The 5,6-epoxycholesterol metabolic pathway in cancer: emergence of new pharmacological targets

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Abstract

Metabolic pathways have emerged as cornerstones in carcinogenic deregulation providing new therapeutic strategies for cancer management. This is illustrated by the recent discovery of a cholesterol metabolic branch involving the biochemical transformation of 5,6-epoxycholesterol (5,6-ECs). 5,6-ECs have been shown to be differentially metabolized in breast cancers (BC) compared to normal breast tissue. 5,6-ECs are metabolized into the tumour promoter oncosterone in BC, while they are transformed into the tumour suppressor metabolite dendrogenin A (DDA) in normal breast tissue. Blocking oncosterone's mitogenic and invasive potential will represent new opportunities for BC treatment. The reactivation of DDA biosynthesis, or its use as a drug, represents promising therapeutic approaches such as DDA-deficiency complementation, activation of BC cell re-differentiation and BC chemoprevention. This review presents current knowledge as to the 5,6-EC metabolic pathway in BC focusing on the 5,6-EC metabolic enzymes ChEH and HSD11B2, and on 5,6-EC metabolite targets LXRβ and GR.

1. Introduction.

Metabolic reprogramming has emerged as a hallmark in cancer, impacting gene expression, cell de-differentiation and tumour microenvironment (Pavlova & Thompson, 2016). It is well established that cancer cells must rewire the cellular metabolism to satisfy the demands of growth and proliferation, most notably by providing energy, reducing equivalents and building blocks while several metabolites exert a signalling functions promoting tumour growth and progression (Danhier et al., 2017). The exploration of cancer metabolism for clinical benefits is required to identify metabolic pathways that are limiting for tumour progression (Vander Heiden & DeBerardinis, 2017). Cholesterol is a major lipid that is crucial as a building block for membrane formation and protein structuration (Grouleff, Irudayam, Skeby & Schiott, 2015). It is also the precursor of steroid hormones, bile acids and oxysterols (Schroepfer, 2000). Cholesterol biosynthesis is a multi-step process (Nes, 2011) that is subject to homeostasis and finely regulated in cells (Luo, Yang & Song, 2020). In cancer cells several deregulations have recently emerged, opening up new therapeutic strategies (Huang, Song & Xu, 2020; Kuzu, Noory & Robertson, 2016).

Recent epidemiological studies have shown that breast cancer (BC) still represent the world leading female cancer in terms of incidence and mortality (Bray, Ferlay, Soerjomataram, Siegel, Torre & Jemal, 2018; Global Burden of Disease Cancer et al., 2019). Thus, there is an urgent need to find and validate new therapeutic targets in order to improve patient survival and tumour recurrence. BC is a heterogeneous pathology and several molecular BC subtypes have been described driving therapeutic treatments. We can distinguish the following three major subtypes: 1) Estrogen receptor positive breast cancers (ER(+)BC) are the most frequent BC and are treated with selective ER modulators (SERM) and aromatase inhibitors (AI). SERM block the mitogenic effects of 17β-oestradiol (E2) at the ER level, and AI inhibit E2 neosynthesis in BC

(Jordan & Brodie, 2007). 2) ER(-)negative and HER2(+) BC are treated using anti-HER2 therapeutic antibodies that block the activation of HER2-dependent mitogenic pathways with or without conventional chemotherapy (Goldhirsch et al., 2011); 3) triple negative BC (TNBC) that do not express steroid hormone receptors and HER2 are treated by conventional chemotherapy with non-selective cytotoxic drugs (Goldhirsch et al., 2011).

Epidemiological population studies have identified links between cholesterol and cancer. Meta-analysis of clinical trials have shown an inverse relationship between circulating cholesterol levels and BC (Touvier et al., 2015), while hypercholesterolemia has been proposed as a risk factor for BC recurrence (Nelson, 2018), implying that cholesterol metabolism deregulations occured in BC and that targeting cholesterol metabolism deregulations may be of interest for BC treatment and chemoprevention (Garcia-Estevez & Moreno-Bueno, 2019).

At the molecular level, recent studies have shpwn that certain oxysterols display either tumour promoter but also tumour suppressor properties (Fig 1A). 27-hydroxycholesterol (27-HC) has been shown to stimulate ER(+)BC proliferation and invasiveness through the modulation of ER and LXRβ respectively (Nelson et al., 2013) . It has also been shown that the pro-metastatic action of 27-HC in mice required myeloid immune cell functions such as polymorphonuclear-neutrophils and γδ-T cells at distal metastatic sites (Baek et al., 2017). In mice, it has been shown that the CXCR2 receptor was involved in this effect (Baek et al., 2017; Raccosta et al., 2013; Raccosta, Fontana, Traversari & Russo, 2013) . It has been shown that other side-chain oxysterols displayed similar properties, possibly after sulfation by the sulfotransferase SULT2B1b (Moresco et al., 2018; Raccosta et al., 2013). These observations led to the proposal that combination therapies associating the inhibition of 27-HC biosynthesis at the 27-hydroxylase level (CYP27A1) and the use of ERα and LXR antagonist could increase the efficacy of treatments against ER(+)-BC (Nelson, 2018). In human, clinical studies from the EPIC-Heidelberg cohort showed that high level of circulating 27-HC were associated with a decreased BC risk in postmenauposal women suggesting that 27-HC could prevent BC in these cases (Le Cornet et al., 2020; Lu, Le Cornet, Sookthai, Johnson, Kaaks & Fortner, 2019). In contrast, strategies aiming to target 27HC biosynthesis as well as its effectors, as proposed by Nelson et al, should be limited to ER(+)-BC patients after an endocrine therapy to protect them against BC recurrence .

Certain B-ring oxysterols such as 7-hydroperoxycholesterol and 5,6-epoxycholesterol (5,6-EC) (Fig.1A) have retained the attention of researchers during the last century as they are major autoxidation and photo-oxidation products of cholesterol (Smith, 1981; Smith & Johnson, 1989), and are suspected to be alkylating substances and thus possibly mutagenic and carcinogenic. These oxysterols were shown to induce mutagenicity in some yeast strains (Ansari, Walker, Smart & Smith, 1982; Smith, Smart & Ansari, 1979) and chinese hamster V79 cells (Chang, Jone, Trosko, Peterson & Sevanian, 1988; Peterson, Peterson, Spears, Trosko & Sevanian, 1988; Sevanian & Peterson, 1984; Sevanian & Peterson, 1986) *in vitro.* However *in vivo* tests failed to show any carcinogenic potencies for 5,6-EC (el-Bayoumy et al., 1996). Meanwhile, recent studies have revealed that 5,6-EC are involved in a metabolic branch clearly involved in carcinogenesis and identified new 5,6-EC metabolites with opposite properties regarding BC oncogenesis. 1) 5,6α-EC can give metabolites with antiproliferative and cancer cell redifferentiation properties: 5,6α-EC can be sulphated by the sulfotransferase SULT2B1b in BC cells to produce 5,6α-epoxy-cholesterol-3β-sulfate (5,6-ECS) (Fig 1A) and 5,6-ECS was shown to induce BC cell death and BC cell redifferentiation activities*in vitro* . In normal breast tissue 5,6α-EC was shown to be conjugated to histamine to give Dendrogenin A (Fig 1A,B), a steroidal alkaloid that displays tumour suppressive properties (de Medina et al., 2013; Poirot & Silvente-Poirot, 2018; Segala et al., 2017; Silvente-Poirot & Poirot, 2014) (Fig 2A). 2) 5,6α-EC can be transformed into a tumour promoter: 5,6-ECs were shown to give a secondary metabolite named oncosterone (6-oxo-cholestan-3,6- diol, cholestan-3,6-diol-6-one, OCDO) (Fig 1A,C) with tumour promoter properties in ER(+)BC and TN BC (Poirot, Soules, Mallinger, Dalenc & Silvente-Poirot, 2018; Silvente-Poirot, Dalenc & Poirot, 2018; Voisin et al., 2017) (Fig 2B).

2. The 5,6-epoxycholesterols (5,6-ECs)

5,6-ECs exist as two different diastereoisomers: the 5,6α-EC and the 5,6β-EC (Fig 1A). They stem from

the mono-oxygenation of cholesterol on its $\Delta^{5,6}$-double bond. In biological systems they can be produced as a mixture via a free radical lipid peroxidation process (Iuliano, 2011; Poirot & Silvente-Poirot, 2013; Porter, Xu & Pratt, 2020; Yin, Xu & Porter, 2011). The stereo-selective synthesis of 5,6β-EC can be achieve using porphyrins, that mimic cytochrome p450 monooxygenases(Poirot & Silvente-Poirot, 2013), and a stereoselective biosynthesis of 5,6α-EC by a yet unidentified cytochrome p450 have been described in bovine adrenals (Watabe & Sawahata, 1979). 5,6-EC are known as major autoxidation and photo-oxidation products of cholesterol (Smith, 1981). They are present in biological fluids and solid tissues from mammals (Poirot & Silvente-Poirot, 2013; Schroepfer, 2000).

