Mitochondrial metabolism drives hypercholesterolemia-induced breast cancer cell migration

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Article

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Mitochondrial metabolism drives hypercholesterolemia-induced breast cancer cell migration

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28
29
30 **Abstract**

31 Most cancer related deaths are due to metastases. Systemic factors, such as
32 lipid-enriched environments (particularly LDL-cholesterol), favor breast cancer
33 (including triple negative breast cancer, TNBC) metastasis formation.
34 Mitochondria metabolism impacts TNBC invasive behavior but its involvement in
35 a lipid-enriched setting is undisclosed. Here we show that LDL-cholesterol
36 induces CD36, increases lipid droplets and augments TNBC cells migration and
37 invasion *in vivo* and *in vitro*. LDL increases mitochondrial mass and mitochondria
38 spread in migrating cells, and energetic analyses revealed that LDL renders
39 TNBC dependent on fatty acids for mitochondrial respiration. Indeed,
40 engagement of fatty acid oxidation, but not glycolysis, is required for LDL-induced
41 migration and mitochondrial remodeling. Moreover, LDL induced reactive oxygen
42 species (ROS) production by TNBC cells. Importantly, CD36 or ROS blockade
43 abolished LDL-induced cell migration and mitochondria metabolic adaptations.
44 Our data suggest that LDL induces TNBC cells migration by reprogramming
45 mitochondrial metabolism, revealing a new vulnerability in metastatic breast
46 cancer.

Introduction

Cancer metabolism is widely recognized as a key aspect of tumor initiation and progression, and several metabolic adaptations carried by cancer cells to support tumor growth and proliferation have been described^{1–3}. Cancer cells are no longer thought to obtain all of their energy solely from glycolysis (the so-called “Warburg’s effect”), and, certainly, not because of mitochondrial defects. Tumors are heterogeneous masses that comprise different cell types, which have the ability to adopt to different metabolic programs. There is compelling evidence, particularly in breast cancer, that migratory/invasive cancer cells specifically favor mitochondrial respiration over aerobic glycolysis. It was shown that triple negative breast cancer (TNBC) cells enhance oxidative phosphorylation (OXPHOS) and mitochondrial biogenesis during metastasis formation and rely in fatty acid oxidation (FAO) for the acquisition of an aggressive phenotype^{4–6}. Lipid metabolism is known to have a crucial role in cancer development and lipid synthesis and consumption in tumors have been under scrutiny. There is a vast epidemiological literature suggesting that obesity, and high cholesterol levels in particular, may result in greater tumor incidence and worse cancer patient outcomes^{7–9}. In particular, our lab has described that elevated cholesterol levels in untreated breast cancer patients strongly correlates with bigger and more metastatic tumors¹⁰. Although this data suggests a strong association between lipid-enriched environments and tumor progression, the exact nature of the lipidic metabolic alterations that fuel tumorigenesis is not well understood. Importantly, results from our lab and others, have shown that lipid-enriched environments (particularly LDL-cholesterol) favor tumor progression in experimental murine models of breast cancer by modulating the tumor microenvironment and favoring

aggressive cancer cell autonomous properties, including increase TNBC cell migration, proliferation and metastatic behavior^{11,12}. Whether the acquisition of such an aggressive, pro-metastatic, phenotype by breast cancer cells exposed to elevated systemic lipid levels - and of LDL-cholesterol in particular- is the result of specific mitochondria and metabolic alterations is undisclosed and was the focus of the present study.

Results

LDL exposed TNBC cells display enhanced metastatic spread to distant (and less vascularized) organs in xenotransplanted zebrafish larvae.

To explore the impact of LDL exposure on the behavior of BC cells in vivo, we xenotransplanted human TNBC MDA-MB-231 cells into zebrafish larvae as a model to study metastasis^{13,14}. In the xenotransplanted zebrafish larvae, we investigated whether pre-exposure of MDA-MB-231 cells to LDL for 2 days would impact their migratory behavior and invasion to secondary organs (tropism) and whether this was accompanied by alterations in the mitochondria network. Since both cell conditions were inoculated in the same larvae, we used different cell tracers (Dil for control and Cy5 for LDL-exposed cells) to discriminate the experimental groups and cells were additionally transiently transfected with the mitochondrial reporter Mito-YFP¹⁵.

We first analyzed the ability of LDL-exposed and control MDA-MB-231 cells to migrate to distant organs in the zebrafish larvae at 4 days post-injection (dpi; corresponding to 6 days post-fertilization) using inverted spinning disk confocal microscopy (Fig. 1a). LDL-exposed MDA-MB-231 cells presented a trend toward a differential migration pattern in the zebrafish larvae as compared to control cells (Chi-square $p=0.06$, $n=11$) (Fig. 1a and Extended Data Fig. 1a), with a significant number of zebrafish larvae accumulating LDL-exposed cells in the eye and control cells in the caudal hematopoietic tissue (CHT), a highly vascularized region with a dense plexus unifying caudal artery and caudal vein¹⁶. Moreover, there was an increased number of zebrafish larvae displaying LDL-exposed MDA-MB-231 cells at distant sites, being the dorsal fin, notochord, ventral fin and tail fin invaded solely by LDL-exposed MDA-MB-231 cells (Fig. 1a).

To assess differential cell distribution in the whole organism, we quantified the number of individual cells in each organ and the presence of tumor cell masses in xenotransplanted zebrafish larvae at 4dpi. Similarly, LDL-exposed MDA-MB-231 cells were found in all organs analyzed (Fig. 1b). Notably, less vascularized organs were invaded preferentially by LDL-exposed MDA-MB-231 cells (Fig. 1b). Of these, some were pierced by collateral vessels (dorsal muscle and ventral muscle), by a single vessel (eye), or lied just beneath CHT (anal fin) and were still invaded by MDA-MB-231 control cells; others (such as notochord, ventral fin, dorsal fin, tail fin) have no detectable vessels in angiographic studies¹⁶ and in xenotransplanted Tg (*fli1:eGFP*) zebrafish larvae at 6dpf (Extended Data Fig. 1b) and were exclusively infiltrated by LDL-exposed MDA-MB-231 cells (Fig. 1b). Although MDA-MB-231 control cells were found in higher proportion in extremely vascularized organs (such as optic vesicle, heart, gills and CHT), LDL-exposed MDA-MB-231 cells could also invade those vascularized organs displaying for instance preferred accumulation in the intestinal tract (Fig. 1b).

We could also assess the colonization capability of MDA-MB-231 cells by quantifying the presence of tumor cell masses (>20 cells) established after organ colonization¹⁷. There was no difference in the total number (LDL-exposed n=8 and control n=7) or distant location of the tumor masses (n=5 for both) established between LDL-exposed and control MDA-MB-231 cells (Extended Data Fig. 1c).

These results indicate that LDL alters the migratory and invasive capacity of MDA-MB-231 cells, with cells invading organs with peripheral and less vascularized location, which differ from the distribution of control cells that preferentially accumulate in central and highly vascularized organs of the

zebrafish larvae.

LDL exposed TNBC cells display differential mitochondrial network in xenotransplanted zebrafish larvae.

Since alterations in mitochondrial mass, network and dynamics have been implicated in the acquisition of more aggressive and migratory behavior of cancer cells^{4,18}, we assessed if LDL-exposed TNBC cells present mitochondrial alterations in the xenotransplanted zebrafish larvae, using the Mito-YFP reporter. We observed an overall increased number of Mito-YFP particles in zebrafish larvae xenotransplanted with LDL-exposed MDA-MB-231 cells (Fig. 1c). This difference was statistically significant in most organs analyzed, including PVS, intestinal tract and brain (Extended Data Fig. 1d), suggesting that LDL exposure cell-autonomously increased the number of mitochondria in MDA-MB-231 cells. Moreover, the average area of Mito-YFP particles, which reflects the size of mitochondria, was overall reduced in LDL-exposed MDA-MB-231 cells xenotransplanted into zebrafish larvae (Fig. 1d) and this difference was particularly prominent in the PVS and intestinal tract (Extended Data Fig. 1e), suggesting that LDL may reduce mitochondrial area per particle in MDA-MB-231 cells migrating *in vivo*. Finally, we quantified the mitochondrial network as the total area occupied by Mito-YFP particles in the cell area delineated by the Dil and Cy5 tracers and in general, LDL-exposed MDA-MB-231 cells presented a higher mitochondrial network in xenotransplanted zebrafish larvae as compared to control MDA-MB-231 cells (Fig. 1e) and this difference was significant in the brain (Extended Data Fig. 1f). Interestingly, LDL-exposed MDA-MB-231 cells also present increased Mito-YFP and cell tracer total area (Extended Data Fig. 1g,h).

