 2 (ACO2), the second enzyme involved in the TCA cycle, could induce metabolic rearrangements and proliferation defects in cancer cells. This enzyme is also known as mitochondrial aconitase as it catalyses the reversible isomerisation of citrate to isocitrate in mitochondria, a reaction that can be also performed in the cytosol by aconitase 1 (ACO1). We have focused the attention on ACO2 because it belongs to a branch of the TCA cycle particularly important for cancer metabolic features as it is interposed between citrate, which plays a role in lipid anabolism, and α-KG that can be replenished by glutamine anaplerosis.\textsuperscript{1,2}

Moreover, other relevant cues in support of a putative involvement of ACO2 in cancer are that (i) its reduced levels in pluripotent stem cells make glutamine a key fuel for the TCA cycle,\textsuperscript{14} (ii) it was found inactivated in fumarate hydratase (FH)-deficient tumours\textsuperscript{15} and (iii) its expression was found downregulated in cancer cells.\textsuperscript{16,17}

Here we show that ACO2 expression is reduced in breast cancer, and increasing the levels of the enzyme in MCF-7 cells can inhibit cell proliferation. By generating cells with stable overexpression of ACO2 gene, we demonstrated that proliferation inhibition is associated with enhanced oxidative metabolism mainly due to redirection of pyruvate to mitochondrial oxidation. The metabolic phenotype of ACO2-expressing cell was favoured by autophagic/mitophagic response to increased production of reactive oxygen species (ROS) by the activation of Forkhead box protein O1 (FoxO1) signalling.

METHODS

Cell cultures, transfections and treatments

Cell lines were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Lonza) supplemented with 10% foetal bovine serum (EuroClone), 10 U/ml penicillin/streptomycin (Lonza) and 2 mM L-glutamine (Lonza). Cells were authenticated and characterised by the supplier. Mycoplasma test was routinely carried out according to protocols from our laboratory. We have focused the attention on ACO2 because it belongs to a branch of the TCA cycle particularly important for cancer metabolic features as it is interposed between citrate, which plays a role in lipid anabolism, and α-KG that can be replenished by glutamine anaplerosis.\textsuperscript{1,2}

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Cell proliferation assays

Cell proliferation was evaluated by Trypan blue exclusion test procedure and by bromodeoxyuridine (BrdU) incorporation assay. For the latter, cells were incubated with 10 µM BrdU for 4 h and then fixed for 30 min in ethanol:acetic acid:water solution (18:1:1). DNA denaturation was obtained by incubation on ice for 10 min with 1 N HCl and then 10 min with 2 N HCl. Treatments with PBS/0.4% Triton X-100 solution for 10 min and then PBS/3% BSA for 1 h were performed before incubation for 16–24 h with an anti-BrdU antibody (Santa Cruz Biotechnology) followed by 1 h of incubation with an Alexa Fluor™ 568 donkey secondary antibody; nuclei were stained with 1 µg/ml of Hoechst 33342 for 5 min. Images of cells were obtained with a Delta Vision Restoration Microscopy System (Applied Precision, Issaquah, WA) equipped with an Olympus IX70 fluorescence microscope (Olympus Italia, Segrate, Milan, Italy).

Fluorescence lifetime microscopy (FLM) of NAD(P)H autofluorescence

FLM data were acquired with a Nikon A1-MP confocal microscope equipped with a 2-photon Ti:Sapphire laser (Maia Ti, Spectra Physics, Newport Beach, CA) by producing 80-fs pulses at a repetition rate of 80 MHz. A PML-SPEC 16 GaAsP (B&H, Germany) multi-wavelength detector coupled to a SPC-830 TCSPC/FLM device (B&H, Germany) was used to collect the decay data. A 60 x oil-immersion objective, 1.2 NA, was used for all experiments. Samples were excited at 750 nm. Signals were integrated into the wavelength region of 408–496 nm. For image acquisition, the pixel frame size was set to 512 x 512 and the pixel dwell time was 60 µs. The average laser power at the sample was maintained at the milliwatt level. In the FLIM images, mitochondrial NAD(P)H responses were separated by the rest of the autofluorescence (cytoplasm, nuclei) by means of fluorescence intensity analysis.\textsuperscript{19}

Colorimetric assays

The aconitase 2 activity was determined by using the Aconitase Activity Assay Kit (Sigma-Aldrich) following the manufacturer's
instructions. Citrate Assay Kit (Sigma-Aldrich), α-Ketoglutarate Assay Kit (Sigma-Aldrich) and Fumarate Assay Kit (Abnova) were used to measure the levels of citrate, α-ketoglutarate and fumarate, respectively. Values were normalised on total protein amount.