The epoxide ring, is well known by chemists for its reactivity towards nucleophilic groups and is widely used for this reason in organic chemistry (Gorzynski Smith, 1984; Parker & Isaacs, 1959). 5,6α -EC was suspected to be involved in skin photocarcinogenesis as it accumulated in skin after UV exposure (Black & Douglas, 1973; Black & Lo, 1971; Lo & Black, 1973; Lo & Black, 1972) and activated a ChEH activity to accelerate the hydrolysis and elimination of 5,6-EC (Chan & Black, 1974). Meanwhile, 5,6-EC were found to be chemically stable towards nucleophiles ruling out a direct carcinogenic activity (Paillasse, Saffon, Gornitzka, Silvente-Poirot, Poirot & de Medina, 2012).

In the presence of a catalyst 5,6α-EC was the only of the two 5,6-ECs that reacts with nucleophiles to give a single product of addition with a 5α-6β stereochemistry through a trans-diaxial ring opening (Paillasse, Saffon, Gornitzka, Silvente-Poirot, Poirot & de Medina, 2012; Poirot & Silvente-Poirot, 2013) (Fig 1B). 5,6-EC were reported to be modulators of nuclear receptors such as Liver-X-Receptors α (LXRα) and β (LXRβ) (Berrodin, Shen, Quinet, Yudt, Freedman & Nagpal, 2010; Song, Hiipakka & Liao, 2001) showing that these oxysterols are biologically active oxysterols. The metabolism of 5,6-EC has been previously reviewed and showed that 5,6α-EC and 5,6β-EC were differentially metabolized in mammals (Poirot & Silvente-Poirot, 2013). Interestingly, 5,6β-EC was reported to accumulate in breast nipple fluids from patients with BC and preneoplastic breast lesions supporting that the 5,6-EC metabolism was linked to breast carcinogenesis (Gruenke, Wrensch, Petrakis, Miike, Ernster & Craig, 1987; Petrakis, Gruenke & Craig, 1981; Wrensch et al., 1989).

3. Metabolism of 5,6-epoxycholesterols

3.1. The cholesterol epoxide hydrolase (ChEH).

ChEH is responsible for the hydration of 5,6-ECs to give cholestane-3β,5α,6β-triol (CT)(Aringer & Eneroth, 1974; Chan & Black, 1974; Nashed, Michaud, Levin & Jerina, 1985; Sevanian & McLeod, 1986) (Fig 1C). Like other epoxide hydrolases, ChEH was initially considered as a type II detoxification enzyme to eliminate toxicants (Morisseau & Hammock, 2005). However, the biological functions epoxide hydrolases have been extended to the control of the production of bioactive lipids (Kodani & Hammock, 2015; Morisseau, 2013; Newman, Morisseau & Hammock, 2005) as observed for other families of type two detoxification enzymes such as glutathione transferases (Board & Menon, 2013). CT was shown to display certain biological properties suggesting it may have a physiological role in mammals. CT is an oxysterol that can induce cytotoxicity *in vivo* and *in vitro* in normal and cancerous cells (Carvalho, Silva, Moreira, Simoes & Sa e Melo, 2010; Carvalho, Silva, Moreira, Simoes & Sa, 2011; Imai, Werthessen, Subramanyam, LeQuesne, Soloway & Kanisawa, 1980; Kandutsch, Chen & Heiniger, 1978) and hypocholesterolemia in animals (Aramaki, Kobayashi, Imai, Kikuchi, Matsukawa & Kanazawa, 1967). As opposed to other oxysterols such as 25-hydroxycholesterol, CT is not an inhibitor of the HMG-coA reductase, the rate-controlling enzyme of the cholesterol pathway, (Cavenee, Gibbons, Chen & Kandutsch, 1979). This hypocholesterolemic property of CT might be due to its inhibition of post-lanosterol cholesterogenic enzymes such as DHCR7 (Witiak, Parker, Dempsey & Ritter, 1971) and C4 lanosterol demethylase (Scallen, Dhar & Loughran, 1971), and to a modulation of the LXRα/SREBP2 axis (Lin et al., 2013). CT stimulates phospholipid biosynthesis and CTP-phosphocholine Cytidyltransferase in mammalian cells (Mahfouz, Smith, Zhou & Kummerow, 1996). CT was shown to inhibit osteoblastic differentiation and the induction of bone marrow stromal cell apoptosis (Liu, Yuan, Xu, Wang & Zhang, 2005). CT was also reported to suppress prostate cancer cell proliferation, migration and invasion (Lin et al., 2013), and to inhibit voltage-gated sodium channels (Tang et al., 2015; Tang et al., 2018). CT displays

chaperone properties for the Niemann-Pick C1 protein, which is an intracellular sterol transporter (Ohgane, Karaki, Noguchi-Yachide, Dodo & Hashimoto, 2014)and is also a blood marker for Niemann-Pick C1 Disease (Porter et al., 2010). This illustrates the fact that CT displays certain biological properties.

ChEH was characterized in 2010 as being carried out by a multiproteinaceous hetero-oligomeric complex. This complex includes enzymes involved in the late stages of cholesterol biosynthesis (de Medina, Paillasse, Segala, Poirot & Silvente-Poirot, 2010). ChEH is composed of the 3β-hydroxyterol-Δ8-Δ7-isomerase (D8D7I) and of the 3β-hydroxyterol-Δ7-reductase (DHCR7). D8D7I also known as the emopamyl binding protein (EBP) is the catalytic subunit, and DHCR7 is a regulatory subunit of ChEH (Fig 3A). ChEH was characterized as a pharmacological target of the antitumour drugs tamoxifen and tesmilifene (de Medina, Paillasse, Segala, Poirot & Silvente-Poirot, 2010; de Medina et al., 2013; Segala et al., 2013; Sola et al., 2013). The different structural classes of drugs that inhibit the ChEH activity were also found to be inhibitors of EBP and/or DHCR7 leading to the accumulation of Δ8- and/or Δ7-cholesterol intermediates in the biosynthesis of cholesterol (Kedjouar et al., 2004; Korade et al., 2016; Segala et al., 2017; Silvente-Poirot & Poirot, 2012) (Fig 3B). Δ8-cholesterol precursors such as zymostenol (cholest-8-ene-3β-ol) were shown to induce BC cell arrest in the G1 phase of the cell cycle (Payre et al., 2008), lysosome biogenesis and autophagy (de Medina et al., 2009; de Medina, Silvente-Poirot & Poirot, 2009; Segala et al., 2017; Sola et al., 2013). Inhibition of EBP has been shown to enhance oligodendrocyte formation and myelination (Allimuthu et al., 2019; Hubler et al., 2018) suggesting that Δ8 intermediates in cholesterol biosynthesis play a role in cell differentiation. Truncated APC-selective inhibitors (TASIN) including EBP and DHCR7 inhibitors that are under development for colorectal cancer treatment applications (Cully, 2016; Theodoropoulos et al., 2020; Wang, Zhang, Morlock, Williams, Shay & De Brabander, 2019; Zhang, Kim, Luitel & Shay, 2018; Zhang et al., 2016). Interestingly, Δ8- and Δ7-sterols including zymostenol are unstable sterols that are prone to autoxidation (Kedjouar et al., 2004; Lamberson, Muchalski, McDuffee, Tallman, Xu & Porter, 2017; Payre et al., 2008; Porter, Xu & Pratt, 2020) to produce B-ring oxysterols (Lamberson, Muchalski, McDuffee, Tallman, Xu & Porter, 2017). On the other hand, B-ring oxysterols are known as endogenous ChEH inhibitors (de Medina, Paillasse, Segala, Poirot & Silvente-Poirot, 2010). This and the fact that EBP carries the ChEH activity, suggests that it will be important to consider these parameters in the cell differentiation effects of TASIN compounds especially if they are developed for clinical applications. The recent elucidation of the EBP structure complexed with tamoxifen (Long, Hassan, Thompson, McDonald, Wang & Li, 2019) by X-ray crystallography will encourage structure-function studies to identify amino acid residues responsible for ChEH activity.

In the clinic, Kaplan-Meier analyses of several BC transcriptome patient datasets showed that EBP and DHCR7 expression were positively correlated and were overexpressed in all BC subtypes compared to normal breast tissue (Voisin et al., 2017). In addition, it was reported that high levels of expression of EBP and DHCR7 were associated with a lower survival rate of patients (Voisin et al., 2017). ChEH can therefore represent an interesting target for the development of anticancer compounds which deserves further exploration with regard to redifferentiation therapies (Bizzarri, Giuliani, Cucina & Minini, 2020).

3.2. Oncosterone

3.2.1. Oncosterone an oncometabolite and a tumour promoter in BC

Studies on the 5,6-ECs metabolism in BC cell lines have shown that they are metabolized into CT by ChEH and that CT was subsequently transformed into a substance structurally characterized as the 6-oxo-cholestan-3β,5α-diol (OCDO, Oncosterone) (Fig 1C). Since ChEH is known to be inhibited by antiproliferative substances (de Medina, Paillasse, Segala, Poirot & Silvente-Poirot, 2010; Silvente-Poirot & Poirot, 2012), and that CT has been reported to display certain carcinogenic properties after oxydation (Cheng, Kang, Shih, Lo & Wang, 2005). This suggested that oncosterone could promote BC cell proliferation. Indeed, oncosterone was shown to dose-dependently stimulate the proliferation of human and mouse ER(+) and TNBC cell lines suggesting a receptor-mediated effect of oncosterone. Importantly, oncosterone was shown to stimulate the growth of BC tumours *in vivo* . The inhibition of ChEH inhibited BC tumour growth, and oncosterone addition rescued tumour growth. Together these data establish that oncosterone is a tumour promoter.