We could observe two phenotypes of mitochondrial network distribution: mitochondrial aggregates (clusters) around the nucleus, often located unilaterally, which we designated perinuclear mitochondria (Fig. 1f,g) and long mitochondrial filaments, located unilaterally or bilaterally around the nucleus and distributed throughout the whole cell, designated as filamentous mitochondria (Fig. 1f,g). We observed that the majority of LDL-exposed MDA-MB-231 migrating cells in zebrafish larvae presented a filamentous distribution (54.7% versus 33.3% for control cells) contrasting with control cells that adopted preferentially a perinuclear mitochondrial network (66,7% versus 45.3% for LDL cells). Filamentous distribution was more prevalent in LDL-exposed MDA-MB-231 cells in most organs analyzed, being particularly evident in the brain, in agreement with the qualitative analysis (Extended Data Fig. 1f, i).

LDL exposed migrating TNBC cells display differential mitochondrial network and increased cristae destabilization.

To explore the mechanistic action of LDL, we went on to characterize the mitochondrial alterations upon LDL exposure in migrating MDA-231 cells in vitro. We have previously reported ¹¹ that LDL exposed MDA-MB-231 cells proliferate two times more and display increased migratory capacity in a wound-healing assay (Extended Data Fig. 2d). In accordance to what was observed in the zebrafish xenotransplanted larvae, MDA-MB-231 migrating cells in the wound-healing assay (for 18-24h with Mitomycin C for cell cycle arrest) exhibit increased number of HSP-60 labelled mitochondria in the presence of LDL (Fig. 2a). Additionally, we observed increase mitochondrial mass as determined by mitochondrial DNA (mtDNA) content and MitoTracker Deep Red staining by flow

cytometry in LDL-exposed MDA-MB-231 migrating cells (Fig. 2b,c). LDL exposure promoted a similar effect in the TNBC cell line MDA-MB-436, with increased mitochondrial mass and migration in the wound healing assay (Extended Data Fig. 2a,b). In agreement with the *in vivo* data, the impact of LDL exposure in the HSP-60 labelled mitochondrial network of MDA-MB-231 migrating cells was very striking (Fig. 2d) with virtually all control cells presenting a perinuclear (around the nucleus) mitochondrial network (96.8% versus 54.8% for LDL cells; Fig. 2d) whereas the majority of LDL-exposed cells exhibited a filamentous (extended through the cell area) network distribution (45.2% versus 3.2% for control cells; Fig. 2d). In order to account for technical artefacts inherent to cell fixation, we confirmed these results using live MitoTracker Deep Red imaging (Extended Data Fig. 2c). In order to investigate if mitochondrial network adaptations in migrating MDA-MB-231 cells was exclusive for LDL exposure or more broadly applies to other agents that induce cell migration, we treated cells with lysophosphatidic acid (LPA), a phospholipid ligand that stimulates cell migration through activation of PI3K/PAK1/ERK signaling¹⁹. As for LDL, MDA-MB-231 cells exposed to LPA present increased migration in the wound-healing assay (Extended Data Fig. 2d). However, and as opposed to LDL, LPA exposure did not significantly alter the number of TOM-20 mitochondrial particles (Extended Data Fig. 2e) or the mitochondrial network adopted by migrating cells compared to the control situation (Extended Data Fig. 2f). We additionally quantified the mitochondrial network as the total area occupied by TOM-20 mitochondrial particles in the cell area (delineated by phalloidin staining) and observed that LDL-exposed MDA-MB-231 migrating cells presented increased

mitochondrial network as compared to the control or LPA-exposed MDA-MB-231 migrating cells (Fig. 2e).

Mitochondria are double membrane composed by an outer membrane (OM) that surrounds the organelle and an inner membrane (IM), which is aligned adjacently to the OM and forms numerous invaginations termed cristae²⁰. In order to obtain further insights into the impact of LDL in the mitochondria network of MDA-MB-231 cells, we used transmission electron microscopy (TEM) to obtain high-resolution micrographs and evaluate the organization of the mitochondrial inner membrane cristae. TEM analysis confirmed that LDL-treated MDA-MB-231 cells present increased number of mitochondria (Fig. 2f,g) and a tendency for decreased average area of mitochondrial particles (Extended Data Fig. 2g), similarly to what was observed in the xenotransplanted zebrafish larvae (Fig. 1d). LDL-exposed cells presented mitochondria with more heterogeneous cristae density compared to control cells, where the number of cristae range only from 1 to 4 cristae per μm^2 (Fig. 2h). Moreover, the mitochondria of control cells presented increased proportion of cristae with smallest incident angle (0° , Fig. 2i), more parallel cristae arrangement, compared to LDL-exposed cells. Overall these results suggest that LDL exposure promoted a cristae disarrangement in the mitochondria inner membrane of MDA-MB-231 cells.

Mitochondria are highly dynamic organelles that undergo continuous changes in morphology, degree of biogenesis and mitophagy²¹. Upon 48h exposure to LDL, the expression of several mitochondrial biogenesis factors, including the master regulator PGC-1 α and the FAO coactivator PPAR α were either unchanged or reduced in migrating MDA-MB-231 cells (Extended Data Fig. 2h) and additionally

PGC-1 α protein levels were pronouncedly reduced (Extended Data Fig. 2i), suggesting that the mitochondrial adaptations in migrating TNBC cells upon LDL exposure do not rely in PGC-1 α expression. Regarding mitochondrial dynamics, LDL-exposed MDA-MB-231 cells display increased expression of the mitochondrial fission protein Drp1 with no difference in the expression of the mitochondrial fusion proteins Mfn1 and Mfn2 (Fig. 2j and Extended Data Fig. 2j), suggesting that LDL-exposure may potentiate mitochondrial fission resulting in smaller mitochondria in MDA-MB-231 cells. Additional mitochondrial morphological parameters can provide insight into mitochondrial dynamics, where for instance, an elongation score closer to 1 is indicative of circular mitochondria and more fragmented network and higher elongation scores are characteristic of fused mitochondria^{22,23}. Quantification of mitochondrial morphology using a macro developed for ImageJ software²³ revealed that MDA-MB-231 cells present an elongation score close to 1 (Fig. 2k) under control or under LDL exposure, suggesting that, and in accordance with the literature²⁴ TNBC cells exhibit fragmented mitochondria and LDL-exposure did not alter this parameter in a statistically significant manner.

LDL-induced TNBC cell migration is mediated by the fatty acid transporter CD36.

