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Targeting Carboxylesterase 1 to counteract metabolic reprogramming in platinum-resistant ovarian cancer cells

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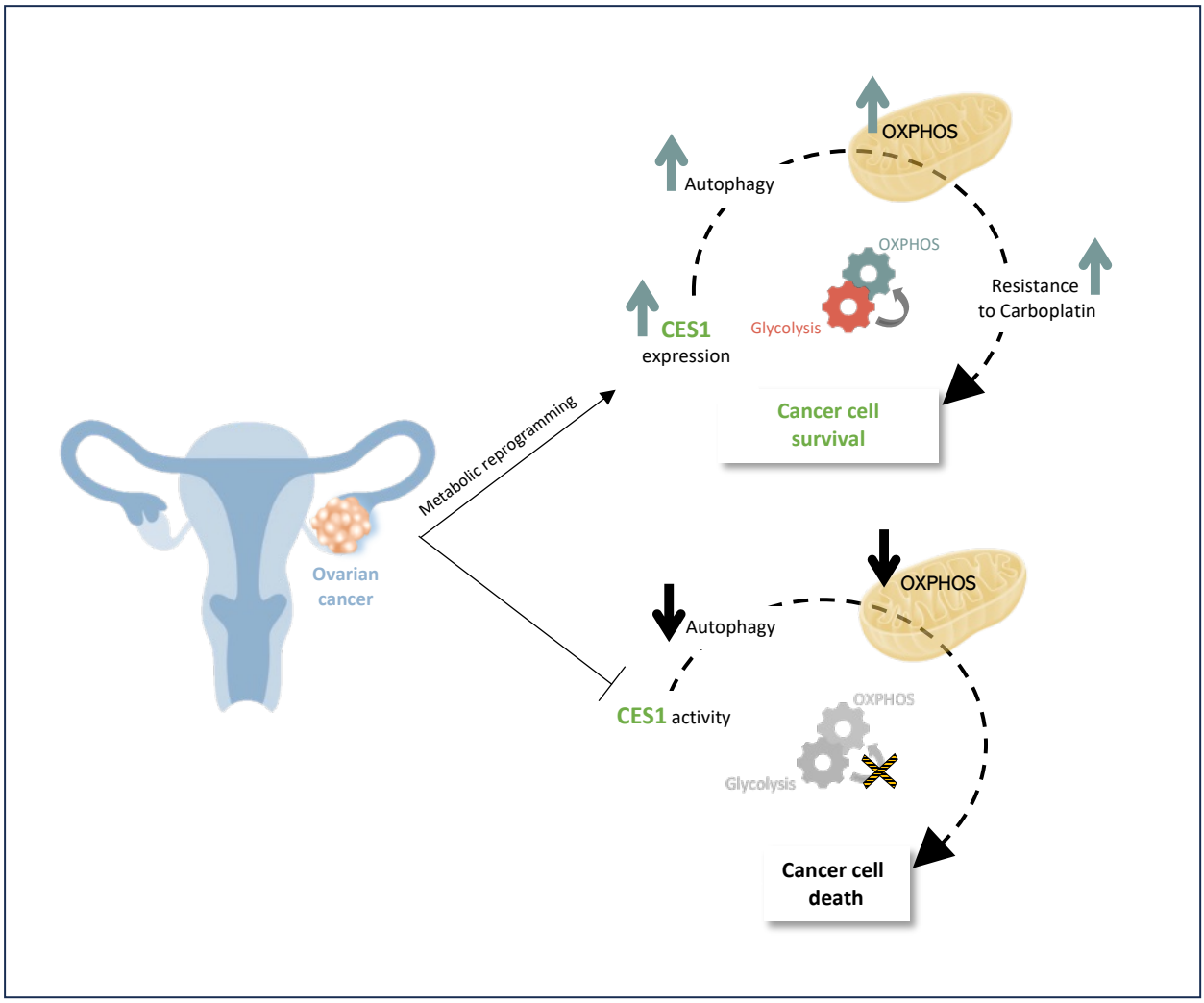
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GRAPHICAL ABSTRACT



SUMMARY

Nowadays, treating ovarian cancer (OC) remains challenging. Hence, while women initially show responsiveness to platinum and taxane-based chemotherapy, the gold standard for OC treatment, they frequently relapse due to platinum resistance. The low availability of validated therapeutic targets further limits the therapeutic options, resulting in a high mortality rate of approximately 70%.