Thus blocking oncosterone biosynthesis or neutralising oncosterone receptor(s) provides a new rational for the pharmacological control of BC growth which is extremely attractive especially for TNBC for which no targeted therapy has been developed to date.

Only a few recent studies have included the dosage of oncosterone (OCDO) in addition to other oxysterols (Dalenc et al., 2017; Iuliano et al., 2015; Soules et al., 2017). Since 5,6-EC and CT have been linked to several pathologies such as cancer and Niemann-Pick C1 disease, it will be interesting to include the dosage of oncosterone in future studies to determine its potential role in these pathologies

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Oncosterone (OCDO) is an enzymatic oxidation product of CT and is not an autoxidation product of cholesterol (Poirot, Soules, Mallinger, Dalenc & Silvente-Poirot, 2018). Oncosterone was shown to be generated from CT by the 3β-hydroxysteroid dehydrogenase type 2 (HSD11B2) (Voisin et al., 2017). HSD11B2 is expressed in BC from patients and BC cell lines, while weakly or not expressed in normal breast tissue, and the expression of HSD11B2 paralleled OCDO levels, establishing that oncosterone is an oncometabolite (Voisin et al., 2017). HSD11B2's primary function is the inactivation of cortisol by oxidation to produce cortisone (Chapman, Holmes & Seckl, 2013). This reaction is known to be reverted by HSD11B1, and HSD11B1 was earlier reported to reduce 7-ketocholesterol into 7β-hydroxycholesterol (Mitic et al., 2013). HSD11B1 was also found to catalyse the conversion of OCDO into CT (Voisin et al., 2017). HSD11B1 requires hexose-6-phosphate dehydrogenase (H6PD) for the regeneration of its cofactor NADPH in order to achieve a reductase activity. In the absence of H6PD, it was reported that HSD11B1 functions as an oxidase like HSD11B2 (Odermatt & Klusonova, 2015). HSD11B1 and H6PD were found weakly or not expressed in the breast and in BC as well as in BC cell lines showing that the eventual equilibrium of CT oncosterone conversion was completely displaced in favour of oncosterone production in BC (Voisin et al., 2017) .

The knock down of HSD11B2 in the ER(+)-BC cell line MCF-7 blocks the endogenous production of oncosterone, cell proliferation and clonogenicity *in vitro* and tumour growth *in vivo* . Addition of exogenous oncosterone reverts the effect of HSD11B2 knock down (Voisin et al., 2017). These data suggest that the oncosterone biosynthesis enzyme HSD11B2 may constitute a target for the development of inhibitors in BC treatment. Natural and synthetic inhibitors of HSD11B2 have been described and it would be of interest to define their impact on OCDO biosynthesis (Latif, Pardo, Hardy & Morris, 2005; Morris, Latif, Hardy & Brem, 2007; Vitku et al., 2016; Zhou, Ye, Wu, Ye & Chen, 2017). An open question and an exciting challenge will be to develop HSD11B2 inhibitors that block oncosterone biosynthesis with little or no impact on the cortisol metabolism to limit side effects such as hypertension. Such inhibitors could display anticancer properties in all BC-subtypes, offering new targeted therapies for TN BC. Such a hypothesis should deserve further exploration.

Analysis of HSD11B2 expression in patient BC samples showed that it was significantly higher than in normal matched tissue. Kaplan-Meier analyses of several BC transcriptome patient datasets showed that high levels of HSD11B2 mRNA were associated with a poor prognosis. High expression levels of both ChEH subunits and HSD11B2 were associated with the risk highest risk of patient mortality (Voisin et al., 2017). These data strongly support the development of ChEH and HSD11B2 inhibitors for BC treatment.

3.2.3. Oncosterone is a ligand and a modulator of nuclear receptors.

Glucocorticoid receptor (GR) and oxysterol receptors LXR have been identified as oncosterone receptors. Oncosterone was shown to activate the nuclearization of GR in MDA-MB231 TN BC cells as observed with cortisol. Oncosterone was found to antagonize cortisol in its stimulation of the expression of two GR-dependent SGK1 and MKP1 genes. Gene reporter assays further confirmed that oncosterone did not modulate other oxysterol receptors such as RORs or FXR (Voisin et al., 2017), and binding assays showed that oncosterone interacted with LXRs and GR. GR knock down in BC cell lines induced a loss of the proliferative effects of oncosterone. A similar effect was also observed with GR ligands such as mifepristone (RU38486) and dexamethasone showing that GR mediates oncosterone mitogenicity. Structural differences

between cortisol and oncosterone are key elements that drive GR activation differently in terms of gene regulation and cell proliferation. This suggests that conformational modifications of GR complexes will affect differently the recruitment of co-regulators, GR dimerization states (Kadmiel & Cidlowski, 2013; Vandewalle, Luypaert, De Bosscher & Libert, 2018) and even GR heterodimerization with other nuclear receptors (De Bosscher, Desmet, Clarisse, Estebanez-Perpina & Brunsveld, 2020).

In contrast, the LXR knock down did not affect OCDO-mediated BC cell proliferation (Voisin et al., 2017). Since LXR was found to control 27HC invasiveness, it was postulated that LXR could mediate the pro-invasive effects of oncosterone. Further investigations will be required to show that genetic and pharmacological inhibition of LXR can block the effects of oncosterone. These observations, in addition to the links that have already established between GR, glucocorticoids and BC (Kanai et al., 2020; Obradovic et al., 2019; Perez Kerkvliet et al., 2020; Tonsing-Carter et al., 2019), highlight the pharmacological interest of GR targeting for the control of BC development. The discovery of oncosterone as an endogenous GR ligand in BC will add a new rational to consider the implication of GR in BC development. Together these data underline the pharmacological importance of GR and LXR as effector of oncosterone and give a new rational for the targeted therapy of BC (Fig 3).

3.3. Dendrogenin A (DDA)

3.3.1. DDA is enzymatically produced in normal tissues

Researchers have hypothesized that 5,6-EC could be metabolized into inducers of cell-differentiation such as dendrogenin A for several reasons: 1) ChEH contains the microsomal anti-oestrogen binding site (AEBS) which controls BC cell death and differentiation, showing that 5,6-EC and 5,6-EC metabolites mediated BC cell differentiation(de Medina et al., 2009; Payre et al., 2008; Segala et al., 2013; Silvente-Poirot & Poirot, 2012); 2) ChEH subunits EBP and DHCR7 are required for human development programs (Herman, 2003; Porter & Herman, 2011) opening up the possibilities that ChEH activity and 5,6-EC metabolites could contribute to development programs as inducers of cell differentiation; 3) at the chemical level, 5,6α-EC was found to be reactive towards nucleophilic groups such as mercaptant and amines only in the presence of a catalyst and give a single conjugated stereoisomer amongst multiple possibilities(Silvente-Poirot, de Medina, Record & Poirot, 2016). This supported the possible existence of a new metabolic branch based on 5,6α-EC stereospecific conjugation since homochirality is a common occurence in life (Blackmond, 2019; Fujii & Saito, 2004); 4) the AEBS binds 5,6-EC and nucleophilic substances such as histamine or polyamines opening up a possibility of conjugation reaction at the ChEH level (Leignadier, Dalenc, Poirot & Silvente-Poirot, 2017). The chemical synthesis of 5,6α-EC conjugation products with certain biogenic amines including histamine and polyamine has been performed(de Medina, Paillasse, Payre, Silvente-Poirot & Poirot, 2009). As expected, 5,6α-EC conjugates displayed strong cell differentiation properties *in vitro* (de Medina, Paillasse, Payre, Silvente-Poirot & Poirot, 2009). Amongst these conjugates, the histaminic adduct called dendrogenin A (DDA), was found to induce cell differentiation properties in pluripotent undifferentiated cells (de Medina, Paillasse, Payre, Silvente-Poirot & Poirot, 2009) and normal progenitor cells (Khalifa, de Medina, Erlandsson, El-Seedi, Silvente-Poirot & Poirot, 2014). DDA was reported to induce cell differentiation and death in mouse and human cells of various tissue origins (Bauriaud-Mallet et al., 2019; de Medina, Paillasse, Payre, Silvente-Poirot & Poirot, 2009; de Medina et al., 2013). More specifically DDA was found to induce BC cell re-differentiation to produce breast epithelial-like cells (Bauriaud-Mallet et al., 2019; de Medina, Paillasse, Payre, Silvente-Poirot & Poirot, 2009; de Medina et al., 2013). The production of chemical tools such as radio- and deuterium-labelled DDA, and the development of analytical methods has made possible the detection and quantification of DDA in mammalian tissues which has led to the demonstration that DDA exists in mammals (de Medina et al., 2013; Soules et al., 2019). DDA can be formed in mammalian tissue from 5,6α-EC and histamine and this reaction was shown to require a proteinaceous enzyme that remains to be identified (de Medina et al., 2013). Cultured normal breast epithelial cells produced DDA, while DDA was not detectable in BC cell lines (de Medina et al., 2013). This suggests that a deregulation in DDA metabolism could occur during oncogenesis and it may result from an alteration of the DDA metabolism in epithelial cells. This was confirmed in patients by comparing DDA levels in BC tumours and normal

adjacent tissues (de Medina et al., 2013). Interestingly, thanks to the improvement of analytical methods for DDA quantification, dendrogenin B which is a 5,6α-EC conjugate of spermidine with neurodifferentiation and neurone regeneration properties (Dalenc, Poirot & Silvente-Poirot, 2015; de Medina, Paillasse, Payre, Silvente-Poirot & Poirot, 2009; Fransson, de Medina, Paillasse, Silvente-Poirot, Poirot & Ulfendahl, 2015; Khalifa, de Medina, Erlandsson, El-Seedi, Silvente-Poirot & Poirot, 2014) was found to exist as a mammalian metabolite (Soules et al., 2019). This showed that a new metabolic branch existed in the cholesterol pathway and this branch is centered around 5,6α-EC conjugation and produces inducers of cell differentiation.