Lipoproteins are complex particles composed of multiple proteins and lipids, including cholesterol, phospholipids and triglycerides. Once internalized, LDL is hydrolyzed in the endosome and lysosome and lipids are stored into lipid

droplets, being subsequently mobilized for several cellular processes, including ATP production through FAO²⁵. Indeed, we observed that exposure of MDA-MB-231 cells to LDL for 48h in vitro resulted in a significant increase in lipid droplets content as assayed by the neutral lipid dye BODIPY 493/503 (Bodipy) by flow cytometry and live imaging (Fig. 3a,b). There are several types of membrane LDL receptors and scavengers that mediate the endocytosis of native and modified LDL, including the LDL receptor (LDL-R), SRBP1 and CD36^{26,27}. We observed that exposure to LDL for 48h promoted a dramatic reduction in the expression of *LDL-R* and *SRBP1* in MDA-MB-231 cells (Extended Data Fig. 3a) whereas the expression of CD36 transcript and protein by flow cytometry is augmented (Fig. 3c and Extended Data Fig. 3a). To further explore the requirement of CD36 for LDL-induced TNBC cell migratory phenotype, we inhibited CD36 using the small-molecule inhibitor sulfosuccinimidyl oleate (SSO)²⁸ and observed that SSO treatment abrogated the LDL-induced migration of MDA-MB-231 cells in the wound-healing assay (Fig. 3d) and this was accompanied by reduction in lipid droplets content (Fig. 3e). Treatment with SSO was also effective at blocking the increased proliferation of MDA-231 cells induced by LDL-exposure (Extended Data Fig. 3b). Similarly, short hairpin RNA (shRNA)-mediated depletion of CD36 (Extended Data Fig. 3c) abrogated the LDL-induced increase migration of MDA-MB-231 cells (Fig. 3f), and the lipid droplet content (Extended Data Fig. 3d). In contrast, a neutralizing antibody against LDL-R had no impact in the LDL-induced migration, proliferation or lipid droplet accumulation in MDA-MB-231 cells (Extended Data Fig. 3e-g). Next, we went on to determine the impact of CD36

inhibition in the LDL-induced mitochondrial adaptations in migrating MDA-MB-231 cells. Treatment with SSO abrogated the LDL-induced increase in mitochondrial mass, as determined by relative mtDNA content (Fig. 3g) and the mitochondrial markers TOM-20 (Fig. 3h) and HSP-60 (Fig. 3i and Extended Data Fig. 3h) in migrating MDA-MB-231 cells. Moreover, CD36 knockdown also abolished the LDL-induced increase in mtDNA content in MDA-MB-231 cells (Extended Data Fig. 3i). SSO treatment abrogated the prevalent filamentous mitochondrial network distribution present in LDL-exposed MDA-MB-231 migrating cells (Fig. 3j and Extended Data Fig. 3i) with an increase proportion of co-treated SSO+LDL cells presenting a perinuclear network distribution (Fig. 3j: perinuclear network 36.8% in LDL versus 45.5% in SSO+LDL). We additionally performed a qualitative assessment of the mitochondrial network determined as the area occupied by TOM-20 particles in the overall cell area delineated by phalloidin staining, and observed that in the presence of SSO, LDL exposure did not increase the mitochondrial network in migrating MDA-MB-231 cells (Fig. 3k). Taken together, these results reveal that CD36 is specifically required for the LDL-induced increase in lipid droplets, migration and mitochondrial network remodeling in migrating MDA-MB-231 cells.

LDL-induced migratory behavior of TNBC cells relies in increased usage of fatty acids and FAO-dependent mitochondrial metabolism.

In order to determine the contribution of fatty acids and FAO in the LDL-induced aggressive properties of TNBC cells, we exposed cells to palmitic acid (PA), a

common saturated fatty acid implicated in the metastatic initiating potential of CD36-positive cells²⁹. Similarly to LDL, exposure to PA produced an increase in the migratory phenotype (Fig. 4a) and mitochondrial mass of MDA-MB-231 cells, as measured by mtDNA content (Fig. 4b) and MitoTracker Deep Red flow cytometry staining (Extended Data Fig. 4a), suggesting that, at least part of the, LDL impact in TNBC cells migration is due to the fatty acids content. To address the requirement of FAO oxidation in the LDL and PA-induced migration of MDA-MB-231 cells, we selectively inhibited the carnitine palmitoyltransferase system (CPT1) that catalyzes the first reaction to shuttle long-chain fatty acids into the mitochondria for FAO. Inhibition of the outer membrane isoform CPT1a with etomoxir abrogated both LDL and PA increased migratory capabilities (Fig. 4a), the augmented mtDNA content (Fig. 4b) and MitoTracker staining (Extended Data Fig. 4a) in migrating MDA-MB-231 cells. Etomoxir treatment produced no major changes in the LDL or PA-induced lipid droplet content and proliferative potential (Extended data Fig. 4b,c).

Inhibition of CPT1a interrupts fatty acids transportation into the mitochondria and thereby, blocks the whole process of long-chain fatty acid metabolism. Quantification of the number of TOM-20 labelled mitochondria in immunostained fixed cells revealed that etomoxir alone produced a dramatic decrease in the number of mitochondria in migrating MDA-MB-231 cells (Fig. 4c) and LDL was still able to increase the number of mitochondrial particles even in the presence of etomoxir (Fig. 4c). Moreover, etomoxir treated cells present increased mitochondria average area and LDL treatment was able to restore it in migrating

MDA-MB-231 cells (Extended Data Fig. 4d). Regarding mitochondrial network, CPT1a blockade produced an increase in the proportion of migrating cells with perinuclear network distribution (Fig. 4d: eto, perinuclear 82.5% and filamentous 17.5%) and LDL co-treatment was still able to increase the proportion of MDA-MB-231 cells displaying filamentous network distribution (Fig. 4d; eto vs. eto+LDL $p<0.0001$) although the mitochondrial network of co-treated cells was significantly reduced as compared to LDL-treated only (Fig. 4e-f; LDL vs. eto+LDL $p<0.0001$). In order to further investigate the reliance on glucose and mitochondrial respiration for the LDL-induced migration, we treated MDA-MB-231 cells with 2-DG, a glucose analogue which blocks the glycolytic enzyme hexokinase 2, and oligomycin, an inhibitor of ATP synthase. 2-DG treatment resulted in suppression of the LDL-induced migratory advantage in MDA-MB-231 cells (Fig. 4g) and reduction in the amount of stored lipid droplets (both control and LDL-exposed cells; Extended Data Fig. 4e), suggestive of increased usage of stored lipids under glycolytic blockade. As expected, treatment of MDA-MB-231 cells with oligomycin produced a dramatic suppression in the migratory capacity of MDA-MB-231 cells (both control and LDL-treated) abrogating the advantage conferred by LDL-exposure (Fig. 4h) and this was accompanied by an increase in lipid droplets especially in LDL-exposed cells (Extended data Fig. 4f). LDL treatment was still able to increase cell proliferation even in the presence of 2-DG (Extended data Fig. 4g) whereas oligomycin totally abolished the proliferative advantage induced by LDL-exposure in MDA-MB-231 cells (Extended data Fig. 4h). Cell migration is a highly energetic process that requires

a dynamic remodeling between mitochondria energetic processes and actin cytoskeleton as mitochondria deliver localized ATP for Arp2/3-F-actin network growth during cell invasion³⁰. Indeed, co-treatment with the Arp2/3 Inhibitor CK666³¹ abolished the LDL-induced migratory effect (Extended data Fig. 4i), and the LDL-induced increase in mitochondrial mass and network distribution in migrating MDA-MB-231 cells (Fig. 4i,j), suggesting that LDL-induced migration is accompanied by actin cytoskeleton-mitochondria network remodeling. Overall, these results suggest that both mitochondrial bioenergetic metabolism (glucose and fatty acid-dependent) and co-ordination with actin cytoskeleton is required for the LDL-induced mitochondrial network adaptations and migratory advantage in TNBC cells.

Increased lipid exposure induces metabolic and bioenergetic dependencies in TNBC cells.

When fatty acids are the most abundant energy substrate, mitochondrial bioenergetics and oxidative phosphorylation (OXPHOS) can be tuned by transcriptional regulation of metabolic enzymes and electron transport chain (ETC) complexes³². In order to assess if LDL exposure impacts the bioenergetic program adopted by TNBC cells, we used gene expression microarray data from control and 48h LDL-exposed MDA-MB-231 cells¹¹. Gene set enrichment analysis revealed several genes significantly reduced upon LDL exposure, including endoplasmic reticulum-related and several metabolic pathways (Fig. 5a), with a significant decrease in the biosynthesis of lipids (steroid, isoprenoid, cholesterol) and membrane structures (nuclear envelope-ER network,

endomembrane system, organelle membrane) (Fig. 5a). In addition, there was a non-significant increase in fatty acid beta-oxidation (FAO) and carbohydrate catabolism (Extended Data Fig. 5a), suggesting that LDL exposure induces remodeling of anabolic and bioenergetic pathways in TNBC cells. These findings were further validated by qPCR analysis which confirmed increased expression of FAO enzymes (*CPT1A*, *ACADVL*), decreased expression of lipid biosynthetic enzymes, including the rate-limiting *FASN* and *HMGCR*, and an overall increase expression of bioenergetic enzymes, both OXPHOS (*COX5b*, *ATP5g1*, *NDUFB5*) and glycolytic (*PKM2*, *PFKFB3*, *LDHA*), in LDL-exposed MDA-MB-231 cells (Fig. 5b).