Citrate synthase activity
Citrate synthase activity was determined spectrophotometrically according to Oexle et al.20 About 2 x 10^6 cells were harvested and washed in ice-cold PBS. Pellets were lysed in 100 mM Tris-HCl, pH 8.1 and 0.25% Triton X-100 supplemented with protease inhibitor cocktail (AMRESCO) for 30 min in ice. A total of 25 μg of proteins were used for each enzymatic reaction performed in 250 μl of reaction buffer (100 mM Tris-HCl, pH 8.1, 0.25% Triton X-100, 0.1 5,5-dithiobis(2-nitrobenzoate) (DTNB), 0.5 mM oxaloacetate and 0.31 mM acetyl-CoA). The principle of the assay is based on the reaction between DTNB and CoA-SH to form TNB with a maximum absorbance at 412 nm. The citrate synthase activity is proportional to the intensity of the absorbance. After a delay of 5 s, the reaction proceeds at 37 °C for a period of 4 min and absorbance recorded at 10-s intervals by an Eppendorf BioSpectrometer®. Enzyme activity was represented as a change in absorbance per minute (U), normalised on total protein amount.

Oxygen consumption and ATP measurement
Oxygen consumption was determined by using a Clark-type oxygen electrode as described in Di Leo et al.11 ATP levels were determined by the ATP Bioluminescence Assay Kit CLS II (Roche Applied Science) according to the manufacturer’s instructions. Values were normalised on the protein amount.

Extracellular lactate assay
The level of extracellular lactate was measured as previously described with minor modifications.11 Following 24 h from cell plating, the medium was replaced with a fresh one, and after 3 h, 500 μl of cell medium was precipitated with 250 μl of 30% trichloroacetic acid. Media were frozen after centrifugation at 14,000×g for 20 min at 4 °C, 10 μl of supernatant was incubated for 30 min at 37 °C in 290 μl of a buffer containing 0.2 M glycine/ hydrazine buffer, pH 9.2, 0.6 mg/ml NAD+ and 17 U/ml LDH. NADH formation was followed at 340 nm by using an Eppendorf BioSpectrometer®.

Cytofluorimetric analysis
Thirty minutes before the end of the experimental time, cells were incubated with 2-NBDG (100 μM) for measurement of glucose uptake, MitoTracker Green (200 nM) or Nonyl Acridine Orange (NAO) (50 nM), MitoTracker Red CMXRos (200 nM) for mitochondrial membrane potential, dihydroethidium (DHE) (50 μM) and MitoSOX Red (5 μM) for ROS determination and Bodipy 493/503 (1 μM). Cells were washed and then collected in PBS, and the fluorescence intensity immediately analysed by means of a FACScalibur instrument. In total, 10,000 events were counted, and mean fluorescence intensity expressed as arbitrary units.