3.3.2. DDA is an oxysterol with chemopreventive and oncosuppressive properties

DDA is a mammalian metabolite present in mammalian tissue including the breast, the levels of which decreased drastically during oncogenesis. Tested on preclinical models of BC in immunocompetent mice, DDA was found to inhibit the growth of aggressive syngeneic tumours at low doses (0.035 µg/kg). This effect was observed on a chemopreventive setting and after 10 days of post implantation of BC cells in mice. Analysis of tumours from treated animals showed that cancer cells harboured features of normal epithelial mammary cells, and that tumours were infiltrated with T lymphocytes and dendritic cells (de Medina et al., 2013). This data suggested that the immune system could contribute to the anti-cancer action of DDA. This was further supported by the fact that the same treatment with the same dose of DDA was inefficient to cure or prevent BC development in immunodepressed nude mice (Sandrine Silvente-Poirot et al, unpublished observations). It has been reported that DDA inhibits ChEH (de Medina et al., 2013) and OCDO production in BC cells (Voisin et al., 2017). This explains the control of OCDO mitogenicity *in vitro* and *in vivo* in BC tumors implanted in immunocompromised mice at DDA doses that induced ChEH inhibition (Voisin et al., 2017). This confirmed the existence of a metabolic balance involving on 5,6-EC and controlled by ChEH (Silvente-Poirot & Poirot, 2014; Voisin et al., 2017). Whether the DDA/oncosterone ratio reflects a pre-cancerous or a cancerous situation in the breast deserves further investigations. Together these data suggest that the use of DDA could be an interesting alternative strategy for BC treatment through a metabolic deficiency complementation- and redifferentiation-therapy.

3.3.3. DDA is an LXR modulator that induces lethal autophagy in cancer cells.

DDA was found to induce cell death and differentiation in mouse and human cancer cells. Further work was done to study the molecular mechanisms involved in DDA cytotoxicity. It was found that DDA induced cytotoxicity in cultured cancer cells in a dose- and time-dependent manner suggesting the implication of a receptor, death was not apoptotic, and required gene expression and protein neosynthesis (Segala et al., 2017), as observed with other ChEH inhibitors (de Medina et al., 2009; Leignadier, Dalenc, Poirot & Silvente-Poirot, 2017; Payre et al., 2008). However, as opposed to other ChEH inhibitors, DDA cytoxicity was not inhibited by anti-oxidants because 5,6-EC do not accumulate and are not second messengers of DDA (de Medina et al., 2009; Leignadier, Dalenc, Poirot & Silvente-Poirot, 2017; Payre et al., 2008; Segala et al., 2017). Studies on the DDA molecular mechanism of cytotoxicity showed that it induced lethal autophagy (Segala et al., 2017) as opposed to other ChEH inhibitors that induced a protective autophagy (Leignadier, Dalenc, Poirot & Silvente-Poirot, 2017). ChEH contributed to autophagy through the accumulation of pro-autophagic Δ8-sterols due the inhibition of its EBP subunit (de Medina, Silvente-Poirot & Poirot, 2009; Silvente-Poirot, Segala, Poirot & Poirot, 2018). DDA was found to be a ligand of LXRα and LXRβ receptors as opposed to other ChEH inhibitors (Segala et al., 2013). Genetic and pharmacological evidences has been given to confirm that LXRβ was required for lethal autophagy. Although DDA was a poor modulator of canonical LXR-dependent genes, it activated via LXRβ the transcription of genes encoding master regulators of lysosome biogenesis and autophagy such as the transcription factor EB (TFEB) (Segala et al., 2017). DDA is, to our knowledge, the first example of an LXR ligand that induces TFEB expression. Such an effect has not been reported to date by other cytotoxic natural LXR ligands belonging to the oxysterols family. It was further established that DDA did not modulate other common nuclear receptors establishing its selectivity to LXRs (Segala et al., 2017).

It was next established that DDA, at cytotoxic doses, actively inhibit the growth of human and mouse tumours implanted into immunocompromized mice with different administration modes, including primary

tumours from patients (Segala et al., 2017). Knock down of LXR receptors in cancer cells using small interfering RNA (siRNA) or single hairpin RNA (shRNA) approaches strongly impaired DDA induction of autophagy and its anticancer activities *in vitro* and *in vivo* (Segala et al., 2017). This showed that LXRβ was required to control the anticancer activity of DDA. The particular cell death induced by DDA compared to conventional ChEH inhibitors and LXR modulators is probably due to its specific LXRβ-dependent regulation of gene expression and induction of Δ8-sterols accumulation as discussed earlier (Poirot & Silvente-Poirot, 2018). Moreover, this suggests that the control of autophagy might be a specific physiological function of the LXRβ isoform when activated by specific ligands such as DDA. This data shows the importance of LXR in cancer cells as targets for anticancer strategies. Due to the established importance of LXR in the tumour micro-enrironment (Ma & Nelson, 2019) and in the control of the immune response (Fessler, 2016), it will be interesting to determine how autophagy and LXR from immune cells can contribute to the anti-tumour action of DDA observed in immunocompetent mice (de Medina et al., 2013). It would be of interest to determine the impact of combination treatments of DDA with other chemotherapeutic agents acting through different molecular mechanisms, and in particular with drugs that have a limited therapeutic outcome or resistance through the induction of protective autophagy.

4. Conclusion:

We report herein that 5,6-ECs are at the center of a newly discovered metabolic branch that controls carcinogenesis (Fig 4). The identification of oncosterone, its biosynthetic pathway and its effectors, highlights the existence of interesting targets for the development of anticancer drugs applicable for TNBC treatment (Fig 5). This is reminiscent of the development of targeted therapeutic strategies against the tumour promoter 17β-oestradiol for ER(+)-BC treatment (Simpson & Santen, 2015). On the other hand, studies of the 5,6α-EC metabolism in normal tissues led to the identification of DDA. Surprisingly DDA was found to display tumour suppressor properties on BC through the activation of cell differentiation and death programs, and the inhibition of oncosterone biosynthesis, highlighting the existence of a metabolic balance between the tumour promoteur oncosterone and the tumour suppressor DDA. This opens up new options for the development of new anticancer agents targeting the oncosterone pathway in BC and new strategies for the chemoprevention of BC. This also provides a new rational at the molecular level to study potential relationships that may exist between cholesterol, diet and BC oncogenesis programs (Silvente-Poirot, Dalenc & Poirot, 2018).

References:

Allimuthu D, Hubler Z, Najm FJ, Tang H, Bederman I, Seibel W*, et al.* (2019). Diverse Chemical Scaffolds Enhance Oligodendrocyte Formation by Inhibiting CYP51, TM7SF2, or EBP. Cell Chem Biol 26**:** 593-599 e594.

Ansari GA, Walker RD, Smart VB, & Smith LL (1982). Further investigations of mutagenic cholesterol preparations. Food Chem Toxicol 20**:** 35-41.

Aramaki Y, Kobayashi T, Imai Y, Kikuchi S, Matsukawa T, & Kanazawa K (1967). Biological studies of cholestane-3beta,5alpha,6beta-triol and its derivatives. 1. Hypocholesterolemic effects in rabbits, chickens and rats on atherogenic diets. J Atheroscler Res 7**:** 653-669.

Aringer L, & Eneroth P (1974). Formation and metabolism in vitro of 5,6-epoxides of cholesterol and beta-sitosterol. J Lipid Res 15**:** 389-398.

Baek AE, Yu YA, He S, Wardell SE, Chang CY, Kwon S*, et al.*(2017). The cholesterol metabolite 27 hydroxycholesterol facilitates breast cancer metastasis through its actions on immune cells. Nat Commun 8**:** 864.

Bauriaud-Mallet M, Vija-Racaru L, Brillouet S, Mallinger A, de Medina P, Rives A*, et al.* (2019). The cholesterol-derived metabolite dendrogenin A functionally reprograms breast adenocarcinoma and undifferentiated thyroid cancer cells. J Steroid Biochem Mol Biol 192**:** 105390.