To understand the impact of LDL exposure in the bioenergetics of TNBC cells, we performed a series of functional mitochondrial stress tests using the Seahorse extracellular flux analysis on cultured MDA-MB-231 cells untreated or LDL-exposed in the presence or absence of the CD36 pharmacological inhibitor SSO. No significant differences were observed in the oxygen consumption rate (OCR) between control and LDL-treated MDA-MB-231 cells, including basal and maximal mitochondrial respiration and ATP production (Fig. 5c and Extended Data Fig. 5b). However, comparing to untreated cells, LDL-exposed cells present a trend for increased proton leak and a significant decrease in spare capacity (Fig. 5c; $p=0.0036$), which is obtained by the differential between the basal and maximal respiration and lower spare capacity has been associated with increase susceptibility to oxidative stress³³. In addition, LDL treatment did not significantly impact the extracellular acidification rate (ECAR) of MDA-MB-231 cells, with only a (non-significant) decrease in the glycolytic reserve observed (Fig. 5d and Extended data Fig. 5c). Blockade of CD36 by SSO treatment alone or combined

with LDL promoted several OCR bioenergetic adaptations, including increased non-mitochondrial respiration, basal respiration, proton-leak (Fig. 5c and Extended Data Fig. 5b) and glycolytic reserve (Fig. 5d and Extended data Fig. 5c) suggesting that decreased cellular lipid uptake upon CD36 blockade potentiates bioenergetics either by recruitment of endogenous lipid storage (see Fig. 3e) or usage of alternative fuels (glucose or glutamine). Next, we decided to investigate if LDL exposure impacts the main energetic fuel preferentially used by TNBC cells. For that, we performed substrate dependency assays (Seahorse Mito Fuel Flex Test) based on OCRs response to glutamine-, glucose-, and FA-oxidation inhibitors (BPTES, UK5099, and etomoxir, respectively) in cultured MDA-MB-231 cells untreated or LDL-exposed cells in the presence or absence of the CD36 inhibitor SSO. We observed that LDL-exposed MDA-MB-231 cells present increase FAO dependency and decreased flexibility for mitochondrial respiration (Fig. 5e) and CD36 blockade did not significantly alter this effect (Fig. 5e). On the contrary, LDL exposure led to a decrease glucose oxidation capacity and flexibility in MDA-MB-231 cells and CD36 blockade was able to revert this effect (Fig. 5f). Overall these results suggest that LDL exposure does not impact the main bioenergetic pathway adopted by MDA-MB-231 cells but promotes a dependency on fatty acids usage for mitochondrial respiration, decreasing the capacity of TNBC cells to adapt to other fuels, such as glucose.

LDL-induced migratory behavior and mitochondrial adaptations rely in reactive oxygen species signaling.

In macrophages, CD36-dependent oxidized LDL import leads to mitochondrial fatty acid accumulation which shifts the mitochondrial function from electron transport chain to reactive oxygen species (ROS) production³⁴. Indeed, ROS are important secondary messengers that regulate ECM, cytoskeleton remodeling and cell motility also in cancer^{35,36}. LDL-exposed MDA-MB-231 cells present several pathways related to ROS production and metabolism deregulated, as repression of Oxidoreductase and Oxidation reduction (Fig. 5a) and induction of disulfide bond and metabolism of xenobiotics by cytochrome P450 (Extended Data Fig. 5a), and a lower respiratory spare capacity (Fig. 5c), raising the possibility that the mitochondria of LDL-treated MDA-MB-231 cells are producing excessive oxidative stress. Using the cellular ROS indicator Cell ROX, we observed that LDL stimulated cellular ROS production in migrating MDA-MB-231 cells (Fig. 6a, vehicle and Extended Data Fig. 6a) in a CD36-dependent manner as SSO treatment (Extended Data Fig. 6b) or CD36 knock-down (Extended Data Fig. 6c) blocked the LDL-induced increase in cellular ROS. Moreover, no differences in ROS production were observed upon LPA treatment (Extended Data Fig. 6a). Consistent with previous studies by others³⁴, ROS production induced by LDL was unlikely due to down-regulation of antioxidant enzymes (Extended Data Fig. 6d) and LDL-exposed cells present even higher levels of catalase, a peroxisome enzyme that converts hydrogen peroxide to water, probably as an attempt to counterbalance the oxidative stress. LDL-induced increase in cellular ROS was abolished using the cellular antioxidant *N*-acetylcysteine (NAC, Fig. 6a) and the mitochondria targeted antioxidant

MitoTEMPO (MitoT, Fig. 6b) suggesting that mitochondria contribute, at least in part, for the increased ROS levels in LDL-exposed MDA-MB-231 cells. Importantly, both cellular and mitochondria-specific antioxidants were able to block LDL-induced migration (Fig. 6c,d) and no effect in cell migration was observed for the cellular antioxidant NAC in CD36-depleted MDA-MB-231 cells (Extended Data Fig. 6e). Antioxidant supplementation also impacted the mitochondrial network of MDA-MB-231 migrating cells, with NAC abolishing the increase in TOM-20-labeled mitochondria (Fig. 6e) and MitoTEMPO blocking the remodeling of mitochondrial network and acquisition of a filamentous distribution (Fig. 6f-h) in LDL-exposed cells. Supplementation with NAC or MitoTEMPO did not alter the mitochondria average area of LDL-treated cells (Extended Data Fig. 6f). This data is consistent with LDL promoting fatty acids trafficking and accumulation into the mitochondria, leading to local ROS production and induction of cell motility^{35,37}. Indeed, LDL-exposed MDA-MB-231 cells treated simultaneously with the CPT1a inhibitor etomoxir, which blocks mitochondrial import of LCFA³⁸, present a significant reduction in the production of cellular ROS (Fig. 6h; LDL vs. etomoxir+LDL $p=0.007$). Moreover, we use the fluorescent analog of palmitic acid (BODIPY FLC16) to visualize exogenous FA uptake together with mitochondria labeling using MitoTracker Deep Red (Extended Data Fig. 6i). Confocal microscopy analysis revealed that in the presence of LDL, shSCR MDA-MB-231 exhibited enhanced uptake of the FA analog relative to the CD36-depleted cells, being FA staining more prominent in the perinuclear region with more intense MitoTracker Deep Red staining.

468 Etomoxir co-treatment decreased the co-localization of FA in the perinuclear
469 MitoTracker Deep Red-stained region, being virtually absent for the shCD36
470 MDA-MB-231 cells (Extended Data Fig. 6g).

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Discussion

The role of hypercholesterolemia in cancer has been under scrutiny by our lab and others^{7–12}. In detail, we have shown that high LDL cholesterol in particular promotes breast cancer progression (by acting directly on cancer cells inducing proliferation and migration^{10,11} and by suppressing immune responses, namely the action of gamma delta T cells¹².

Given the role of lipids in several metabolic processes, most notably in mitochondria (where lipid oxidation mainly occurs), here, we exploited the hypothesis that LDL cholesterol induces breast cancer cells migration by modulating mitochondrial metabolism. In particular, CD36 mediated metabolic adaptations have recently been implicated in lipid-dependent breast cancer aggressive phenotypes, including metastasis, resistance to HER2-targeted therapy and modulation of the tumor microenvironment by T cells dysfunction^{29,39–41}. In macrophages, CD36-dependent import of lipids induces mitochondrial reprogramming, with increase accumulation of fatty acids leading to ROS production and activation of inflammatory patterns³⁴.

Our data shows in vivo and in vitro that LDL exposure induces cell migration and invasion and that this is accompanied by altered (spread) network distribution throughout the migrating cells, classified here as filamentous. The resulting alterations in cellular metabolism indicate that breast cancer cells exposed to high lipid levels depend on lipid oxidation (OXPHOS) as their main energy source and that this results in increased oxidative stress (similar to what is, systemically, seen in hyperlipidemia patients⁴². Accordingly, ROS levels (cellular and mitochondrial

ROS) are significantly elevated in LDL exposed breast cancer cells. Our studies further show that blocking LDL uptake and the resulting lipid entry into mitochondria via CD36 and CPT1a blockade, respectively, or dumping ROS levels by antioxidants completely abrogate cell migration and mitochondria metabolism reprogramming.

