Going off the grid: ERα Breast cancer beyond estradiol

Ylenia Perone¹ and Luca Magnani¹*

¹Department of Surgery and Cancer, Imperial Centre for Translational and Experimental Medicine

Short title
Metabolic reprogramming in luminal breast cancer

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*To whom correspondence should be addressed: l.magnani@imperial.ac.uk

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Abstract

Novel studies have linked cholesterol biosynthesis to drug resistance in luminal breast cancer. Structural data suggest that cholesterol metabolites, including 27-hydroxycholesterol (27-HC), can act as ERα ligands in these cells. Additionally, hypercholesterolemia has now been linked to breast cancer progression. The focus of this review is to briefly summarize these recent findings and discuss how epigenetic reprogramming is definitively connected to endogenous cholesterol biosynthesis. We elaborate on how these data support a working model in which cholesterol biosynthesis promotes autocrine, pro-invasive signaling via activation of a series of closely related transcription factors. Importantly, we discuss how this mechanism of resistance is specifically associated with aromatase inhibitors. Finally, we examine how the field is now considering the development of anti-cholesterol therapeutics and companion biomarkers to stratify and treat ERα breast cancer patients. In particular, we review recent progress in pharmaceutical strategies targeting the cholesterol molecular machinery in primary and secondary breast cancers.
Breast cancer is the most common cancer in women and, despite significant progress in detection and treatments, incidence has been steadily increasing in the last 40 years (Torre et al. 2015). Over the past decades, it has become clear that breast cancer is a heterogeneous disease characterized by many distinct molecular subtypes. The largest subgroup of breast cancer patients is found in the luminal subtype, representing around 70% of all breast cancer cases. These tumours are positive for estrogen receptor α (ERα), a nuclear-receptor transcription factor promoting cell proliferation and tumor growth. Multiple drugs, commonly referred to as endocrine therapies, have been developed to inhibit ERα activation, representing one of the first examples of targeted therapy. Endocrine therapies can reduce mortality and relapse rate up to 50% (Early Breast Cancer Trialists' Collaborative Group (EBCTCG) et al. 2011).

Though endocrine therapies target the same nuclear receptor (NR), they are characterized by different mechanisms of action. For example, selective estrogen receptor modulators, such as Tamoxifen, antagonize the receptor in breast tissue but can have agonistic effect in the endometrium. Aromatase Inhibitors (AI), such as Letrozole, Anastrozole and Exemestane, are used primarily in postmenopausal women and block peripheral aromatase activity thus lowering circulating estrogen levels. Finally, Faslodex/Fulvestrant is a selective estrogen receptor down regulator commonly used in the metastatic breast cancer setting. Although these drugs are particularly effective, over 30% of these patients eventually develop disease recurrence, often due to endocrine therapy resistance (Early Breast Cancer Trialists' Collaborative Group (EBCTCG) et al. 2011). Resistance to endocrine therapies is multifactorial and involves several molecular events. However, it was not clear if the specific type of treatment would play a significant role in this process. In a recent study, we have characterized, for the first time, the net effect of each treatment with a
particular focus on the epigenetic changes occurring in response to each therapy (Nguyen et al. 2015). By using epigenomic, genome-wide analyses, we have uncovered how cells developing resistance to AI endogenously trigger cholesterol biosynthesis leading to sustained estrogen independent ERα activation (Nguyen et al. 2015). The link between hypercholesterolemia and breast cancer was previously known since obesity and metabolic disease are two of the strongest risk factors for this tumor development. In particular, recent evidence suggest that endogenous oxysterols including 27-hydroxysterol (27-HC) link hypercholesterolemia and breast cancer pathophysiology (Nelson et al. 2013; Wu et al. 2013).