Berrodin TJ, Shen Q, Quinet EM, Yudt MR, Freedman LP, & Nagpal S (2010). Identification of 5alpha, 6alpha-epoxycholesterol as a novel modulator of liver X receptor activity. Mol Pharmacol 78**:**1046-1058.

Bizzarri M, Giuliani A, Cucina A, & Minini M (2020). Redifferentiation therapeutic strategies in cancer. Drug Discov Today 25**:**731-738.

Black HS, & Douglas DR (1973). Formation of a carcinogen of natural origin in the etiology of ultraviolet light-induced carcinogenesis. Cancer Res 33**:** 2094-2096.

Black HS, & Lo WB (1971). Formation of a carcinogen in human skin irradiated with ultraviolet light. Nature 234**:** 306-308.

Blackmond DG (2019). The Origin of Biological Homochirality. Cold Spring Harb Perspect Biol 11**:** a002147.

Board PG, & Menon D (2013). Glutathione transferases, regulators of cellular metabolism and physiology. Biochim Biophys Acta 1830**:**3267-3288.

Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, & Jemal A (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 68**:** 394-424.

Carvalho JF, Silva MM, Moreira JN, Simoes S, & Sa e Melo ML (2010). Sterols as anticancer agents: synthesis of ring-B oxygenated steroids, cytotoxic profile, and comprehensive SAR analysis. J Med Chem 53**:** 7632-7638.

Carvalho JF, Silva MM, Moreira JN, Simoes S, & Sa EMML (2011). Selective cytotoxicity of oxysterols through structural modulation on rings A and B. Synthesis, in vitro evaluation, and SAR. J Med Chem 54**:** 6375-6393.

Cavenee WK, Gibbons GF, Chen HW, & Kandutsch AA (1979). Effects of various oxygenated sterols on cellular sterol biosynthesis in Chinese hamster lung cells resistant to 25-hydroxycholesterol. Biochim Biophys Acta 575**:** 255-265.

Chan JT, & Black HS (1974). Skin carcinogenesis: cholesterol-5alpha,6alpha-epoxide hydrase activity in mouse skin irradiated with ultraviolet light. Science 186**:** 1216-1217.

Chang CC, Jone C, Trosko JE, Peterson AR, & Sevanian A (1988). Effect of cholesterol epoxides on the inhibition of intercellular communication and on mutation induction in Chinese hamster V79 cells. Mutat Res 206**:** 471-478.

Chapman K, Holmes M, & Seckl J (2013). 11beta-hydroxysteroid dehydrogenases: intracellular gate-keepers of tissue glucocorticoid action. Physiol Rev 93**:** 1139-1206.

Cheng YW, Kang JJ, Shih YL, Lo YL, & Wang CF (2005). Cholesterol-3-beta, 5-alpha, 6-beta-triol induced genotoxicity through reactive oxygen species formation. Food Chem Toxicol 43**:**617-622.

Cully M (2016). Anticancer drugs: Exploiting a weakness in colorectal cancers. Nat Rev Drug Discov 15**:** 820-821.

Dalenc F, Iuliano L, Filleron T, Zerbinati C, Voisin M, Arellano C*, et al.* (2017). Circulating oxysterol metabolites as potential new surrogate markers in patients with hormone receptor-positive breast cancer: Results of the OXYTAM study. J Steroid Biochem Mol Biol 169**:** 210-218.

Dalenc F, Poirot M, & Silvente-Poirot S (2015). Dendrogenin A: A Mammalian Metabolite of Cholesterol with Tumor Suppressor and Neurostimulating Properties. Curr Med Chem 22**:** 3533-3549.

Danhier P, Banski P, Payen VL, Grasso D, Ippolito L, Sonveaux P*, et al.* (2017). Cancer metabolism in space and time: Beyond the Warburg effect. Biochim Biophys Acta Bioenerg 1858**:** 556-572.

De Bosscher K, Desmet SJ, Clarisse D, Estebanez-Perpina E, & Brunsveld L (2020). Nuclear receptor crosstalk - defining the mechanisms for therapeutic innovation. Nat Rev Endocrinol in press.

de Medina P, Paillasse MR, Payre B, Silvente-Poirot S, & Poirot M (2009). Synthesis of new alkylaminooxysterols with potent cell differentiating activities: identification of leads for the treatment of cancer and neurodegenerative diseases. J Med Chem 52**:**7765-7777.

de Medina P, Paillasse MR, Segala G, Poirot M, & Silvente-Poirot S (2010). Identification and pharmacological characterization of cholesterol-5,6-epoxide hydrolase as a target for tamoxifen and AEBS ligands. Proc Natl Acad Sci U S A 107**:** 13520-13525.

de Medina P, Paillasse MR, Segala G, Voisin M, Mhamdi L, Dalenc F*, et al.* (2013). Dendrogenin A arises from cholesterol and histamine metabolism and shows cell differentiation and anti-tumour properties. Nat Commun 4**:** 1840.

de Medina P, Payre B, Boubekeur N, Bertrand-Michel J, Terce F, Silvente-Poirot S*, et al.* (2009). Ligands of the antiestrogen-binding site induce active cell death and autophagy in human breast cancer cells through the modulation of cholesterol metabolism. Cell Death Differ 16**:** 1372-1384.

de Medina P, Silvente-Poirot S, & Poirot M (2009). Tamoxifen and AEBS ligands induced apoptosis and autophagy in breast cancer cells through the stimulation of sterol accumulation. Autophagy 5**:** 1066-1067.

el-Bayoumy K, Ji BY, Upadhyaya P, Chae YH, Kurtzke C, Rivenson A*, et al.* (1996). Lack of tumorigenicity of cholesterol epoxides and estrone-3,4-quinone in the rat mammary gland. Cancer Res 56**:**1970-1973.

Fessler MB (2016). The Intracellular Cholesterol Landscape: Dynamic Integrator of the Immune Response. Trends Immunol 37**:** 819-830.

Fransson A, de Medina P, Paillasse MR, Silvente-Poirot S, Poirot M, & Ulfendahl M (2015). Dendrogenin A and B two new steroidal alkaloids increasing neural responsiveness in the deafened guinea pig. Front Aging Neurosci 7**:** 145.

Fujii N, & Saito T (2004). Homochirality and life. Chem Rec 4**:**267-278.

Garcia-Estevez L, & Moreno-Bueno G (2019). Updating the role of obesity and cholesterol in breast cancer. Breast Cancer Res 21**:** 35.

Global Burden of Disease Cancer C, Fitzmaurice C, Abate D, Abbasi N, Abbastabar H, Abd-Allah F*, et al.* (2019). Global, Regional, and National Cancer Incidence, Mortality, Years of Life Lost, Years Lived With Disability, and Disability-Adjusted Life-Years for 29 Cancer Groups, 1990 to 2017: A Systematic Analysis for the Global Burden of Disease Study. JAMA Oncol 5**:** 1749-1768.

Goldhirsch A, Wood WC, Coates AS, Gelber RD, Thurlimann B, Senn HJ*, et al.* (2011). Strategies for subtypes–dealing with the diversity of breast cancer: highlights of the St. Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2011. Ann Oncol 22**:** 1736-1747.

Gorzynski Smith J (1984). Synthetically Useful Reactions of Epoxides. Synthesis 1984**:** 629-656.

Grouleff J, Irudayam SJ, Skeby KK, & Schiott B (2015). The influence of cholesterol on membrane protein structure, function, and dynamics studied by molecular dynamics simulations. Biochim Biophys Acta 1848**:** 1783-1795.

Gruenke LD, Wrensch MR, Petrakis NL, Miike R, Ernster VL, & Craig JC (1987). Breast fluid cholesterol and cholesterol epoxides: relationship to breast cancer risk factors and other characteristics. Cancer Res 47**:** 5483-5487.

Herman GE (2003). Disorders of cholesterol biosynthesis: prototypic metabolic malformation syndromes. Hum Mol Genet 12 Spec No 1**:**R75-88.

Huang B, Song B-l, & Xu C (2020). Cholesterol metabolism in cancer: mechanisms and therapeutic opportunities. Nat Metab 2**:** 132-141.

Hubler Z, Allimuthu D, Bederman I, Elitt MS, Madhavan M, Allan KC*, et al.* (2018). Accumulation of 8,9-unsaturated sterols drives oligodendrocyte formation and remyelination. Nature 560**:**372-376.

Imai H, Werthessen NT, Subramanyam V, LeQuesne PW, Soloway AH, & Kanisawa M (1980). Angiotoxicity of oxygenated sterols and possible precursors. Science 207**:** 651-653.

Iuliano L (2011). Pathways of cholesterol oxidation via non-enzymatic mechanisms. Chem Phys Lipids 164**:** 457-468.

Iuliano L, Crick PJ, Zerbinati C, Tritapepe L, Abdel-Khalik J, Poirot M*, et al.* (2015). Cholesterol metabolites exported from human brain. Steroids 99**:** 189-193.

Jordan VC, & Brodie AM (2007). Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. Steroids 72**:** 7-25.

Kadmiel M, & Cidlowski JA (2013). Glucocorticoid receptor signaling in health and disease. Trends Pharmacol Sci 34**:** 518-530.