Increasing evidence suggests that the metabolic adaptation of cancer cells facilitates their growth, invasiveness, and metastatic potential. Therefore, the reprogramming of energy metabolism is undoubtedly in the eye of the storm in the context of cancer research and treatment.

Specifically, OC cells show addiction to lipid metabolism in advanced stages, supporting the hypothesis that fat availability plays a crucial role in promoting aggressiveness and chemoresistance. Notably, obesity is one of the main risk factors that contributes to OC poor prognosis, reinforcing the crucial role of lipids in this context.

The NF-κB signaling pathway is majorly implicated in OC advanced disease, where it drives chemoresistance, stemness, metastasis and immune evasion. Although NF-κB signaling is upregulated in OC tumors and correlates with poorer clinical outcomes, there are no clinically useful specific NF-κB inhibitors. The conundrum in therapeutically blocking NF-κB has been achieving cancer-cell specificity, given the ubiquitous and pleiotropic functions of the NF-κB pathway. To overcome the toxicity associated with systemic NF-κB blockade, both academia and pharma are focused on targeting essential cancer-specific downstream effectors of oncogenic NF-κB signalling rather than NF-κB itself.

Recently, CES1 has been depicted as a NF-κB-regulated triacylglycerol (TAG) lipase linking obesity-associated inflammation with lipid catabolism and metabolic adaptation to nutrient depletion-driven energy stress (ES) in aggressive colorectal carcinoma (CRC). Specifically, CES1 promotes cancer cell survival and metabolic adaptation in CRC cells under ES via two distinct cell-autonomous mechanisms: 1) First, it enables CRC cells to meet their energy demand by increasing the breakdown of TAGs stored in lipid droplets (LD) and providing free fatty acids for fatty acid oxidation (FAO) and oxidative phosphorylation (OXPHOS); 2) Second, it prevents the toxic build-up of TAGs that results in reactive oxygen species (ROS) production and lipid peroxidation leading to cell death by apoptosis and ferroptosis.

Alike CRC, OC shows addiction to both lipid metabolism and NF-κB and the tendency to preferentially metastasize toward the peritoneal cavity and the omentum, a fat-rich organ, making this tumor a promising setting in which investigate CES1 role.

Hence, this project aimed at understanding whether CES1 could support the metabolic adaptation and survival of OC cells, to potentially identify a new druggable target to counteract aggressive OC disease.

We demonstrated that OC cells mostly rely on glucose to meet their energetic demand in basal conditions, but they reprogramme their metabolism toward OXPHOS upon ES. Notably, the OC cells upregulated FAO and CES1 mRNA when cultured under ES and became resistant to platinum, suggesting that lipid metabolism and CES1 could be relevant to cope with nutrient fluctuations and this metabolic reprogramming could increase chemoresistance.

Indeed, unlike carboplatin, pharmacological CES1 blockade by commercially available GR-148672X inhibitor impaired bioenergetic parameters, blocked autophagy flux and is effective in killing carboplatin-resistant OC cells. The clinical relevance of CES1 blockade is supported by the inverse correlation between CES1 expression and worse prognosis in OC patients and the capacity of CES1 inhibitors to kill primary OC cells, thus resembling OC cell lines behaviour.

All together, these data underscore that CES1-driven reprogramming is mostly relevant during the metabolic reprogramming towards an oxidative phenotype and sustain the actionability of CES1 inhibition to impede autophagy, lipid catabolism and survival of platinum-resistant OC cells.