Taken together, we reveal novel and detailed mechanistic cellular and metabolic changes underlying the migratory behavior of breast cancer cells exposed to systemic lipids, and propose that CD36 and/or ROS signaling may provide a novel therapeutic strategy for subsets of invasive breast cancer.

Fig.1 - LDL-exposed TNBC cells show different invasion potential, metastatic tropism and mitochondrial network distribution in xenotransplanted zebrafish larvae at 4dpi (6dpf). **a**, Top, Schematic representation of MDA-MB-231 cell invasion potential throughout the zebrafish body in xenotransplanted larvae at 2dpf and analyzed at 4dpi (6dpf) with Dil-labelled MDA-MB-231 control (red) and Cy5-labelled LDL-exposed (grey) cells detected in the indicated organs (grey and red depicts organs with increase tropism for LDL-exposed and control cells, respectively, and each dot represents one xenograft). Bottom, Quantification of the invasive potential depicted as the number of zebrafish larvae xenografts ($n=11$ from 1 independent experiment) with Dil-labelled MDA-MB-231 control (red) and Cy5-labelled LDL-exposed (grey) cells in the indicated organs. **b**, Top, Schematic representation of the total distribution of labelled MDA-MB-231 control (red, $n=1567$) and Cy5-labelled LDL-exposed (grey, $n=931$) cells throughout the zebrafish larvae ($n=11$), each circle represents the proportion of cells of each condition in that organ (grey and red depicts organs with statistical significance for LDL-exposed and control cells, respectively). Bottom, Quantification of cells distribution depicted as the percentage of Dil-labelled MDA-MB-231 control (red) and Cy5-labelled LDL-exposed (grey) cells present in the indicated organs over total. **c**, **d**, **e**, Total Mito-YFP number (**c**), average area (**d**) and mitochondria network (**e**) determined as the Mito-YFP area by cell tracer Dil or Cy5 area (for control and LDL cells respectively) in control ($n=33/35$) and LDL-exposed ($n=46/55$) MDA-MB-231 cells throughout the body of immunolabelled xenotransplanted zebrafish larvae

($n=11$). Each circle represents individual cell measurement, lines represent the mean $\pm$ sem for each condition. **f**, Chart representing Mito-YFP-labelled mitochondrial network distribution of control ($n=39$) and LDL-exposed ($n=64$) MDA-MB-231 cells in the xenotransplanted zebrafish larvae. **g**, Representative images of maximum intensity projection of Dil-labelled MDA-MB-231 control (red) and a Cy5-labelled MDA-MB-231LDL (grey) cells expressing Mito-YFP (green) and displaying a perinuclear (control) and filamentous (LDL) mitochondrial network distribution acquired from the PVS (control) and eye (LDL) of xenotransplanted zebrafish larvae at 4dpi. Nuclei are labelled with DAPI (blue). Scale bar, 10 μ m.

Legend: A. (anal), CHT (caudal hematopoietic tissue), D. (dorsal), H. kidney (head kidney), Intestinal t. (intestinal tract), Optic v. (optic vesicle), PVS (perivitelline space), S. Bladder (swim bladder), T. (tail), V. (ventral). Statistical analysis was performed by applying the Fisher's exact test (a, b, f) and two-tailed Student's *t*-test (c-e). * $p<0.05$, ** $p<0.01$, *** $p<0.0001$.

Fig.2 - LDL exposed migrating TNBC cells display differential mitochondrial network and increased cristae destabilization.

a, Total HSP-60-labelled mitochondria number in control and LDL-exposed migrating MDA-MB-231 cells ($n=20$ each). **b**, Mitochondrial DNA (mtDNA) content accessed by qPCR analysis of the human mitochondrial ND1 gene relative to the nuclear β 2-microglobulin gene in DNA samples from untreated (control) or LDL-exposed MDA-MB-231 cells ($n=7$ each). **c**, Flow cytometry quantification of MitoTracker Deep Red staining depicted as relative median fluorescence intensity (MFI) (left) in untreated (control) or LDL-exposed MDA-

MB-231 cells and histograms (right; $n=5$ each from a representative experiment). **d**, HSP-60-labelled mitochondrial network distribution of control and LDL-exposed migrating MDA-MB-231 cells (left; $n=31$ each) and representative images of cells displaying perinuclear (control) and filamentous (LDL) mitochondrial network distribution (right; 63x objective). Nuclei are labelled with DAPI (blue). Scale bar, 10 μ m. **e**, Mitochondrial network determined as the TOM-20 total area over the phalloidin area of immunolabelled control, LDL or LPA-exposed MDA-MB-231 migrating cells ($n=50/82$ cells per condition). **f,g,h,i**. Representative electron microscope images (TEM) images (left 1500x and right 3000x; scale bar equals 2 μ m; M: mitochondria) (**f**) and quantitative plots of mitochondria number per cell ($n=15/18$ cells) (**g**), cristae density as the number of cristae per μ m² mitochondrial area ($n=100$ mitochondria) (**h**) and percentage of cristae in the angles displayed ($n=12/27$ mitochondria) (**i**) in untreated (control) or LDL-exposed MDA-MB-231 cells. **j**, Western blot for Drp1, Mfn1 and Mfn2 protein in untreated (control) or LDL-exposed MDA-MB-231 cells (representative images, uncropped images of blots are shown in Extended Data Fig. 7). **k**, Mean elongation of HSP-60-labelled mitochondria number in control and LDL-exposed migrating MDA-MB-231 cells ($n=20$ each) in the wound-healing assay. Data are presented as mean $\pm$ s.d. Each circle in the plot represents individual cell measurement. Statistical analysis was performed by the Fisher's exact test (**d**), One-way ANOVA with multiple comparison correction (**e,h**) and two-tailed Student's *t*-test. * $p<0.05$, ** $p<0.01$, *** $p<0.0001$.

Fig.3 - LDL-induced migration of TNBC cells is mediated by the fatty acid transporter CD36.

a, Flow cytometry quantification of BODIPY 493/503 (Bodipy) staining depicted as relative median fluorescence intensity (MFI) (left) in untreated (control) or LDL-exposed MDA-MB-231 cells ($n=5$ each) and representative histograms (right). **b**, Representative live fluorescent microscopy images of control and LDL-treated MDA-MB-231 cells stained with BODIPY 493/503 (Bodipy, 63x objective, scale bar 20 μm) **c**, Flow cytometry quantification of CD36 cell surface expression depicted as fold median fluorescence intensity (MFI) (left) in untreated (control) or LDL-exposed MDA-MB-231 cells and histograms (right; $n=4$ each). **d**, Migratory capacity represented as percentage of wound closure at 24h of control or LDL-exposed MDA-MB-231 cells in the absence (vehicle) or presence of sulfosuccinimidyl oleate (SSO, 50 μM) ($n=4$ each) and representative images of wound closure at 0h and 20h by optical microscopy (4x objective). **e**, Flow cytometry quantification of BODIPY 493/503 (Bodipy) staining of control or LDL-exposed MDA-MB-231 cells in the absence (vehicle) or presence of SSO ($n=4$ each). **f**, Wound closure at 18h of shSCR or shCD36 MDA-MB-231 cells in the absence (control) or presence of LDL ($n=5/7$ each). **g**, Relative mitochondrial DNA (mtDNA) content from control or LDL-exposed MDA-MB-231 cells in the absence (control) or presence of SSO ($n=6/8$ each). **h**, Number of TOM-20 labelled mitochondria in control or LDL-exposed migrating MDA-MB-231 cells in the absence (vehicle) or presence of SSO ($n=44/78$ cells per condition). **i**, Representative images of control, LDL and LDL+SSO treated MDA-MB-231 migrating cells immunolabelled for HSP-60 (red) and lipid droplets (Bodipy, green). Nuclei are labelled with DAPI (blue). 63x objective, scale bar 20 μm . **j**,

Chart representing TOM-20-labelled mitochondrial network distribution of control or LDL-exposed MDA-MB-231 migrating cells in the absence or presence of SSO ($n=44/87$ cells per condition). **k**, Mitochondrial network determined as the TOM-20 total area over the phalloidin area of immunolabelled control or LDL-exposed MDA-MB-231 migrating cells in the absence or presence of SSO ($n=44/82$ cells per condition). Data are presented as mean $\pm$ s.d. Each circle in the plot represents individual cell measurement. Statistical analysis was performed by One-way ANOVA with multiple comparison correction (h,k), Fisher's exact test (j), and two-tailed Student's *t*-test. * $p<0.05$, ** $p<0.01$, *** $p<0.0001$.