Metabolite determination by HPLC
Cell cultures were collected after 24 h from plating, washed with ice-cold PBS and subsequently centrifuged at 1890×g for 10 min at 4 °C. Cell pellets were depurinated as described in detail elsewhere.21 Briefly, cell pellets were treated with a precipitating solution composed of CH3CN 75% and KH2PO4 25% (10 mM) at pH 7.4 and then centrifuged at 20,690×g for 15 min at 4 °C. Supernatants were collected and subjected to two chloroform washings in order to obtain an upper aqueous phase that was directly injected onto the HPLC, and analysed to determine concentrations of uridine diphosphate (UDP), uridine-diphosphate galactose (UDP-Gal), cytosine, uridine-diphosphate-n-acetylgalactosamine (UDP-GalNAc), uridine-diphosphate-N-acetylgalactosamine (UDP-GlcNAc), glutamate (Glu), glutamine (Gln) and taurine (Tau). Amino acids were analysed as ortophtalaldehyde (OPA) derivatives by using a method with precolumn derivatisation,22 whilst cytosine and UDP derivatives were separated and quantified according to an ion-pairing HPLC method previously set up.21 For both analyses, the HPLC apparatus consisted of a SpectraSystem P4000 pump and a highly sensitive UV6000LP diode array detector (ThermoElectron Italia), equipped with a 5-cm light-path flow cell, set up between 200- and 400-nm wavelength for acquisition of chromatographic runs. Data were acquired and analysed by Chrom-Quest® software package provided by the HPLC manufacturer. Separation of the various compounds was carried out by using a Hypersil 250 3 4.6-mm, 5 mM particle-size column, which was provided with its own guard column (ThermoElectron Italia). Species identification and quantification in depurinised cell extracts were determined by matching retention times, peak areas and absorption spectra of those of freshly prepared ultrapure standards. If needed, cochromatograms were performed by adding proper standards with known concentration to the medium samples. Concentrations of UDP, UDP-Gal, cytosine, UDP-GalNAc and UDP-GlcNAc were determined at 260-nm wavelength and those of NO2 and NO3 were calculated at 206-nm wavelength. Differently, concentrations of OPA-derivatised amino acids were calculated at 338-nm wavelength. Intracellular glutathione levels were determined as previously described.23

Determination of protein carbonylation
Carbonylated proteins were detected by the OxyBlot Kit (Millipore, S7150) as previously described,24 by using 15 μg of total proteins that were resolved in 12% SDS-polyacrylamide gels.

Chromatin immunoprecipitation (ChIP) analysis
ChIP assays were performed on nuclear lysates as previously described.25 Briefly, cells were cross-linked with 1% formaldehyde for 10 min at room temperature of 37%, and the reaction was quenched by 5 min of incubation in 0.125 M glycine. Cell monolayer was harvested in ice-cold PBS containing protease inhibitors, and nuclei isolation performed as previously described.18 Chromatin sonication was achieved by using Bioruptor NextGen (Diagenode) to high power. Sonicated DNA of ~500–1000 bp was pre-cleared with Protein A-coupled Sepharose beads pre-saturated with Salmon Sperm DNA and then immunoprecipitated with anti-FoxO1 antibody (Cell Signaling Technology) or normal rabbit IgGs (Santa Cruz Biotechnology) for 16 h at 4 °C. Immunoprecipitated DNA amplification was performed by using SYBR® Premix Ex Taq (Tli RNase H Plus) (Takara) on a StepOne real-time PCR System (Applied Biosystems). All reactions were run as triplicates. The results are expressed as fold enrichment with respect to IgG control. Primers used are listed in Supplementary Table 2.

Bioinformatic analyses
ACO2 expression was assessed by Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo) with accession numbers GSE15852 (n = 43 breast tumours vs normal counterpart) and GSE294318 (n = 54 breast tumours vs n = 12 healthy breast samples) through an Affymetrix Human Genome Array. Relapse-free survival (RFS) analysis referred to breast cancer was obtained from the Pan-cancer RNA-seq present in the Kaplan–Meier Plotter database. Mutational analysis of the ACO2 gene was performed by consulting the TGCA database.

Statistical analysis
The results are shown as means ± SEM and derive from at least three independent experiments. Statistical evaluation was conducted by using the unpaired two-tailed Student’s t test for metabolic analysis and enzymatic activity assays, paired Student’s t test for cytofluorimetric analysis and RT-qPCR analysis.
and one-way ANOVA with post hoc Tukey test for cell counts after treatments.