1. INTRODUCTION

1.1 Ovarian Cancer

Ovarian cancer (OC) is the seventh most common cancer in women worldwide but also the deadliest gynaecological malignancy. Nomura and colleagues reported in 2017 that OC is confined to the ovary at diagnosis in 15% of patients, while in 19% of women is spread to regional lymph and in 60% is metastasized beyond the primary site, indicating that more than half of all OC cases are normally undetected until advanced stages (III or IV), probably due to the non-existence of a reliable, specific screening procedure for detecting OC at an earlier stage (Nomura *et al.*, 2017). According to the latest Globocan update, an online database providing global cancer statistics, 313,959 cases of OC have been identified in 2020 and have been found responsible for 207,252 cases of death (Sung *et al.*, 2021). In 2023, the American Cancer Society estimated 19.710 new OC cases and 13.270 deaths in the United States; these statistics underlie that the poor prognosis is often due to the diagnosis at late stages that occurs in 50% of cases, while only 20% of women discover OC in the primary site (Siegel *et al.*, 2023).

Nowadays, most efforts are focused on the early detection of OC and the development of new and more effective chemotherapeutic drugs.

1.1.1 Criteria to classify OC

"Ovarian cancer" is a term that refers to a heterogeneous group of malignancies differing in aetiology, pathogenesis, prognosis, pathology, and molecular features. Added to this, OC is difficult to diagnose and a definite diagnosis can be given only after a postoperative biopsy, because the only clinical investigations are physical examination, transvaginal ultrasonography, and measurement of non-specific biomarkers, such as cancer antigen 125 (Braat, 1997).

In 2014, the new International Federation of Gynecology and Obstetrics (FIGO) classification was published to better classify ovarian, fallopian tube and primary peritoneal lesions into specific groups (Table 1) (Meinhold-Heerlein *et al.*, 2015). Together with FIGO classification focusing on the differentiation into tumor stages, the Shimizu/Silverberg system examines three parameters: the glandular architecture, degree of nuclear atypia, and mitotic index, while the WHO classification is used to indicate the histopathological and molecular tumor type.

Stages	***FIGO classification***
I	Confinement of the tumour to the ovaries or fallopian tubes
II	Involvement of ovaries or fallopian tubes with pelvic extension or primary peritoneal cancer
III	Spread of the tumour to the peritoneum outside of the pelvis and/or metastasis to the retroperitoneal lymph nodes
IV	Confirmation of distant metastasis excluding peritoneal metastases

Table 1: stages of OC disease during progression according to FIGO classification.

Nowadays, histotype classification is the principal method to sub-classify OCs. OC can be subdivided into two main histological subtypes: Epithelial Ovarian Cancers (EOCs), which account for ~90% of OCs, and non-EOCs, which account for ~10% of OCs. In 2020, according to the fifth edition of the WHO Classification of Female Genital Tumours, five principal histotypes were depicted among EOCs: high-grade serous carcinoma (HGSC), low-grade serous carcinoma (LGSC), mucinous carcinoma (MC), endometrioid carcinoma (EC) and clear cell carcinoma (CCC). The different histotypes differentially express a panel of four immunohistochemical markers (Wilson tumor protein-1 (WT-1)/p53/napsin A/progesteron receptor (PR)) (Köbel and Kang, 2022).

HGSC is the most common type of OC that occurs in the 80% of cases but it does not origin from the ovarian surface epithelium. Probably the precursor of HGSCs is a lesion of fallopian tube called tubal intraepithelial carcinoma (TIC) that shares with HGSC the immunoreaction for p53, BRCA1, WT-1, a marker of serous differentiation, and p16. Ki-67 proliferation index is also usually elevated. In fact, HGSCs are characterized by increased nuclear size, mitotic activity and apoptotic bodies (Prat, 2012).