Kanai A, McNamara KM, Iwabuchi E, Miki Y, Onodera Y, Guestini F*, et al.* (2020). Significance of glucocorticoid signaling in triple-negative breast cancer patients: a newly revealed interaction with androgen signaling. Breast Cancer Res Treat 180**:** 97-110.

Kandutsch AA, Chen HW, & Heiniger HJ (1978). Biological activity of some oxygenated sterols. Science 201**:** 498-501.

Kedjouar B, de Medina P, Oulad-Abdelghani M, Payre B, Silvente-Poirot S, Favre G*, et al.* (2004). Molecular characterization of the microsomal tamoxifen binding site. J Biol Chem 279**:**34048-34061.

Khalifa SA, de Medina P, Erlandsson A, El-Seedi HR, Silvente-Poirot S, & Poirot M (2014). The novel steroidal alkaloids dendrogenin A and B promote proliferation of adult neural stem cells. Biochem Biophys Res Commun 446**:** 681-686.

Kodani SD, & Hammock BD (2015). The 2014 Bernard B. Brodie award lecture-epoxide hydrolases: drug metabolism to therapeutics for chronic pain. Drug Metab Dispos 43**:** 788-802.

Korade Z, Kim HY, Tallman KA, Liu W, Koczok K, Balogh I*, et al.*(2016). The Effect of Small Molecules on Sterol Homeostasis: Measuring 7-Dehydrocholesterol in Dhcr7-Deficient Neuro2a Cells and Human Fibroblasts. J Med Chem 59**:** 1102-1115.

Kuzu OF, Noory MA, & Robertson GP (2016). The Role of Cholesterol in Cancer. Cancer Res 76**:** 2063-2070.

Lamberson CR, Muchalski H, McDuffee KB, Tallman KA, Xu L, & Porter NA (2017). Propagation rate constants for the peroxidation of sterols on the biosynthetic pathway to cholesterol. Chem Phys Lipids 207**:**51-58.

Latif SA, Pardo HA, Hardy MP, & Morris DJ (2005). Endogenous selective inhibitors of 11beta-hydroxysteroid dehydrogenase isoforms 1 and 2 of adrenal origin. Mol Cell Endocrinol 243**:** 43-50.

Le Cornet C, Walter B, Sookthai D, Johnson TS, Kuhn T, Herpel E*, et al.* (2020). Circulating 27-hydroxycholesterol and breast cancer tissue expression of CYP27A1, CYP7B1, LXR-beta, and ERbeta: results from the EPIC-Heidelberg cohort. Breast Cancer Res 22**:** 23.

Leignadier J, Dalenc F, Poirot M, & Silvente-Poirot S (2017). Improving the efficacy of hormone therapy in breast cancer: The role of cholesterol metabolism in SERM-mediated autophagy, cell differentiation and death. Biochem Pharmacol 144**:** 18-28.

Lin CY, Huo C, Kuo LK, Hiipakka RA, Jones RB, Lin HP*, et al.*(2013). Cholestane-3beta, 5alpha, 6beta-triol suppresses proliferation, migration, and invasion of human prostate cancer cells. PLoS One 8**:** e65734.

Liu H, Yuan L, Xu S, Wang K, & Zhang T (2005). Cholestane-3beta,5alpha,6beta-triol inhibits osteoblastic differentiation and promotes apoptosis of rat bone marrow stromal cells. J Cell Biochem 96**:** 198-208.

Lo W, & Black HS (1973). Inhibition of carcinogen formation in skin irradiated with ultraviolet light. Nature 246**:** 489-491.

Lo WB, & Black HS (1972). Formation of cholesterol-derived photoproducts in human skin. J Invest Dermatol 58**:** 278-283.

Long T, Hassan A, Thompson BM, McDonald JG, Wang J, & Li X (2019). Structural basis for human sterol isomerase in cholesterol biosynthesis and multidrug recognition. Nat Commun 10**:** 2452.

Lu DL, Le Cornet C, Sookthai D, Johnson TS, Kaaks R, & Fortner RT (2019). Circulating 27-Hydroxycholesterol and Breast Cancer Risk: Results From the EPIC-Heidelberg Cohort. J Natl Cancer Inst 111**:** 365-371.

Luo J, Yang H, & Song BL (2020). Mechanisms and regulation of cholesterol homeostasis. Nat Rev Mol Cell Biol 21**:** 225-245.

Ma L, & Nelson ER (2019). Oxysterols and nuclear receptors. Mol Cell Endocrinol 484**:** 42-51.

Mahfouz MM, Smith TL, Zhou Q, & Kummerow FA (1996). Cholestane-3 beta, 5 alpha, 6 beta-triol stimulates phospholipid synthesis and CTP-phosphocholine cytidyltransferase in cultured LLC-PK cells. Int J Biochem Cell Biol 28**:** 739-750.

Mitic T, Shave S, Semjonous N, McNae I, Cobice DF, Lavery GG*, et al.* (2013). 11beta-Hydroxysteroid dehydrogenase type 1 contributes to the balance between 7-keto- and 7-hydroxy-oxysterols in vivo. Biochem Pharmacol 86**:** 146-153.

Moresco MA, Raccosta L, Corna G, Maggioni D, Soncini M, Bicciato S*, et al.* (2018). Enzymatic Inactivation of Oxysterols in Breast Tumor Cells Constraints Metastasis Formation by Reprogramming the Metastatic Lung Microenvironment. Front Immunol 9**:** 2251.

Morisseau C (2013). Role of epoxide hydrolases in lipid metabolism. Biochimie 95**:** 91-95.

Morisseau C, & Hammock BD (2005). Epoxide hydrolases: mechanisms, inhibitor designs, and biological roles. Annu Rev Pharmacol Toxicol 45**:** 311-333.

Morris DJ, Latif SA, Hardy MP, & Brem AS (2007). Endogenous inhibitors (GALFs) of 11beta-hydroxysteroid dehydrogenase isoforms 1 and 2: derivatives of adrenally produced corticosterone and cortisol. J Steroid Biochem Mol Biol 104**:** 161-168.

Nashed NT, Michaud DP, Levin W, & Jerina DM (1985). Properties of liver microsomal cholesterol 5,6-oxide hydrolase. Arch Biochem Biophys 241**:** 149-162.

Nelson ER (2018). The significance of cholesterol and its metabolite, 27-hydroxycholesterol in breast cancer. Mol Cell Endocrinol 466**:** 73-80.

Nelson ER, Wardell SE, Jasper JS, Park S, Suchindran S, Howe MK*, et al.* (2013). 27-Hydroxycholesterol links hypercholesterolemia and breast cancer pathophysiology. Science 342**:** 1094-1098.

Nes WD (2011). Biosynthesis of cholesterol and other sterols. Chem Rev 111**:** 6423-6451.

Newman JW, Morisseau C, & Hammock BD (2005). Epoxide hydrolases: their roles and interactions with lipid metabolism. Prog Lipid Res 44**:** 1-51.

Obradovic MMS, Hamelin B, Manevski N, Couto JP, Sethi A, Coissieux MM*, et al.* (2019). Glucocorticoids promote breast cancer metastasis. Nature 567**:** 540-544.

Odermatt A, & Klusonova P (2015). 11β-Hydroxysteroid dehydrogenase 1: Regeneration of active glucocorticoids is only part of the story. J Steroid Biochem Mol Biol 151**:** 85-92.

Ohgane K, Karaki F, Noguchi-Yachide T, Dodo K, & Hashimoto Y (2014). Structure-activity relationships of oxysterol-derived pharmacological chaperones for Niemann-Pick type C1 protein. Bioorg Med Chem Lett 24**:** 3480-3485.

Paillasse MR, Saffon N, Gornitzka H, Silvente-Poirot S, Poirot M, & de Medina P (2012). Surprising unreactivity of cholesterol-5,6-epoxides towards nucleophiles. J Lipid Res 53**:** 718-725.

Parker RE, & Isaacs NS (1959). Mechanisms of epoxide reactions. Chem Rev 59**:** 737-799.

Pavlova NN, & Thompson CB (2016). The Emerging Hallmarks of Cancer Metabolism. Cell Metab 23**:** 27-47.

Payre B, de Medina P, Boubekeur N, Mhamdi L, Bertrand-Michel J, Terce F*, et al.* (2008). Microsomal antiestrogen-binding site ligands induce growth control and differentiation of human breast cancer cells through the modulation of cholesterol metabolism. Mol Cancer Ther 7**:** 3707-3718.

Perez Kerkvliet C, Dwyer AR, Diep CH, Oakley RH, Liddle C, Cidlowski JA*, et al.* (2020). Glucocorticoid receptors are required effectors of TGFbeta1-induced p38 MAPK signaling to advanced cancer phenotypes in triple-negative breast cancer. Breast Cancer Res 22**:** 39.

Peterson AR, Peterson H, Spears CP, Trosko JE, & Sevanian A (1988). Mutagenic characterization of cholesterol epoxides in Chinese hamster V79 cells. Mutat Res 203**:** 355-366.

Petrakis NL, Gruenke LD, & Craig JC (1981). Cholesterol and cholesterol epoxides in nipple aspirates of human breast fluid. Cancer Res 41**:** 2563-2565.

Poirot M, & Silvente-Poirot S (2013). Cholesterol-5,6-epoxides: chemistry, biochemistry, metabolic fate and cancer. Biochimie 95**:** 622-631.