Our work demonstrates that cholesterol biosynthesis is activated by changing the chromatin environment around potential SREBP1 (sterol response element binding protein 1) binding sites (Nguyen et al. 2015). This was revealed by profiling AI resistant breast cancer cells using DNAseI hypersensitivity assays coupled with next generation sequencing. In response to intracellular cues, SREBP1 translocate from endoplasmic reticulum to golgi and from here to the nucleus activating cholesterol genes by binding to their promoters (i.e. hydroxyl-methyl-glutaryl CoA reductase (HMGCOAR) and squalene epoxidase (SQLE)). Recently, a new series of compounds (fatostatins) have been designed to block SREBP1 nuclear translocation (Kamisuki et al. 2009). Treating AI resistant cells with fatostatins was sufficient to reduce ERα and block cell invasion in AI resistant cells (Nguyen et al. 2015).

Strikingly, these data indicate that epigenetic reprogramming might induce new transcription factor dependencies including SREBP1/2. Before formulating therapeutic strategies based on epigenetic modulators, several basic questions need to be addressed. How is SREBP1 activated in endocrine therapy resistant breast cancer?
At what stage is this activation required? Is CB only acting through ERα or are there other receptors involved? Finally, can we exploit this finding in the clinical setting?

Interestingly, 27-HC has agonistic activity on members of the NR superfamily (Umetani et al. 2007) including the liver X receptor (LXR). In the last 40 years NRs have been widely studied but, beside ERα, whether other NRs are pivotal in breast cancer is still deeply debated. We know that NRs are activated by several hormones, metabolites, dietary lipids and vitamins and regulate gene transcription after binding transcription factors (Gadaleta & Magnani 2014). A growing body of evidence shows that not only ERα but also other cholesterol and oxysterol activated NRs (CANRs) play a significant role in ERα breast cancer, in particular LXR and estrogen-related receptor-alpha (ERRα). This suggests that anti-cholesterol therapy in several diseases might be mediated by a larger network of NRs. Our work supports the hypothesis that intra-tumoral CB is constitutively activated after chronic exposure to AI leading to the local accumulation of metabolic ligands for ERα and possibly for other CANRs such as LXR and FXR (Farnesoid X receptor). Indeed, DNA motifs for LXR and FXR are enriched with AI-resistant super-enhancers (Nguyen et al. 2015).

Super-enhancers are the key regulatory regions driving cell identity both during development and carcinogenesis (Whyte et al. 2013). Active LXR has been characterized as a controversial player. While some studies have reported that LXR activation might be anti-proliferative (reviewed in (Lin & Gustafsson 2015), others have suggested that it is involved in breast cancer progression (Nelson et al. 2013).

CB might also have agonistic effects on ERRα activation, another NR-family member (Wei et al. 2016). ERRs are a family of NRs homologous to ERα mediating cholesterol regulation of bone resorption, regulation of fatty acid oxidation, mitochondrial biogenesis, oxidative phosphorylation and a modulator of ERα
signaling. Interestingly, Wei and colleagues have recently demonstrated that pharmacological regulation by statins in osteoclasts is ERRα dependent. Similarly, we have shown that statins might antagonize breast cancer progression by interfering with ERα activation (Nguyen et al. 2015).