RESULTS

ACO2 expression is reduced in breast cancer and increasing its levels dampens cell proliferation of MCF-7 cells

To assess the expression of ACO2 in breast cancer cells, we compared the non-tumorigenic human breast epithelial cell line MCF10A with a panel of breast cancer cell lines, most of which exhibit a dramatic reduction of ACO2 protein levels (Fig. 1a). The decreasing trend observed for ACO2 in these cell lines was not a common feature for all the aconitase isoforms or TCA cycle enzymes, as stated by the levels of ACO1 and IDH2 proteins, respectively. Moreover, such changes were not ascribed to relevant fluctuations in mitochondrial mass, as demonstrated by the expression of GRP75 (Fig. 1a). RT-qPCR demonstrated that the transcriptional downregulation of ACO2 was also effective along with decreased protein levels (Supplementary Fig. 1a). The analysis of publicly available gene expression datasets deposited in the Gene Expression Omnibus (GEO) highlighted that ACO2 expression levels were reduced in human breast cancer biopsies (T) when either compared with non-tumoural adjacent counterpart (NT) or with normal breast tissue from other individuals (H) (Fig. 1b, c). The Kaplan–Meier Plotter demonstrated that the high expression of ACO2 in human tumour specimens is associated with good clinical outcomes in terms of relapse-free survival (RFS) (Supplementary Fig. 1b–d). Less than 1% of genetic alterations were instead identified for the ACO2 gene in breast cancer according to the TCGA data set (Supplementary Fig. 1e).

Prompted by the evidence that ACO2 levels are downregulated in breast cancer cells and in human breast cancer specimens, we transiently overexpressed myc-tagged ACO2 in MCF-7 cells (Supplementary Fig. 1f), demonstrating that high levels of ACO2 reduced cell proliferation (Supplementary Fig. 1g). To corroborate this result, we generated MCF-7 cells with stable overexpression of ACO2, and we choose to perform our analyses on two independent clones (#2 and #4) with different degrees of ACO2 overexpression as well as enzymatic activity (Fig. 1d, e). Trypan blue exclusion test (Fig. 1f), bromodeoxyuridine (BrdU) incorporation (Fig. 1g; Supplementary Fig. 1h) and analysis of cytosine levels (Supplementary Fig. 1i) demonstrated dose-dependent inhibition

![Figure 1](image_url)
of cell proliferation in ACO2-overexpressing clones with respect to control MCF-7 cells bearing the empty vector (EV).

ACO2 overexpression boosts mitochondrial metabolism

Mitochondrial metabolism was then monitored to reveal any influence due to the stable increase of ACO2 expression in MCF-7 cells. We observed an increase in the citrate synthase (CS) activity (Fig. 2a) and consequently of the levels of the TCA cycle intermediates citrate, α-KG and fumarate in ACO2-overexpressing cells (Fig. 2b). To investigate whether increased TCA cycle flux could result in activation of ETC, we performed lifetime imaging of NADH, which is extensively used to monitor changes in metabolism. NADH has either a short or long fluorescence lifetime component depending on whether it is in a

![Image](https://example.com/fig2a.png)