Poirot M, & Silvente-Poirot S (2018). The tumor-suppressor cholesterol metabolite, dendrogenin A, is a new class of LXR modulator activating lethal autophagy in cancers. Biochem Pharmacol 153**:** 75-81.

Poirot M, Soules R, Mallinger A, Dalenc F, & Silvente-Poirot S (2018). Chemistry, biochemistry, metabolic fate and mechanism of action of 6-oxo-cholestan-3beta,5alpha-diol (OCDO), a tumor promoter and cholesterol metabolite. Biochimie 153**:** 139-149.

Porter FD, & Herman GE (2011). Malformation syndromes caused by disorders of cholesterol synthesis. J Lipid Res 52**:** 6-34.

Porter FD, Scherrer DE, Lanier MH, Langmade SJ, Molugu V, Gale SE*, et al.* (2010). Cholesterol Oxidation Products Are Sensitive and Specific Blood-Based Biomarkers for Niemann-Pick C1 Disease. Sci Transl Med 2**:** 56ra81.

Porter NA, Xu L, & Pratt DA (2020). Reactive Sterol Electrophiles: Mechanisms of Formation and Reactions with Proteins and Amino Acid Nucleophiles. Chemistry 2**:** 390-417.

Raccosta L, Fontana R, Maggioni D, Lanterna C, Villablanca EJ, Paniccia A*, et al.* (2013). The oxysterol-CXCR2 axis plays a key role in the recruitment of tumor-promoting neutrophils. J Exp Med 210**:**1711-1728.

Raccosta L, Fontana R, Traversari C, & Russo V (2013). Oxysterols recruit tumor-supporting neutrophils within the tumor microenvironment: The many facets of tumor-derived oxysterols. Oncoimmunology 2**:**e26469.

Scallen TJ, Dhar AK, & Loughran ED (1971). Isolation and characterization of C-4 methyl intermediates in cholesterol biosynthesis after treatment of rat liver in vitro with cholestan-3 beta, 5 alpha,6 beta-triol. J Biol Chem 246**:** 3168-3174.

Schroepfer GJ, Jr. (2000). Oxysterols: modulators of cholesterol metabolism and other processes. Physiol Rev 80**:** 361-554.

Segala G, David M, de Medina P, Poirot MC, Serhan N, Vergez F*, et al.* (2017). Dendrogenin A drives LXR to trigger lethal autophagy in cancers. Nat Commun 8**:** 1903.

Segala G, de Medina P, Iuliano L, Zerbinati C, Paillasse MR, Noguer E*, et al.* (2013). 5,6-Epoxy-cholesterols contribute to the anticancer pharmacology of tamoxifen in breast cancer cells. Biochem Pharmacol 86**:** 175-189.

Sevanian A, & McLeod LL (1986). Catalytic properties and inhibition of hepatic cholesterol-epoxide hydrolase. J Biol Chem 261**:** 54-59.

Sevanian A, & Peterson AR (1984). Cholesterol epoxide is a direct-acting mutagen. Proc Natl Acad Sci U S A 81**:** 4198-4202.

Sevanian A, & Peterson AR (1986). The cytotoxic and mutagenic properties of cholesterol oxidation products. Food Chem Toxicol 24**:** 1103-1110.

Silvente-Poirot S, Dalenc F, & Poirot M (2018). The Effects of Cholesterol-Derived Oncometabolites on Nuclear Receptor Function in Cancer. Cancer Res 78**:** 4803-4808.

Silvente-Poirot S, de Medina P, Record M, & Poirot M (2016). From tamoxifen to dendrogenin A: The discovery of a mammalian tumor suppressor and cholesterol metabolite. Biochimie 130**:** 109-114.

Silvente-Poirot S, & Poirot M (2012). Cholesterol epoxide hydrolase and cancer. Curr Opin Pharmacol 12**:** 696-703.

Silvente-Poirot S, & Poirot M (2014). Cancer. Cholesterol and cancer, in the balance. Science 343**:** 1445-1446.

Silvente-Poirot S, Segala G, Poirot MC, & Poirot M (2018). Ligand-dependent transcriptional induction of lethal autophagy: a new perspective for cancer treatment. Autophagy 14**:** 555-557.

Simpson E, & Santen RJ (2015). Celebrating 75 years of oestradiol. J Mol Endocrinol 55**:** T1-20.

Smith LL (1981) *Cholesterol Autoxidation* . Plenum press: New York.

Smith LL, & Johnson BH (1989). Biological activities of oxysterols. Free Radic Biol Med 7**:** 285-332.

Smith LL, Smart VB, & Ansari GA (1979). Mutagenic cholesterol preparations. Mutat Res 68**:** 23-30.

Sola B, Poirot M, de Medina P, Bustany S, Marsaud V, Silvente-Poirot S*, et al.* (2013). Antiestrogen-binding site ligands induce autophagy in myeloma cells that proceeds through alteration of cholesterol metabolism. Oncotarget 4**:** 911-922.

Song C, Hiipakka RA, & Liao S (2001). Auto-oxidized cholesterol sulfates are antagonistic ligands of liver X receptors: implications for the development and treatment of atherosclerosis. Steroids 66**:**473-479.

Soules R, Audouard-Combe F, Huc-Claustre E, de Medina P, Rives A, Chatelut E*, et al.* (2019). A fast UPLC-HILIC method for an accurate quanti fi cation of dendrogenin A in human tissues. J Steroid Biochem Mol Biol 194**:** 105447.

Soules R, Noguer E, Iuliano L, Zerbinati C, Leignadier J, Rives A*, et al.* (2017). Improvement of 5,6alpha-epoxycholesterol, 5,6beta-epoxycholesterol, cholestane-3beta,5alpha,6beta-triol and 6-oxo-cholestan-3beta,5alpha-diol recovery for quantification by GC/MS. Chem Phys Lipids 207**:** 92-98.

Tang L, Wang Y, Leng T, Sun H, Zhou Y, Zhu W*, et al.* (2015). Cholesterol metabolite cholestane-3beta,5alpha,6beta-triol suppresses epileptic seizures by negative modulation of voltage-gated sodium channels. Steroids 98**:** 166-172.

Tang L, Yan M, Leng T, Yin W, Cai S, Duan S*, et al.* (2018). Cholestane-3beta, 5alpha, 6beta-triol suppresses neuronal hyperexcitability via binding to voltage-gated sodium channels. Biochem Biophys Res Commun 496**:** 95-100.

Theodoropoulos PC, Wang W, Budhipramono A, Thompson BM, Madhusudhan N, Mitsche MA*, et al.* (2020). A Medicinal Chemistry-Driven Approach Identified the Sterol Isomerase EBP as the Molecular Target of TASIN Colorectal Cancer Toxins. J Am Chem Soc 142**:** 6128-6138.

Tonsing-Carter E, Hernandez KM, Kim CR, Harkless RV, Oh A, Bowie KR*, et al.* (2019). Glucocorticoid receptor modulation decreases ER-positive breast cancer cell proliferation and suppresses wild-type and mutant ER chromatin association. Breast Cancer Res 21**:** 82.

Touvier M, Fassier P, His M, Norat T, Chan DS, Blacher J*, et al.*(2015). Cholesterol and breast cancer risk: a systematic review and meta-analysis of prospective studies. Br J Nutr 114**:** 347-357.

Vander Heiden MG, & DeBerardinis RJ (2017). Understanding the Intersections between Metabolism and Cancer Biology. Cell 168**:**657-669.

Vandewalle J, Luypaert A, De Bosscher K, & Libert C (2018). Therapeutic Mechanisms of Glucocorticoids. Trends Endocrinol Metab 29**:**42-54.

Vitku J, Starka L, Bicikova M, Hill M, Heracek J, Sosvorova L*, et al.* (2016). Endocrine disruptors and other inhibitors of 11beta-hydroxysteroid dehydrogenase 1 and 2: Tissue-specific consequences of enzyme inhibition. J Steroid Biochem Mol Biol 155**:** 207-216.

Voisin M, de Medina P, Mallinger A, Dalenc F, Huc-Claustre E, Leignadier J*, et al.* (2017). Identification of a tumor-promoter cholesterol metabolite in human breast cancers acting through the glucocorticoid receptor. Proc Natl Acad Sci U S A 114**:** E9346-E9355.

Wang W, Zhang L, Morlock L, Williams NS, Shay JW, & De Brabander JK (2019). Design and Synthesis of TASIN Analogues Specifically Targeting Colorectal Cancer Cell Lines with Mutant Adenomatous Polyposis Coli (APC). J Med Chem 62**:** 5217-5241.

Watabe T, & Sawahata T (1979). Biotransformation of cholesterol to cholestane-3beta,5alpha,6beta-triol via cholesterol alpha-epoxide (5alpha,6alpha-epoxycholestan-3beta-ol) in bovine adrenal cortex. J Biol Chem 254**:** 3854-3860.

Witiak DT, Parker RA, Dempsey ME, & Ritter MC (1971). Inhibitors and stimulators of cholesterolgenesis enzymes. A structure-activity study in vitro of amino and selected N-containing analogs of 5α-cholestane-3β ,5α ,6β-triol. J Med Chem 14**:** 684-693.