Statins might not be the only lipids lowering drugs with potential effects on breast cancer. A recent meta-analysis conducted from the Early Breast Cancer Trialists’ Collaborative group (EBCTCG 2015) displayed the benefits of bisphosphonates treatment in postmenopausal women, reporting significant reductions not only in bone metastasis but also in any distant recurrence site and breast cancer mortality. We know that both lipids lowering drugs and bisphosphonates block osteoclast function and the cholesterol pathway by decreasing the bioavailability of the endogenous ERRα agonist cholesterol (Wei et al. 2016). The effects on bone recurrence emphasize the potential importance of blocking CB in highly invasive metastatic breast cancer cells. For example, it might be possible that in addition to bisphosphonates effect on osteoclasts, lowering CB in micro metastases might also antagonize ERα, ERRα and other CANRs. Consequently, disrupting lipid metabolic pathways could induce tumor regression and inhibit metastatic spread. Indeed, several lipids lowering drugs are under preclinical and clinical studies in several cancer types. There are several ways to block directly SREBPs: our group studied fatostatin in breast cancer showing inhibition of SREBP lowers ERα activation in AI resistant cells (Nguyen et al. 2015). Other drugs, namely sphingomab and nelfinavir, have been designed to block SREBP release and translocation via Site-1 and Site-2 proteases. Furthermore, metformin, an anti-diabetic drug, showed to inhibit breast cancer cell proliferation and the expression of SREBP1-c. On the other hand, statins block HMG-CoAR, while terbinafine is a SQLE inhibitor and
bisphosphonates are designed to interfere with Farnesyl-PP synthase (as reviewed by
Beloribi-djefa et al. 2016). HMG-CoAR, SQLE and Farnesyl-PP synthase are three
critical enzymes in the CB pathway. Additionally, molecules such as orlistat block
Fatty Acid Synthase (FASN), another enzyme of the CB pathway, whereas methyl-b-
cyclodextrine modulates lipid raft components to induce cell death signaling. Finally,
there are some other molecules under investigation able to modulate NRs such as
LXR, or key enzymes like Stearoyl-CoA desaturase 1 (SCD1) and cyclooxygenase 2
(COX-2) (Beloribi-Djefaflia et al. 2016). Overall, the results from our study represent
a step towards the AI resistance mechanisms providing evidence that anticancer
effects can be potentiated by combining different types of endocrine therapy with
strategies depriving the tumor of cholesterol. The rationale for this assumption has its
grounds on a study showing that some drugs already on the market for treating ERα
breast cancer are also able to block Cytochrome P45027A1 (CYP27A1) (Mast et al.
2015). This is the only enzyme in humans converting cholesterol to 27-HC, the
oxysterol modulating NRs like ERα and LXR. During the menopause 27-HC
increases while estrogens decrease suggesting a potential switch in ERα agonists
(Umetani et al. 2007). In breast cancer, CYP27A1 enzyme expression increases and it
correlates with tumor grade (Nelson et al. 2013). Mast et al tested several drugs to
target this enzyme and found that Anastrozole, for example, not only inhibits
CYP19A1 but it is also the strongest inhibitor of CYP27A1. So far, we discussed the
development of compounds able to antagonize breast cancer escape mechanisms with
endocrine treatment. On the other hand, if our hypothesis is correct, another important
potential application of our study could be the translational applicability of using
SREBP1 and CB as potential biomarkers to predict AI resistance.
All these pathways converge to a unique scenario where the reprogrammed metabolism plays a key role for growth and proliferation of invasive cancer cells. The first tumor-specific metabolic alteration was described by Warburg that observed proliferating ascites-derived tumor cells convert the majority of glucose to lactate, even in oxygen-rich conditions (aerobic glycolysis) (Warburg. 1956). He set the foundations for a traditional model based on metabolic alterations as an indirect response to external signaling. Recently, a reverse Warburg has been proposed (Pavlides et al. 2009), in which the stroma can provide metabolites useful for cancer cells. Similarly, many infiltrating tumor cells can act as a source of cholesterol derivatives including macrophages and fibroblasts (Nelson et al. 2013). These phenomenon parallel the astrophysics concept of “Terraforming”, based on the hypothetical modifications of a planet environment in order to be similar to the Earth. Similarly, translating this idea at a biological level, cancer cells could change the microenvironment in order to create a more habitable one suggesting that the tumor might “farm” the stroma for CANRs ligands. However, an alternative model might exist. Evidences have been recently gained supporting a supply-based model in which proliferating cells are reprogrammed to meet the challenges of colonizing different environments such as lung, bones, liver or brain (Ward & Thompson 2012). In this model, metabolic reprogramming is crucial to provide endogenous nutrients (in BC namely ligand for CANRs) in order to metastasize. To further support the hypothesis that invasive cells work toward becoming microenvironment independent, a subgroup of reprogrammed cancer cells behave like pioneers becoming invasive and able to colonize and adapt to different microenvironments. In this scenario, CB could operate as a survival toolkit in order for the pioneer metastatic cell to be able to invade a new environment and survive. Indeed, supplying the necessary fuel, we suggested CB
playing a key role in drug resistant relapses making the cell autonomous. Consistent with this hypothesis and in agreement with others, our data showed that epigenetic reprogramming underlies metabolic changes playing a critical role in estrogen independent cancer cells and promoting self-sustaining signal transduction mechanisms to foster growth and survival (Wu et al. 2013; Nguyen et al. 2015).