ACO2 overexpression boosts mitochondrial metabolism and biogenesis. a Spectrophotometric determination of citrate synthase (CS) activity ($n = 3$; **$p < 0.01$, ***$p < 0.001$ vs EV). b Determination of TCA cycle intermediates by colorimetric assay. Data are shown as fold change vs EV, represented by a solid line ($n = 3$; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs EV). c Average mitochondrial NAD(P)H lifetime retrieved from the NAD(P)H FLIM images (*$p < 0.05$ vs EV). d Representative FLIM images where mitochondrial NAD(P)H responses were separated by the rest of the autofluorescence (cytoplasm, nuclei) by means of fluorescence intensity analysis (scale bar = 20 µm). e Cytofluorimetric analysis in FL-2 channel of mitochondrial membrane potential by incorporation of MitoTracker Red. MFI mean fluorescence intensity, a.u. arbitrary unit ($n = 3$; *$p < 0.05$ vs EV). f Measurement of oxygen consumption by Clark’s electrode ($n = 3$; *$p < 0.05$, **$p < 0.01$ vs EV). g Determination of ATP content by fluorometric assay ($n = 3$; *$p < 0.05$, **$p < 0.01$ vs EV). h Representative western blot ($n = 3$) showing levels of the mitochondrial protein TOM20 as a marker of mitochondrial mass in comparison with ACTB used as loading control. i Cytofluorimetric analysis in FL-1 channel of mitochondrial mass by incorporation of MitoTracker Green. MFI mean fluorescence intensity, a.u. arbitrary unit ($n = 3$; *$p < 0.05$ vs EV). j Representative western blot ($n = 3$) showing nuclear levels of transcription factor/co-activators involved in mitochondrial biogenesis. Histone H3 and LAMIN B1 were used as loading controls.
free or protein-bound state, respectively. Since metabolic states can change the extent of NADH enzymatic binding, fluorescence lifetime imaging microscopy (FLIM) permits us to investigate, with submicrometric resolution and organelle specificity, whether the induced alteration in the TCA cycle was associated with increased binding of the reduced cofactor NADH. Figure 2c shows a significant increase in the average mitochondrial NAD(P)H lifetime, retrieved from the NAD(P)H FLIM images reported in Fig. 2d, in ACO2-overexpressing cells, thus indicating an increase in the enzyme-bound state of NADH species corresponding to higher mitochondrial NADH levels used by ETc. The elevated mitochondrial membrane potential (Fig. 2e), oxygen respiration (Fig. 2f) and ATP production (Fig. 2g) corroborated that ACO2 overexpression boosts OXPHOS in MCF-7 cells in a dose-dependent fashion. To test whether these changes were associated with an increase in mitochondrial mass, we assessed the relative amount of the mitochondrial translocase TOM20 with respect to the cytosolic protein ACTB (Fig. 2h) and the fluorescence intensity of mitochondrial-selective labels (Fig. 2i; Supplementary Fig. 2a). These assays demonstrated that ACO2-overexpressing cells have an increased mitochondrial content along with expression of TCA cycle enzymes (Supplementary Fig. 2b, c) and of both mitochondrial- and nuclear-encoded genes of the OXPHOS complexes (Supplementary Fig. 2d–f). Consistently, the analysis of nuclear factors involved in mitochondrial biogenesis, including PGC-1α and ser129-phosphorylated CREB (p-CREB), were more highly detectable in ACO2-overexpressing clones (Fig. 2). The unaltered expression of the mitochondrial chaperones HSP60 and HSP75 suggested a preserved mitochondrial homeostasis in ACO2-overexpressing cells (Supplementary Fig. 2c).

ACO2 overexpression affects metabolic features of cancer cells and differentiated mammary epithelial cells

To assess whether improved mitochondrial metabolism promoted changes in typical features of cancer metabolism, we focused our attention on aerobic glycolysis and glutamine addiction in MCF-7 cells. After ACO2 overexpression, we observed the reduction of extracellular lactate release (Fig. 3a) that was not ascribable to altered glucose uptake, as demonstrated by the unaffected incorporation of the glucose fluorescent analogue 2-NBDG (Fig. 3b). Since this pattern was paralleled by an upregulation of the pyruvate dehydrogenase complex (i.e., β-subunit of the E1 component, PDHB) (Fig. 3c) and augmented acetyl-CoA levels (Fig. 3d), a more efficient way to funnel pyruvate towards mitochondria seemed to occur in ACO2-overexpressing cells. To demonstrate that the enhancement of oxidative metabolism is the cause of MCF-7 decreased proliferation observed after ACO2 overexpression, we replaced glucose with galactose to force cells to use OXPHOS for ATP production.23 In this condition, control MCF-7 cells decreased their proliferation (Fig. 3e), whereas ACO2-overexpressing cells underwent cell death (Fig. 3f).

Concerning glutamine metabolism, no alteration of glutamine and of glutamate levels was evidenced in our clones (Supplementary Fig. 3a). However, when cells were subjected to glutamine deprivation, we observed a significant decrease of cell proliferation in control cells with respect to ACO2-overexpressing cells (Fig. 3g) and no signal of apoptosis (data not shown). Treatments with the allosteric inhibitor of glutaminase, BPTES, had similar effects, thus highlighting that MCF-7 cells are less dependent on glutamine metabolism after ACO2 overexpression (Fig. 3h).