Fig.4 - LDL-induced migratory behavior of TNBC cells relies in increased usage of fatty acids and FAO-dependent mitochondrial metabolism.

a, Migratory capacity represented as percentage of wound closure at 18h of control, PA (0.4 mM) or LDL-exposed MDA-MB-231 cells in the absence (vehicle) or presence of etomoxir (200 μ M, $n=4$ each) and representative images of wound closure at 0h and 18h by optical microscopy (4x objective). **b**, Relative mitochondrial DNA (mtDNA) content from control, PA or LDL-exposed MDA-MB-231 cells in the absence (control) or presence of etomoxir ($n=4$ each). **c**, Number of TOM-20 labelled mitochondria in control or LDL-exposed migrating MDA-MB-231 cells in the absence or presence of etomoxir ($n=61/78$ cells per condition). **d**, Chart representing TOM-20-labelled mitochondrial network distribution of control or LDL-exposed MDA-MB-231 migrating cells in the absence or presence of etomoxir ($n=63/87$ cells per condition). **e**, Mitochondrial network determined as the TOM-20 total area over the phalloidin area of immunolabelled control or LDL-

exposed MDA-MB-231 migrating cells in the absence or presence of etomoxir ($n=63/82$ cells per condition). **f**, Representative images of control, LDL and LDL+eto treated MDA-MB-231 migrating cells immunolabelled for TOM20 (green) and phalloidin (red). Nuclei are labelled with DAPI (blue). 63x objective, scale bar 20 μm . **g,h**, Migratory capacity represented as percentage of wound closure at 18h of control or LDL-exposed MDA-MB-231 cells in the absence (vehicle) or presence of 2-DG (2 mM, $n=4$ each) (**g**) and oligomycin (2 μM , $n=4$ each) (**h**). **i,j** Number of TOM-20 labelled mitochondria (**i**) and mitochondrial network determined as the TOM-20 total area over the phalloidin area (**j**) of immunolabelled control or LDL-exposed MDA-MB-231 migrating cells in the absence or presence of CK666 ($n=10/15$ cells per condition). Data are presented as mean $\pm$ s.d. Each circle in the plot represents individual cell measurement. Statistical analysis was performed by One-way ANOVA with multiple comparison correction (c,e,i,j), Fisher's exact test (d), and two-tailed Student's *t*-test. * $p<0.05$, ** $p<0.01$, *** $p<0.0001$.

Fig.5 - Increased lipid exposure induces metabolic and bioenergetic dependencies in TNBC cells.

a, Gene set enrichment analysis (GSEA) of transcriptomes of MDA-MB-231 control cells compared to LDL-exposed for 48h depicting repressed signaling pathways. **b**, qPCR analysis of the relative expression of the indicated genes in untreated (control) or LDL-exposed MDA-MB-231 cells ($n=4/5$ each). **c**, Oxygen consumption rate (OCR) of control or LDL-exposed MDA-MB-231 cells cultured

in the absence (vehicle) or presence of SSO for 48h ($n=14/15$ each from 3 independent experiments). **d**, Extracellular acidification rate (ECAR) of control or LDL-exposed MDA-MB-231 cells cultured in the absence (vehicle) or presence of SSO for 48h ($n=12/15$ each from 3 independent experiments). **e,f**, Fuel dependency depicting oxygen consumption rate for fatty acid oxidation (**e**) and glucose oxidation (**f**) of control or LDL-exposed MDA-MB-231 cells cultured in the absence (vehicle) or presence of SSO ($n=3$ each).

Data are presented as mean $\pm$ s.d. Statistical analysis was performed by the two-tailed Student's *t*-test (b) and One-way ANOVA with multiple comparison. For (a), statistic cut-off [$-\log_{10}(0.05)$] was applied for Benjamini false discovery rate (FDR) correction. * $p<0.05$, ** $p<0.01$, *** $p<0.0001$.

Fig.6 - LDL-induced migratory behavior and mitochondrial adaptations rely in reactive oxygen species signaling.

a,b, Flow cytometry quantification of Cell ROX Deep Red staining depicted as relative median fluorescence intensity (MFI) (left) in control or LDL-exposed MDA-MB-231 cells in the absence (vehicle) or presence of *N*-acetylcysteine (NAC, 5 mM) (**a**) or MitoTEMPO (MitoT, 100 μ M) (**b**) ($n=3$ each) and representative histograms (right). **c,d** Migratory capacity represented as percentage of wound closure at 18h of control or LDL-exposed MDA-MB-231 cells in the absence (vehicle) or presence of *N*-acetylcysteine (NAC, 5 mM, $n=3$ each) (**c**) or MitoTEMPO (MitoT, 100 μ M, $n=3$ each) (**d**). **e,f,g**, Number of TOM-20 labelled mitochondria ($n=36/78$ cells per condition) (**e**), mitochondrial network determined as the TOM-20 total area over the phalloidin area ($n=36/82$ cells per

condition) (**f**) and TOM-20-labelled mitochondrial network distribution ($n=36/87$ cells per condition) (**g**) in control or LDL-exposed migrating MDA-MB-231 cells in the absence or presence of *N*-acetylcysteine (NAC, 5 mM) or MitoTEMPO (MitoT, 100 μ M). **h**, Representative images of control, LDL and LDL+eto treated MDA-MB-231 migrating cells immunolabelled for TOM20 (green) and phalloidin (red). Nuclei are labelled with DAPI (blue). 63x objective, scale bar 20 μ m. **i**, Flow cytometry quantification of Cell ROX Deep Red staining depicted as relative median fluorescence intensity (MFI) (left) in control or LDL-exposed MDA-MB-231 cells in the absence (vehicle) or presence of etomoxir (200 μ M, $n=3/6$ each). Data are presented as mean $\pm$ s.d. Each circle in the plot represents individual cell measurement. Statistical analysis was performed by One-way ANOVA with multiple comparison correction (e,f), Fisher's exact test (g), and two-tailed Student's *t*-test. * $p<0.05$, ** $p<0.01$, *** $p<0.0001$.

Methods

Zebrafish welfare and handling. Zebrafish (*Danio rerio*) casper, nacre, and Tg (*fli1:eGFP*) fish were handled and maintained according to the standard protocols of the European Animal Welfare Legislation, Directive 2010/63/EU (European Commission, 2016) and Champalimaud Fish Platform. All protocols were approved by the Champalimaud Animal Ethical Committee and Portuguese institutional organizations—ORBEA (Orgão de Bem-Estar e Ética Animal/ Animal Welfare and Ethics Body) and DGAV (Direção Geral de Alimentação e Veterinária/Directorate General for Food and Veterinary).