Epigenetic changes promote overexpression of several oncogenic metabolic genes, nonetheless, genetic changes might also contribute to similar metabolic switches. For example, it has been recently shown that breast cancer cells with p53 gain-of-function mutations are characterized by up-regulation of CB (Freed-Pastor et al. 2012). Indeed, the up-regulation of the initial steps of CB is necessary for the effects of mutant p53 on tissue architecture. Mutant p53 might act as a co-activator for SREBPs proteins. Mutated p53 binding enhances SREBPs activation resulting in augmented CB in malignant cells. Furthermore, the authors showed that fatostatin significantly reduced the level of mutant p53 binding to the HMGCoAR gene promoter. Interestingly, CB up-regulation significantly correlated with a higher rate of p53 mutations in breast cancer patients and it also correlates with a poor prognosis.

There are several studies testing lipids lowering drugs anticancer potential but their protective role in the clinical setting is still greatly debated. For example, a nation-wide study (Ahern et al. 2011), found that ERα positive breast cancer patients who were treated with statins were less likely to develop relapses. Interestingly, pharmacodynamic studies showed no effect of these drugs on estradiol levels or Anastrozole metabolism, laying the first stone at further investigating the possibility of a combined therapy in metastatic breast cancer patients. Clinical trials combining lipids lowering drugs and AI are needed in order to clarify at a clinical level the new molecular insights that our study highlighted.
Collectively, our data warrants an in-depth investigation towards potential new biomarkers to predict AI resistance *a priori* and to stratify ERα patients to the most appropriate form of therapy while considering lipids lowering drugs and endocrine combined treatment in breast cancer.
Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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Author contributions

Y P and L M wrote the review jointly.

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**Figure legend**

**Figure 1: Schematic model of Aromatase Inhibitors resistant ERα breast cancer.**

(A) ERα breast cancer proliferates in response to circulating estrogens. ERα activates gene transcription (arrow) by binding to epigenetically bookmarked distal enhancers (asterisks).

(B) In post-menopausal women, inhibition of androgens peripheral conversion to estrogens is sufficient to impair ERα binding and block ERα driven transcription.

(C) Long-term exposure to aromatase inhibitors can lead to permanent epigenetic reprogramming especially near genes involved in cholesterol biosynthesis. In this scenario the chromatin structure near these genes become accessible to transcription factors such as SREBP1 leading to endogenous cholesterol biosynthesis. Ultimately cholesterol is transformed into ERα ligands (i.e. 27-hydroxil cholesterol) and become an alternative fuel for ERα mediated transcription. Potential therapeutic molecules targeting the cholesterol machinery are included in brackets.

DNA is represented in black and grey, red arrow indicates gene transcription. The key molecules involved in this working model are shown in the scheme (estradiol, E2; Estrogen receptor 1, ESR1; Sterol regulatory binding element 1, SREBP1; 27 hydroxycholesterol, 27HC).

**Figure 2: Schematic supply-based model of resistant ERα breast cancer cell.**

In response to Aromatase Inhibitors resistant cancer cells endogenously trigger cholesterol biosynthesis leading to sustained oestrogen independent ERα activation. Metabolic epigenetic reprogramming induces new transcript factor dependencies including SREBP1. Metastatic cells behave like pioneers becoming invasive and able to colonise and adapt to different microenvironments. Cancer cells with p53 gain-of-function (GOF p53) are characterized by up-regulation of CB with mutant p53 potentially acting as co-activator for SREBPs proteins. Several drugs are under study in order to block the cholesterol pathway (red arrows).
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