According to the Bowtell's model, HGSCs arise from *TP53* mutations followed by BRCA loss, that cause a weakening of the mechanism of double-strand break repair, leading to chromosomal instability and widespread copy number (CN) changes, that are the peculiar features of HGSC (Bowtell, 2010). The cascade of secondary and tertiary molecular events facilitates tumor evolution and dictates its response to therapy.

On the other hand, LGSCs originate from benign ovarian serous cysts, which can progress to serous borderline tumor (SBT) and then to invasive LGSC. Moreover, LGSCs do not express the same genetic alterations of HGSCs (*TP53* and BRCA), neither the chromosomal instability, but LGSCs and HGSCs share, at least in part, the biomarker expression profile (Prat, 2012; Babaier *et al.*, 2022). Specifically, unlike HGSCs, LGSCs express less Ki-67 and overexpress PAX-2 in most of the cases (Babaier *et al.*, 2022).

MC is sporadic (3%) and benign in the 80% of cases when identified at early stages. Contrary to HGSCs, diagnosing primary MCs is possible, but given the histological heterogeneity, making extensive sampling is necessary (Babaier and Ghatage, 2020). The origin of MCs is unknown, but the cells of MCs show gastrointestinal differentiation. Anyway, the immunoreactivity for specific markers permit to distinguish gastrointestinal carcinomas from ovarian MCs. Hence, MCs are immunoreactive for cytokeratin 7 (CK7) and Dpc4 while the staining for CK20 and CDX-2 is weak and focal. Moreover, MCs are uniformly negative for ER and WT1, in contrast to endometrioid (ER^+) and serous (ER^+ and $WT1^+$) carcinomas (Prat, 2012; Borella *et al.*, 2023).

Instead, according to the Sampons's theory, EC originates from retrogradation of endometrium in the peritoneal cavity due to its implantation into the peritoneum and pelvic organs, including the ovaries (Lamceva, Uljanovs and Strumfa, 2023). Women with endometriosis undergo to a higher risk to develop EC over years but only in concomitance with others predisposing factors. In a retrospective study of 2021, it was demonstrated that 12.3% of patients with EC had coexisting endometrial atypical hyperplasia and 20.5% of patients with EC had also endometrial cancer (Zhou *et al.*, 2021). EC is also susceptible to changes of microbiota in the reproductive tract where anaerobic bacteria interfere with immune and metabolic signalling by causing inflammation (Chen *et al.*, 2021). Recently, Soovares and colleagues depicted biomarkers of EC to distinguish histological grade: good-prognosis markers are PR, estrogen receptor (ER), β-catenin and vimentin while poor-prognosis markers are p53, p16 and L1CAM that gradually increased from grade 1 to 3 (Soovares *et al.*, 2022).

CCC is a distinct genomic and epigenomic subtype of OC and was originally called mesonephroma because it was thought to originate from the mesonephric gland, considering the similarity with renal clear cell carcinoma (Willis *et al.*, 2017; Sun and Jiang, 2023). Immunohistochemistry assesses the lack of ARID1A protein expression as it occurs in 50% cases of CCC and, according to Yamamoto and colleagues, loss of ARID1A frequently coexists with *PIK3CA* mutations

(Yamamoto *et al.*, 2012a). Also in CCC of the ovary, endometriosis is considered a precursor of this tumor (Gadducci *et al.*, 2021). Ovarian endometriosis characterized by PD-L1 overexpression and copy number variation cause the transition from a non-invasive precursor lesion to CCC (Sun and Jiang, 2023).

1.1.2 Epidemiology and risk factors in OC

OC mainly develops in older women after menopause. In fact, about half of the women who are diagnosed with ovarian cancer are 63 years or older. Lower age at diagnosis is probably related to the hereditary status of breast and ovarian cancer or to the race of women with OC: African Americans are characterized by a lower median age at diagnosis than the international median. However, family history of OC is a predisposing factor. In fact, it was reported a three- to four-fold higher rate of OC among first-degree relatives diagnosed with the disease. The risk to develop OC subsists even if there are other cancer history among relatives because of the sharing of genetic basis of cancer and probably the exposition to the same environmental factors (Ali, Al-Ani and Al-Ani, 2023).