Cell culture. Human triple negative breast cancer cells lines (MDA-MB-231, MDA-MB-436) were purchased from ATCC between 2012 and 2016, tested as being mycoplasma free and authenticated by examination of morphology and consistent in vitro performance. Cells were cultured in DMEM (ThermoFisher) supplemented with 10% (v/v) heat-inactivated FBS (Gibco) and 1% Antibiotic-Antimycotic (Gibco) at 37°C and 5% CO₂ atmosphere. For cell proliferation and wound healing assays, cells were seeded in round bottom 24-well plates, after 24h the medium was replaced by DMEM 1% FBS-LPF (fetal bovine serum-lipoprotein free) (Bio West) for 12h, after which fresh DMEM 1% FBS-LPF growth medium only (control) or supplemented with LDL (Merck Milipore, 100 ug/mL) or palmitic acid (PA):BSA (Sigma, 0,4mM) was added alone or with the following compounds pre-incubated 1 h before: 2-DG (Calbiochem, 2mM), Oligomycin (Sigma, 2µM), Etomoxir (Sigma, 150-200µM), SSO (Sigma, 50µM), LPA (Sigma, 10µM), NAC (Sigma, 5mM), MitoTempo (Sigma, 100µM), CK666 (Sigma, 50-

75µM), anti-human LDL receptor blocking antibody (AF2148, R&D Systems, 5µg/ml). Palmitic acid:BSA solution was prepared according to¹⁸. Briefly, sodium palmitate (Sigma) was dissolved in 0.1M NaOH and warmed at 80 °C until clear. The solution was complexed with fatty acid-free BSA (Sigma) in a molar ratio fatty acid:BSA of 5:1, where BSA was dissolved in 0.9% NaCl, and the mixture was warmed to 45 °C. The clear solution of palmitate was added drop-by-drop by pipette with agitation and the final solution was filtered at 0.45 µm. Cell count ratio was determined by Trypan Blue (0.4% solution, Amresco) exclusion and cell counts (with the aid of a hemocytometer) in a Burker chamber (Blau Brand) after 48h. For the wound-healing assay, two hundred microliters tips were used to make a scratch ("wound") in the center of the well, followed by washing with PBS, and replacement with DMEM 1% FBS-LPF with Mitomycin C (Merck Milipore, 0,5µM) and the appropriate experimental conditions and images were acquired with 4x objective on a Zeiss Primovert microscope coupled with a Zeiss AxioCam ERc or a EVOS M500 microscope at time 0h and after 18/24h. The migrated distance was by subtracting the wound length at 18/24h from 0h and expressed as percentage of wound closure. Lentiviral shRNA construct targeting *CD36* and a non-targeting scramble shRNA sequence (TRCN0000056999 Sigma; kindly provided by Dr. Salvador Aznar Benitah) were used as before ²⁹. For shRNA transduction, lentiviruses were produced in HEK293T cells with co-vectors (pCMV delta R8.2, pMD2.G) using X-tremeGENE DNA Transfection Reagent (Roche). Lentivirus particles were collected 48h post-transfection and used to transduce MDA-MB-231 cells in the presence of 8µg/ml polybrene. Transduced

cells were selected for during 3 days before experiments through addition of 1µg/ml of puromycin (In vivoGen) in the culture media. Cells were transfected with 2-6µg Mito-YFP using FuGENE (ThermoFisher) and according to the manufacturer instructions.

Zebrafish xenografts. For cell preparation, MDA-MB-231 cells were seeded at day 1 at a density of 6×10^6 (control) or $4,5 \times 10^6$ (LDL) cells in a T-175 flask with complete DMEM and 6h later, growth medium was replaced by DMEM supplemented with 1% FBS-LPF. At day 2, growth medium was replaced by DMEM with 1% FBS-LPF alone or supplemented with LDL for a total of 48h. At day 3, cells were transfected with Mito-YFP and in the next day, cells were detached with non-enzymatic methods (Cell Dissociation Buffer, enzyme-free, PBS; ThermoFisher), washed with Dulbecco's phosphate-buffered saline (DPBS) 1X (Bio West) and stained separately with the lipophilic dyes Vybrant CM-Dil (4 µl/ml in DPBS 1X) or Deep Red Cell Tracker (1 µl/ml in DPBS 1X, 10mM stock) (ThermoFisher), for 10 min at 37°C, in the dark. Fluorescently labeled control and LDL-treated cells were mixed (1:1 proportion at 0.5×10^6 cells/µl) and injected using borosilicate glass microcapillaries under a fluorescence scope (Zeiss Axio Zoom V16) with a mechanical micropipetor attached (World Precision Instruments, Pneumatic Pico pump PV820). Approximately 500–1000 cells were injected into the perivitelline space (PVS) of 2 dpf zebrafish embryo, previously anesthetized with Tricaine 1X (Sigma). After injection, zebrafish xenografts remained for ~10 min in Tricaine 1X and then transferred to E3 medium and kept at 34 °C. Every day xenografts were checked—dead ones removed and E3

medium refreshed. Four days after injection zebrafish xenografts were sacrificed, fixed with 4% (v/v) Formaldehyde (FA) (Thermo Fisher) at 4°C overnight and preserved at -20° C in 100% (v/v) methanol (MetOH).

Immuno and cell staining. Zebrafish larvae whole-mount immunofluorescence was performed starting hydration through methanol series (75% > 50% > 25%).

Next, xenografts were permeabilized with 0.1% (w/v) Triton X-100 (Sigma) in PBS 1x and blocked with a mixture of PBS 1X, 1% BSA , 1% DMSO (Sigma), 0.05% Triton X-100, 1.5% goat serum (DAKO) for 1 h at room temperature (RT).

The xenografts were then incubated with primary anti-GFP rabbit polyclonal antibody Alexa 488 conjugate (Invitrogen, 1:100) and DAPI (50 µg/ml; Merck Millipore) by overnight at 4°C. Wash and fixation steps were performed, and

xenografts mounted between two coverslips, allowing double side acquisition using Mowiol mounting media (Sigma). For immunofluorescence in cultured cells,

cells were seeded in glass coverslips fixed with 4% Paraformaldehyde (PFA) (ThermoFisher) following by permeabilization with 0,5% Triton X-100 and

blocking with 0.5% BSA-PBS1x. For BODIPY 493/503 co-staining, cells were permeabilized with 0.2M glycine (Sigma), 0.1mg/ml saponin (Sigma) and 3%

BSA. Cells were then incubated with primary antibody anti-human mouse HSP60 antibody (BD Biosciences,1:250) or mouse TOM20 (F10, Santa Cruz, 1:200)

over-night at 4°C and followed by incubation of the secondary antibody donkey anti-mouse Alexa 488 (Invitrogen, 1:400), donkey anti-mouse Alexa 594,

(Invitrogen; at 1:500), Alexa Fluor 594 Phalloidin (ThermoFisher, 1:200) or BODIPY 493/503 (ThermoFisher; 0,2µg/mL) for 1h at RT. For BODIPY FLC16

and MitoTracker co-staining, seeded cells were washed with 0.1% free fatty acid-BSA (Sigma) in pre-warmed Hank's Balanced Salt Solution (HBSS 1x, ThermoFisher), followed by incubation with BODIPY FLC16 (2 μ M) and Mitotracker Deep Red (25nM) (ThermoFisher) at 37°C for 20 minutes and fixation with 4% PFA. Mounting was performed using Vectashield with DAPI mounting medium (vectorlabs).

Microscopy acquisition and analysis. For zebrafish xenografts imaging and analysis, initial screening was performed using an inverted fluorescence widefield microscope Zeiss Axiovert 200M, with 10x amplification. For the tropism evaluation, tiles of zebrafish larvae were acquired using the spinning disk confocal microscope Zeiss Cell Observer SD, with 10x magnification. Generated images were processed using the Fiji software. Acquired z-stacks were projected using maximum intensity projection. Number of cells was quantified with ImageJ software Cell counter plugin. For the quantification of cells per organ, Imaris 9.0.1 was used. Tiles were converted into three-dimensional reconstructions, and cancer cells in each organ were quantified. For mitochondrial network quantification, confocal microscopy was performed in a Zeiss LSM 880 microscope and the images were acquired with a 63x oil objective (with 1.2-1.6x zoom). For live cell imaging, MitoTracker Deep Red (50 nM) and BODIPY 493/503 (0,2 μ g/mL) (ThermoFisher) were added in complete DMEM for 30 minutes and imaging was performed in a Zeiss Cell Observer Microscope with 63x oil objective. Mitochondrial morphological parameters, as number of mitochondria, mitochondria average area, mitochondria network (the area

occupied by mitochondria particles by the cell tracer area or phalloidin staining area) and mean elongation were quantified using Fiji software. Acquired z-stacks of the various channels were projected using maximum intensity projection, merged, converted to binary and the “Analyze particles” tool was used.