Wrensch MR, Petrakis NL, Gruenke LD, Miike R, Ernster VL, King EB*, et al.* (1989). Breast fluid cholesterol and cholesterol beta-epoxide concentrations in women with benign breast disease. Cancer Res 49**:** 2168-2174.

Yin H, Xu L, & Porter NA (2011). Free radical lipid peroxidation: mechanisms and analysis. Chem Rev 111**:** 5944-5972.

Zhang L, Kim SB, Luitel K, & Shay JW (2018). Cholesterol Depletion by TASIN-1 Induces Apoptotic Cell Death through the ER Stress/ROS/JNK Signaling in Colon Cancer Cells. Mol Cancer Ther 17**:** 943-951.

Zhang L, Theodoropoulos PC, Eskiocak U, Wang W, Moon YA, Posner B*, et al.* (2016). Selective targeting of mutant adenomatous polyposis coli (APC) in colorectal cancer. Sci Transl Med 8**:** 361ra140.

Zhou C, Ye F, Wu H, Ye H, & Chen Q (2017). Recent advances in the study of 11beta-Hydroxysteroid dehydrogenase type 2 (11beta-HSD2)Inhibitors. Environ Toxicol Pharmacol 52**:** 47-53.

List of abbreviations: DDA: dendrogenin A; 5,6-EC: 5,6-epoxycholesterol; 5,6α-EC: 5,6α-epoxycholesterol; 5,6β-EC: 5,6β-epoxycholesterol; CT: Cholestane-3β,5α,6β-triol; OCDO: 6-oxo-cholestan-3β,5α-diol ; AEBS: microsomal antioestrogen binding site; D8D7I: 3β-hydroxyterol-Δ8-Δ7-isomerase; EPB: emopamyl binding

protein; DHCR7: 3β-hydroxyterol-Δ7-reductase; ChEH: cholesterol-5,6-epoxide hydrolase; HSD11B1: 11β-hydroxysteroid dehydrogenase type 1; H6PD, HSD11B2: 11β-hydroxysteroid dehydrogenase type 2; ER: estrogen receptor; LXR: liver-X-receptor; GR: glucocorticoid receptor; BC: breact cancer; ER(+)BC: oestrogen receptor positive BC; TNBC, triple negative BC; HER2: Epithelial Growth Factor Receptor of type 2; SERM: selective ER modulators; AI: aromatase inhibitors; 27-HC: 27-hydroxycholesterol; CXCR2: C-X-C Motif Chemokine Receptor 2; SULT2B1b: hydroxysteroid sulfotransferase 2B1b; CYP27A1: Cytochrome P450 Family 27 Subfamily A Member 1; 5,6-ECS: 5,6α-epoxy-cholesterol-3β-sulfate; zymostenol: cholest-8-ene-3β-ol.

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Figure legends:

Figure 1: A) Detailed chemical structure of: cholesterol, 27-hysdroxycholesterol (27-HC), 5,6α-epoxycholesterol (5,6α-EC), 5,6β-epoxycholesterol (5,6β-EC), 5,6α-epoxy-cholesterol-3β-sulfate (5,6-ECS), DDA: dendrogenin A, Cholestane-3β,5α,6β-triol (CT), 6-oxo-cholestan-3β,5α-diol (oncosterone). B) 5,6α-EC can react with histamine to give dendrogenin A in the presence of a chemical catalyst or an enzyme.C) 5,6-EC is hydrated by the cholesterol-5,6-epoxide hydrolase (ChEH) to give CT. CT is transformed into OCDO by the 11β-hydroxysteroid dehydrogenase type 2 (HSD11B2) to give oncosterone. The reverse reaction is catalysed by the 11β-hydroxysteroid dehydrogenase type 1 (HSD11B1) and the hexose-6-phosphate dehydrogenase (H6PD), the enzyme that produces the cofactor NADPH necessary for the reductase activity of HSD11B1.

Figure 2: A) DDA is a mammalian metabolite produced in normal tissues. DDA is a tumour suppressor metabolite that induces cancer cell re-differentiation into normal-like cells and kills cancer cells via a mechanism of lethal autophagy. DDA inhibits oncosterone biosynthesis at the ChEH step. B) Oncosterone is a tumour promoter that stimulates cancer cell proliferation.

Figure 3: A) ChEH is made of two subunits involved in the late steps of cholesterol biosynthesis. ChEH catalyses the trans-hydration of the epoxide ring 5,6-EC to produce CT which can be subsequently transformed into oncosterone. B) DDA inhibit ChEH which blocks the hydration of 5,6-EC and induces the accumulation of zymostenol.

Figure 4: Snapshot of the 5,6-EC metabolism in normal and pathological breast.

Figure 5: Diagram showing different pharmacological strategies to block the enzymes responsible of oncosterone biosynthesis from 5,6-EC and the receptors that are effectors of oncosterone tumour promoter activity. DDA can control oncosterone biosynthesis and action at the ChEH and LXR levels.

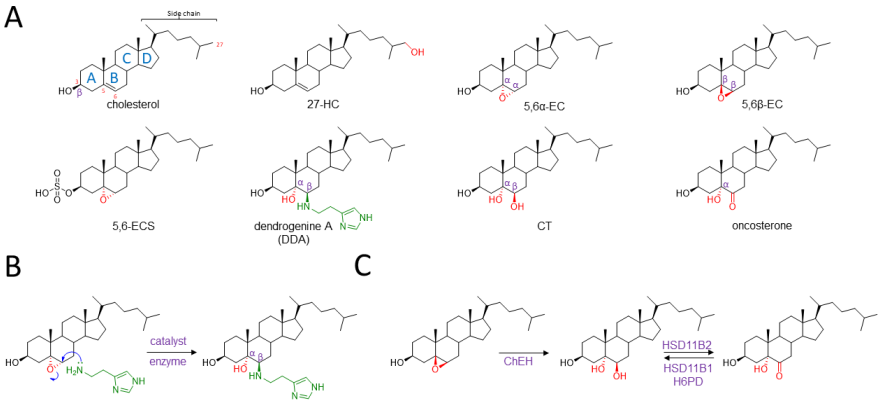

Figure 1

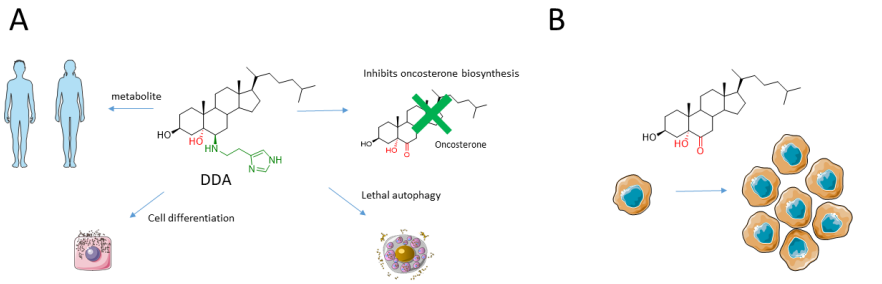

Figure 2

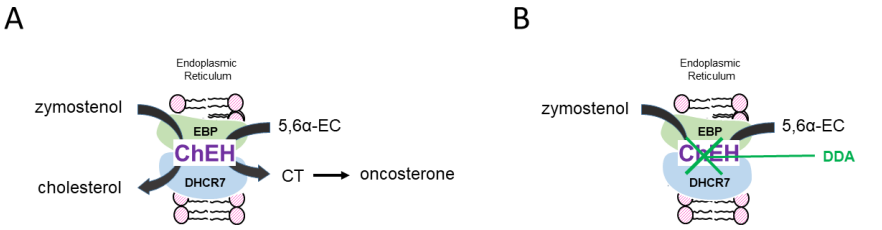

Figure 3

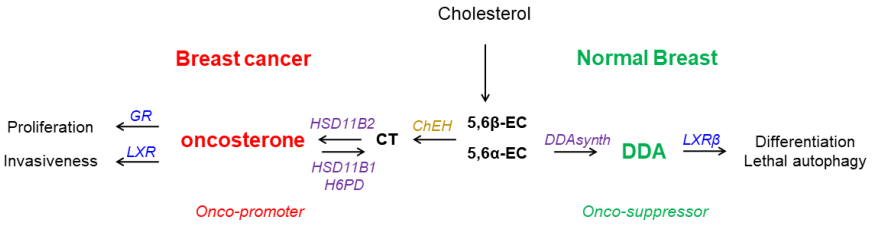

Figure 4

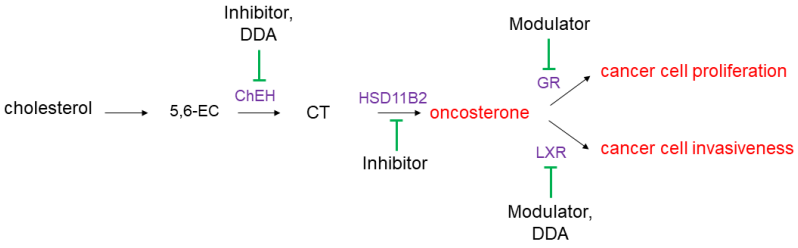

Figure 5