Generally, BRCA1 and BRCA2 variants in the germline account for around 75% of hereditary disease by representing the most common form of hereditary OC. The Lynch syndrome is a predisposing factor and is the cause of 10-15% of hereditary cases of OC associated to defects in genes involved in single stranded mismatch DNA repair (MMR) such as MLH1, MSH2, MSH6 and PMS2. Instead, inherited TP53 mutations are associated to Li-Fraumeni syndrome responsible for the remaining hereditary OC cases (3%) (Hollis and Gourley, 2016; Nomura *et al.*, 2017).

On the other hand, the use of oral contraceptive pills (OCP) reduces the risk of OC by 30 to 50%, establishing a positive relationship between the duration of OCP use and the degree of the protective effect (Tsilidis *et al.*, 2011; Havrilesky *et al.*, 2013; Michels *et al.*, 2018). Besides the effects of OCP on the general population, the BRCA1 and BRCA2 mutation carriers show a decreased risk of OC the more they use OCP (Schrijver *et al.*, 2021). Moreover, a retrospective cohort study conducted in Minnesota from 2000 through 2013 on OC patients revealed that the OCP use versus the non-use is associated to a better overall survival and better progression-free survival (Jatoi *et al.*, 2015).

Late menopause is associated to an increased risk of developing OC together with nulliparity. The risk of ovarian cancer is 24% higher among nulliparous women compared with women who have

even only one child (Gaitskell *et al.*, 2018). In the Million Women Study conducted among UK women it was also demonstrated that nulliparity is closely associated to more cases of clear cell OC and, albeit to a lesser extent, of endometroid OC while there is no association with the most common high grade serous OC (Gaitskell *et al.*, 2018). Breastfeeding reduces OC risk without affecting the histological phenotype.

Multiple studies were conducted to determine a relationship between OC occurrence and food habits. Despite some methodological limitations characterizing these studies, the findings suggest that women consuming dietary fat and animal fat as well as animal-source nitrate are more susceptible to OC (Crane *et al.*, 2014). Not only dietary fat alone, but also the combination with serum lipid levels triggers OC according to a meta-analysis mostly from North America and Europe published in 2023 (Zhang *et al.*, 2023). Interestingly, overweight women suffer of 6% increased risk for OC, while in obese women this risk increases to 19% (Ellwanger *et al.*, 2022). In fact, higher incidence of OC is associated to obesity, metabolic, and lipid disorders causing increased susceptibility even among younger women (Huang *et al.*, 2022). Recent evidence underlies that the up-regulation of specific obesity and lipid metabolism-related genes, such as CD36 and TGF-β, is responsible for poorer outcome among women with similar cancer driver mutation profiles by negatively impacting on chemosensitivity (Cuello, Kato and Liberona, 2018). In a diet-induced obesity OC allograft model, all the high fat diet (45% fat) mice had detectable remaining tumor burden on the omentum, ovaries/uterus, mesentery, and fat at the end-point after treatment with combination regimen of paclitaxel and carboplatin compared to tumors from low fat diet (10%) mice, suggesting how obesity reduces the efficacy of standard-of-care chemotherapy (Liu *et al.*, 2023).

Therefore, identify the predisposing conditions and thus the population at high risk is imperative to prevent OC.