Flow cytometry. For lipid droplets, cells were stained with BODIPY 493/503 (ThermoFisher; 0,2µg/mL) for 10 minutes at RT in the dark, followed by analysis in a LSR Fortessa or BD Accuri (BD Biosciences) flow cytometers. For mitochondrial mass, cells were incubated with MitoTracker Deep Red (ThermoFisher; 2 nM) for 15 minutes at RT followed and for ROS, cells were incubated with Cell ROX (ThermoFisher; 2 µM) for 20 minutes at 37°C, followed by a similar type of analysis. For detection of CD36 cell surface, cells were blocked with PBS-0.5% BSA and incubated with primary antibody anti-human mouse CD36 (Biolegend; 1:100) in the same solution for 1h at RT, followed by incubation with secondary antibody goat anti-mouse Alexa 633 (Invitrogen, 1:400) and analysis in a LSR Fortessa flow cytometer. All data was analyzed using the FlowJo software (LLC).

Quantitative Real-Time PCR. For RNA analysis, total RNA from cells was extracted using TRIZOL (Life Technologies) and samples were reverse transcribed using random priming and Superscript Reverse Transcriptase (Life Technologies), according to the manufacturer’s instructions. Quantitative real-time PCR (qPCR) was performed using DNA master SYBR Green I mix (Applied Biosystems) in an ABI PRISM 7700 thermocycler. Quantifications were made applying the ΔC_t method (C_t of gene of interest- C_t of housekeeping gene)

843 followed by $2^{(-\Delta\Delta Ct)}$. For mtDNA determination, total DNA was isolated from
844 cells using phenol:chloroform:isoamyl alcohol (Sigma) and measured by
845 assessing the levels of the human mitochondrial ND1 (mtND1) relative to nuclear
846 β 2-microglobulin gene. Quantifications were made applying the ΔCt method (Ct
847 of nuclear DNA gene - Ct of mitochondrial DNA gene) followed by $2^{(\Delta Ct)}$
848 according to ⁴³ and all primer sequences are described in Supplementary Table
849 1.

850 **Transmission electron microscopy.** Cells were were fixed with a solution
851 containing 2.5% glutaraldehyde (Electron Microscopy Sciences) plus 0.1%
852 formaldehyde (ThermoFisher) in 0.1 M cacodylate buffer (Sigma), pH7.3 for 1 h.
853 After fixation, samples were washed and treated with 0.1% Millipore filtered
854 cacodylate buffered (Sigma), post-fixed with 1% Millipore-filtered osmium
855 tetroxide (Electron Microscopy Sciences) for 30 min, and stained en bloc with 1%
856 Millipore-filtered uranyl acetate (Agar scientific). Samples were dehydrated in
857 increasing concentrations of ethanol, infiltrated and embedded in EMBed- 812
858 medium (Electron Microscopy Sciences). Polymerization was performed at 60°C
859 for 2 days, and ultrathin sections were cut in a Reichert supernova microtome,
860 stained with uranyl acetate and lead citrate (Sigma) and examined in an H-7650
861 transmission electron microscope (Hitachi) at an accelerating voltage of 100 kV.
862 Digital images were obtained using a XR41M mid mount AMT digital camera
863 (Advanced Microscopy Techniques Corp). For quantification, cells were
864 examined at 3000x magnification and the number of mitochondria per cell was
865 counted in the sectioned image.

Western blot. Whole-cell extracts were prepared using RIPA buffer (1% Triton X-100, 0.1 % SDS, 150 mM NaCl, 50 mM Tris-HCl pH 7.8, 5 mM EDTA, 0.5% NaDOC) containing proteinase inhibitors (Roche) and resolved using SDS-PAGE (10%, 12% Tris-HCl gels or 4-15% gradient gels (Mini-PROTEAN TGX, BioRad) under reducing conditions (in the presence of β -mercaptoethanol, BioRad) and Precision Plus Protein Standards (dual color, BioRad) was used. Proteins were subsequently blotted onto a nitrocellulose membrane (BioRad) following conventional protocols and hybridized using anti-human antibodies against β -Actin (Sigma; 1:15000), Drp1 (Santa Cruz; 1:200), Mfn1 (Abcam; 1:1000), PGC-1 α (Calbiochem; 1:000) and HRP-coupled secondary antibodies (1:6000; Promega) were used. The Pierce ECL (Thermo Fisher) detection system and Fuji Medical X-ray Films (FUJIFILM) was used. Quantification of densitometric units was performed using the Scion Image software.

Seahorse energetic assays. MDA-MB-231 cells were seeded into the specialized XF24 cell culture microplate (Seahorse Bioscience), about at 24,000 cells/well and maintained in DMEM 1% FBS-LPF (control) or LDL-exposed cells in the presence or absence of SSO (Sigma, 50 μ M) for 36h. All measurements were performed using a Seahorse Bioscience Extracellular Flux Analyzer (Agilent).

For the Seahorse Cell Mito Stress Test profile for mitochondrial respiration and glycolytic function, cells were incubated for 1h at 37°C in XF Seahorse medium supplemented with glucose, glutamine and sodium pyruvate and without CO₂. Oxygen consumption rate (OCR) was measured under standard conditions and after the addition of 1 μ M oligomycin, 1,5 μ M FCCP and 1 μ M rotenone/1 μ M

Antimycin A. Extracellular acidification rate (ECAR) was measured under standard conditions and after the addition of 10mM glucose, 1µM oligomycin and 20mM 2-DG. Real time measurements (quintuplicates) of the OCR (picomoles per minute) and ECAR (mpH per minute) were plotted over time. Cells were then lysed in RIPA buffer and subjected to Pierce BCA protein assay (ThermoFisher). OCR and ECAR values were normalized by protein values in each well.

For the Seahorse Mito Fuel Flex Test, cells were switched to XF Seahorse medium containing glucose and glutamine, and CO₂ privation, 1h previous the assay at 37°C. Glucose dependency and capacity assays were measured under standard conditions and after the addition of 6µM UK5099, 8µM etomoxir/4µM BPTES and 1µM rotenone/1µM Antimycin A and for the FAO dependency and capacity assays after addition of 8µM etomoxir, 6µM UK5099/4µM BPTES and 1µM rotenone/1µM Antimycin A.

Statistical analysis. For zebrafish xenografts tropism and mitochondrial network statistical analyses was performed using Fisher's exact test and Chi-square. The remaining statistical analyses was performed using two-tailed Student's *t*-test and one-way ANOVA followed by Tukey's post test for multiple comparisons using the Graph-Pad Prism 8.0 (GraphPad software), and data are presented as mean ± s.d. * *p*<0.05, ** *p*<0.01, *** *p*<0.001.

Pathway analysis of transcriptomes from microarrays data¹¹ obtained from MDA-MB-231 cells control of LDL-exposed for 48h was performed using the DAVID Functional Annotation tool^{44,45} and applying a statistic cut-off [*-log10 (0.05)*] for Benjamini false discovery rate (FDR) correction.

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920 **Data availability**

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Author contributions

S.N.-P. designed and executed most of the experiments and performed data analyses. T.S., A.P.L. and M.S. contributed for experimental work and data analysis. F.C. and V.M. performed the Seahorse energetic assays. T.S., B.B.J., V.M. contributed to manuscript preparation. S.N.-P. and S.D. conceived the project and experimental design, the writing and preparation of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at

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Figures

Nóbrega-Pereira S_Fig. 1

Figure 1

LDL-exposed TNBC cells show different invasion potential, metastatic tropism and mitochondrial network distribution in xenotransplanted zebrafish larvae at 4dpi (6dpf).

Figure 2

LDL exposed migrating TNBC cells display differential mitochondrial network and increased cristae destabilization.

Figure 3

LDL-induced migration of TNBC cells is mediated by the fatty acid transporter CD36.

Nóbrega-Pereira S_Fig. 4

Figure 4

LDL-induced migratory behavior of TNBC cells relies in increased usage of fatty acids and FAO-dependent mitochondrial metabolism.

Figure 5

Increased lipid exposure induces metabolic and bioenergetic dependencies in TNBC cells.

Figure 6

LDL-induced migratory behavior and mitochondrial adaptations rely in reactive oxygen species signaling.

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