In parallel to metabolic aspects of cancer cells, we also monitored a typical metabolic trait of differentiated epithelial mammary cells, consisting of the accumulation of milk components or precursors. In fact, a decreased proliferation of breast cancer cells can be accompanied by cell differentiation, including MCF-7.20 The increased levels of UDP-galactose, combined with UTP decline, indicated the activation of the lactose biosynthetic pathway after ACO2 overexpression (Supplementary Fig. 3b). In support of a different utilisation of UDP galactose, we found an impairment of glucosamine pathways as shown by the reduction of UDP-GlcNAc and UDP-GalN (Supplementary Fig. 3c). In this scenario, the increase in the human milk components taurine (Supplementary Fig. 3d) and albumin (Supplementary Fig. 3e) together with the accumulation of neutral lipids (Supplementary Fig. 3f) demonstrated that ACO2-overexpressing clones have a more differentiated phenotype.

ACO2 overexpression induces oxidative stress-mediated autophagic/mitophagic flux

The enhancement of mitochondrial metabolism in ACO2-overexpressing cells may be associated with augmented oxidative stress. Consistently, we observed an increase in intracellular (Fig. 4a) and mitochondrial ROS (Fig. 4b), as well as carbonylated proteins (Supplementary Fig. 4a) after ACO2 overexpression. This pattern was accompanied by variations in the antioxidant system as demonstrated by the upregulation of GCLC and SOD2 genes (Supplementary Fig. 4b), by decreased levels of reduced glutathione (Supplementary Fig. 4c) and by increased NADP+/NADPH ratio (Supplementary Fig. 4d). No sign of nitrosative stress was instead identifiable as suggested by the unchanged levels of intracellular nitrite and nitrate (Supplementary Fig. 4e, f). We then tested the functional role of oxidative stress in cancer-related processes by incubating MCF-7 cells with the ROS scavenger N-acetylcysteine (NAC). We observed that ROS abrogation is associated with increased cell growth and with molecular features of epithelial–mesenchymal transition phenotype (i.e. upregulation of VIM and downregulation of CDH1) in ACO2-overexpressing cells (Supplementary Fig. 4g, h).

ROS production by mitochondria is known to promote autophagy, which is necessary for the removal of damaged/exhausted mitochondria via mitophagy.31 Therefore, we measured the levels of lipitated LC3B protein (LC3B-II) as a marker of autophagy activation. The increased levels of LC3B-II in our clones and the further accumulation observed in the presence of the autophagy inhibitor chloroquine (CQ) demonstrated that ACO2 overexpression triggers an active autophagic flux (Fig. 4c). We further revealed an increase in mitophagy, as shown by the high levels of ubiquitinated proteins (Fig. 4d) and of PARKIN recruitment (Fig. 4e) in mitochondrial fractions. These results were corroborated by the co-localisation of mitochondrial matrix-targeted mitoDsRed with GFP-LC3 (Supplementary Fig. 5a) and by the high expression of the mitochondria fission protein DRP132 in ACO2 clones (Supplementary Fig. 5b). We definitely demonstrated that autophagic induction in ACO2-overexpressing cells was a consequence of ROS production by the treatment with the ROS scavengers NAC, MitoTEMPO and ascorbate that were able to inhibit the accumulation of LC3B-II protein (Fig. 4f; Supplementary Fig. 5c). To define the relevance of autophagy induction in our cells, we inhibited it by preventing the acidification of lysosomes with bafilomycin A1 (BafA1). The reduction in the number of ACO2-overexpressing cells (Supplementary Fig. 5d) and the appearance of the cleaved fragment of PARP-1 protein, a typical marker of apoptosis, after BafA1 (Supplementary Fig. 5e) suggested an important contribution of autophagy/mitophagy in the maintenance of cell homeostasis buffering ROS.

Autophagy induction downstream of ACO2 overexpression is associated with ROS/FoxO1 signalling