1.1.3 Genetic and molecular changes triggering OC

In the era of personalized medicine, the identification of specific genetic mutations not only could allow the setting up of personalized surveillance programs, including prophylactic surgery (salpingectomy or oophorectomy) that family history by itself would not be sure to justify, but also promote the detection of potential vulnerabilities to target with specific drugs. According to the

model of Shih and Kurman, EOCs can be subdivided in two broad categories: Type I and Type II tumors (Shih and Kurman, 2004). These two categories are genetically different: type I tumors include LGSOCs, borderline serous tumors, ECs, MCs and CCCs that are genetically stable and mostly present mutations in KRAS, BRAF, PTEN, PIK3CA, b-catenin gene (CTNNB1), and ARID1A. The type II comprises mainly HGSOCs characterized by genomic instability due to TP53 mutation in 95% of patients and BRCA1 (55%) and BRCA2 (30%) (Toss *et al.*, 2015; Kurman and Shih, 2016).

A description of the principal genes linked to OC susceptibility and frequently related to specific OC subtype is reported below.

BRCA1 and BRCA2 are the most common mutated genes in OC and characterized HGSOCs. BRCA1 and 2 have unique roles in homologous recombination (HR) in presence of double strand break (DSB) (Neff, Senter and Salani, 2017). BRCA1 interacts with tumor suppressors, DNA repair proteins and cell cycle regulators to activate DNA damage checkpoints and repair the DNA, while BRCA2 regulates RAD51, which is the molecular effector required for HR repair (Roy, Chun and Powell, 2012; Liontos *et al.*, 2021). In particular, HR involves damage recognition by the kinases ATM and ATR and by CHK2 and BRCA1 as mediators. Then, the DNA repair is prompted by the effectors BRCA2 and RAD51 (Roy, Chun and Powell, 2012; Liontos *et al.*, 2021). Moreover, there are also several facilitators of the HR pathway, beyond BRCA1 and 2 and the already mentioned checkpoint kinases and RAD51, such as PALB2 and BRIP1. A meta-analysis performed on 29400 cases of OC revealed the pathogenicity of BRIP1, RAD51C, and RAD51D mutations that are associated with high risk of OC (Suszynska, Ratajska and Kozlowski, 2020). Carriers of PALP2 mutations also suffer an increased risk of developing OC, according to a study whose 14135 samples of diseased women come from a UK biobank (H. Song *et al.*, 2021).

The abnormalities in mismatch repair (MMR) genes are the most common cause of hereditary OC after BRCA mutations and frequently co-occur with Lynch syndrome, that is a predisposing condition of OC, as said above (Infante *et al.*, 2022).

A study on 215 OC patients recruited from 2015 to 2018 in Ontario (Canada) revealed that 39% of cases were associated with MMR mutations, including MSH6, MLH1, PMS2, and MSH2 (Kim *et al.*, 2022). MMR is a highly conserved pathway responsible of genomic stability. In fact, its functionality is associated with an improved overall survival in OC patients. Specifically, the study conducted on 1335 serous cancers and ECs showed that the upregulation of mRNA expression of

MSH6, MLH1 and PMS2 correlated with better chance to survival also in late-stages (Zhao *et al.*, 2018).

Alterations in the MAPK signaling transduction genes are added to the genetic alterations in homologous recombination proteins and in mismatch repair genes. Aberrant MAPK signalling results in uncontrolled proliferation, differentiation, and invasion. LGSCs are driven by mutations in the MAPK signaling pathway. In fact, mutations in BRAF, KRAS, or NRAS occur in the 60% of cases (Emmanuel *et al.*, 2014; Manning-Geist *et al.*, 2022).

A retrospective analysis conducted by Chui and colleagues revealed that the most frequent $BRAF^{V600E}$ hotspot mutation represents only two-thirds of BRAF mutations in the analysed cohort, suggesting that other less-depicted mutations could be driver as well, such as: non-V600E mutations, gene fusions with MKRN1, AGAP3, and AGK, amplifications and the co-existence with RAS mutations (Chui *et al.*, 2021). Different to BRAF mutations that are associated to good overall survival, KRAS mutation is a known biomarker for poor outcome and chemoresistance in OC (Manning-Geist *et al.*, 2022; Uddin *et al.*, 2022). Specifically, mutation in KRAS is a common and earlier event in different histotypes of OC at stages FIGO I and II (Uddin *et al.*, 2022). For example, RAS mutant V12 confers chemoresistance through induction of autophagy, as shown by the upregulation of specific autophagic markers (ATG5, ATG12, BECLIN1, and LC3B) and the increased tumor growth rate *in vivo* (Liang *et al.*, 2019).