To identify the molecular pathways behind autophagic response in ACO2-overexpressing cells, we firstly focused on the key autophagy inhibitor mTOR.33 The unchanged levels of Ser2448-phosphorylated mTOR and its target Thr389-phosphorylated p70S6 kinase excluded nutrient-limiting conditions as determinants of ROS-mediated autophagic flux in our clones (Fig. 5a). Such evidence is corroborated by the low levels of Thr172 phosphorylation of the energy sensor AMPK (Fig. 5b). In support of
this, we showed that our cells retain the ability to trigger autophagy when challenged with amino acid starvation (achieved by EBSS medium incubation), which is a canonical stimulus impinging on mTOR activity (Supplementary Fig. 5f). Then, we demonstrated that the activation of autophagic flux was associated with transcriptional upregulation of the key autophagic genes LC3B and ATG4B as well as of the mitophagic gene PINK1 (Fig. 5c). Considering that all autophagic genes here analysed are targets of FoxO proteins and that we have previously demonstrated that FoxO1 is a key transcription factor known to connect metabolism-associated oxidative stress with autophagy/mitophagy induction,24 we assessed the levels of FoxO1 in our experimental conditions. We first revealed that FoxO1 is highly abundant in the nuclear fractions of ACO2-overexpressing cells with respect to those of control (Fig. 5d). To determine whether FoxO1 nuclear localisation was a consequence of ROS buildup, we performed NAC treatment, but we did not evidence any specific change in its subcellular distribution (Fig. 5e). We thus analysed...
the levels of Ser256-phosphorylated FoxO1 demonstrating that this modification is less marked in nuclear fractions of untreated ACO2-overexpressing cells while it increases after NAC treatment (Fig. 5e). To evidence whether this scenario was associated with changes in FoxO1 recruitment at autophagic gene promoters, we tested sequences known to be targeted by FoxO1 performing ChIP analysis after NAC treatment. This experiment demonstrated that FoxO1 is highly enriched at LC3B, ATG4B and PINK1 promoters (Fig. 5f) in untreated ACO2-overexpressing cells, whereas its binding is largely disrupted after ROS removal by NAC.

**DISCUSSION**

The specialised metabolic landscape that is established in each cancer cell derives from the activation of oncogenic pathways or abrogation of tumour-suppressor signalling and is continuously shaped by the interaction with tumour microenvironment comprising stromal/immune cells and nutrient/oxygen availability. In some cases, cancer metabolic reprogramming can be a direct consequence of oncogenic driver mutations affecting metabolic genes as shown for half of the enzymes belonging to the TCA cycle: fumarate hydratase, succinate dehydrogenase, isocitrate dehydrogenase 2 and malate dehydrogenase 2. All these mutations are responsible for cellular transformation due to accumulation of TCA cycle intermediates or aberrant production of oncometabolites. Changes in the expression levels of several TCA cycle enzymes, such as CS and IDH3α, have also been shown to affect tumour phenotype suggesting that alteration of any step of TCA cycle can be detrimental for cell metabolic homoeostasis. Although no mutation in ACO2 sequence has been associated with tumour susceptibility, in this paper, we have demonstrated that expression levels of ACO2 are deregulated in breast cancer and that its overexpression in MCF-7 cell line is able to dampen cell proliferation. On the contrary, the modulation of the cytosolic ACO1 was shown to have no impact on breast cancer proliferation. Besides our present work, a direct contribution of ACO2 in tumorigenesis was exclusively demonstrated in prostate cancer, and this is mainly justified by the importance of citrate metabolism in non-malignant prostate epithelial cells. In fact, prostate tissue physiologically accumulates high amount of citrate, thanks to a zinc-mediated inhibition of ACO2. Even though it is well known that ACO2 is not a rate-limiting enzyme of the TCA cycle...
cycle, our data pointed out the importance of its reaction in promoting cancer metabolic rewiring. In fact, we demonstrated that increased levels of ACO2 in MCF-7 cells are able to affect aerobic glycolytic rate as demonstrated by the reduction of extracellular lactate flux. This evidence could give effort to the MCF-7 glycolytic shift observed under defective assembly of iron–sulfur clusters, which are essential for aconitase activity.\(^{39}\)

The unaffected glucose assimilation along with increased level of acetyl-CoA and PDH complex demonstrated a different fate for pyruvate, prevalently directed to mitochondria, in cells with increased ACO2 levels. The same changes were observed during treatment of colorectal cancer cells with dichloroacetate (DCA). This compound induces cell growth arrest by inhibiting pyruvate dehydrogenase kinase (PDK) with consequent activation of PDH, thus promoting the entry of pyruvate into the TCA cycle.\(^{40}\) We also demonstrated that ACO2 expression affects glutamine addiction of MCF-7 cells, indicating that feeding TCA cycle with pyruvate and channelling it for aerobic oxidation in the presence of adequate ACO2 levels dislodge cancer cells from using glutamine for anaplerosis.

The re-routing of glucose-derived pyruvate towards mitochondria can be considered as the guiding force involved in the...
proliferation inhibition of ACO2-overexpressing cells. Consistently, we observed a decrease of cell proliferation in control cells forced to use OXPHOS by galactose treatment.29 The enhancement of mitochondrial metabolism and the reduction of the Warburg effect also underlie the anti-proliferative effects observed after alanine aminotransferase inhibition,41 improved triacylglycerol catabolism in hepatocellular carcinoma42 and DCA treatment in colorectal cancer.40 Moreover, the increase in mitochondrial biogenesis due to PGC-1α activation was shown to be another efficient strategy against the Warburg effect.42–44 It has to be noticed that after ACO2 overexpression, we also observed an increase in mitochondrial mass associated with high levels of PGC-1α and Ser129-phosphorylated CREB. This would imply that ACO2-mediated metabolism is able to trigger a mitonuclear retrograde response culminating in mitochondrial biogenesis that needs further investigations. Whatever the causes, pyruvate redirection, mitochondrial biogenesis or both, our data support that ACO2 can boost OXPHOS weakening the Warburg effect.

Mitochondria are the major physiological source of ROS and can cause an increase in oxidative stress following enhanced metabolic rate or electron leakage to oxygen when they are dysfunctional.45,46 The contribution of ROS in breast cancer aetiology and progression is dual when they act as signal molecules in metabolic adaptations and proliferation pathways, as well as when they act as harmful factors, eliciting pro-apoptotic or mutagenic effects by damaging macromolecules, primarily nucleic acids.47 In general, low concentrations of ROS are believed to promote cancer cell survival as demonstrated for the maintenance of breast cancer stem cells and resistance to radiotherapy.48 High concentrations of ROS instead can promote oxidative damage and eradication of tumour cells via programmed cell death, a mechanism frequently underpinning the action of chemotherapeutic agents that promote oxidative stress after enhanced ROS levels.49 The loss of the essential autophagic protein Beclin1 in mammalian epithelial cells induces tumorigenesis as a consequence of genome instability.51 Another tumour-suppressor role of Beclin1 is present in the DNA-binding domain restraining FoxO1 transcriptional activity.52 Consistently, we observed a reduced FoxO1 occupancy at autophagic/mitophagic promoters after ROS abrogation. Therefore, FoxO1 may be a key player in the communication between mitochondria and the nucleus in cancer cells for the maintenance of oxidative metabolism through mitochondrial quality control. Ongoing research is devoted to the evaluation of other redox-sensitive transcription factors in the metabolic adaptation induced by ACO2 overexpression.

Overall, this work has evidenced that ACO2 expression modifies metabolic features of MCF-7 cells imposing repression of the Warburg effect and repurposing pyruvate in the aerobic route speeding OXPHOS. This scenario is accompanied by a decrease in cell proliferation associated with ROS/FoxO1 signalling necessary for preserving cellular homeostasis. These data suggest that ACO2 can be included in metabolic reprogramming exploited by cancer cells, considering that it can be found deregulated in breast, gastric and prostate tumour biopsies.53,54 Future studies are necessary to enlarge this evidence on other cell systems or in vivo models with the final aim to evaluate whether ACO2 can be a suitable therapeutic target or a biomarker of metabolic vulnerability. In fact, it is worth to highlight that this neglected enzyme of the TCA cycle seems to interfere with different aspects of cancer metabolism from aerobic glycolysis to glutamine addiction and autophagy, without excluding the relevant implication it may have in cancer immunometabolism by supplying cis-aconitate for the production of the anti-inflammatory metabolite itaconate.73

AUTHOR CONTRIBUTIONS
F.C. conceived the study, designed and performed the experiments, interpreted the data and wrote the paper; I.L.D.L performed activity assays and TCA cycle/ATP/oxygen measurements; G.L. and B.T. performed HPLC analysis; G.M. and F.D.G. performed fluorescence lifetime imaging of NADH; M.R.C. conceived and supervised the study, interpreted the data and revised the paper. All authors reviewed and approved the paper.

ADDITIONAL INFORMATION
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