Abnormal PI3K pathway is also involved in OC insurgence and spread. Genetic alterations for PIK3CA have been retraced, albeit to varying degrees, in all types of OCs. Amplification of AKT isoforms, or deletion or inactivation of PTEN characterized OCs too (Rinne *et al.*, 2021). In CCC, three hotspot mutations in PIK3CA have been identified: E542K, E545K, and H1047R that frequently co-occur with mutations in ARID1A; both events seem to be early, as they lack prognostic significance. ARID1A loss of function characterized 56% of CCC cases and results in altered SWI/SNF chromatin remodeling complex, that controls the accessibility of promoters to transcriptional sites and is involved in the regulation of many cellular processes, including development, differentiation, proliferation, and DNA repair (Yamamoto *et al.*, 2012b).

In the attempt to identify the tumor suppressor PTEN as a biomarker of OC, a study of 2020 revealed that PTEN loss is a driver mutation in all OC subtypes but mainly characterized CCCs and ECs, while in HGSOCs it is involved in the context of chromosomal instability (Martins *et al.*, 2020). Moreover, PTEN and PIK3CA mutations co-occurr in 67% of ECs, and in the 53% of cases

a third mutation in the exon 3 of CTNNB1 gene is present along with the other two and define the molecular profile of early-stage ECs (Mcconechy *et al.*, 2014). CTNNB1 encodes for β-catenin, a key protein in the Wnt signaling pathway, that after nucleus translocation promotes cell proliferation. However, the accumulation of β-catenin into the nucleus due to activating mutations of CTNNB1 gene can trigger carcinogenesis (Travaglino *et al.*, 2022).

Terakawa and colleagues demonstrated that the co-existance of PTEN, PIK3CA and CTNNB1 mutations is necessary to boost the growth potential of mutant cells to develop preneoplastic lesions. Specifically, CTNNB1 is responsible to trigger myometrial invasion (Terakawa *et al.*, 2019).

The genetic heterogeneity of OC further complicates a scenario characterized by delayed diagnosis and several histologic subtypes. Certainly, the identification of driver mutations by exploiting NGS technology, and the characterization of their role in tumor development and progression are key points to improve OC treatment.

1.1.4 Treatment landscape in OC

The treatment plan chosen for women affected by OC is strictly related to disease stage, specific histological subtype, and genetic and epigenetic features. In this regard, surgery is necessary to define the stage according to the FIGO and TNM system and, of course, to try a complete resection of OC by removing the ovaries, uterus and fallopian tubes and exploring the abdomen, pelvis, and omentum. Generally, surgical cytoreduction is followed by adjuvant chemotherapy, but often chemotherapy is given before surgery and called “neoadjuvant chemotherapy” to reduce the size of the tumor and increase the probability of surgery success (Berek *et al.*, 2021).

The combinatorial regimen of cisplatin-cyclophosphamide was initially introduced in the treatment of OC thanks to a study by Lambert and Berry (Lambert and Berry, 1985). Then, even if cisplatin-paclitaxel was considered more effective, cisplatin has been progressively replaced by carboplatin because is associated to less toxicity. At present, platinum and taxane-based chemotherapy is the standard treatment for OC but, although the initial effectiveness of platinum-based chemotherapy, 70% of women relapse due to drug-resistance (Pepa *et al.*, 2015; Mai *et al.*, 2022). The problem of cancer progression related to platinum resistance is still an open question to date. Many efforts are focused on improving the response to platinum and understand the molecular mechanisms that are

responsible for recurrence of OC. Specifically, OC cells develop the so called multidrug resistance (MDR) by showing resistance to multiple drugs, different in structure and in mechanism of action (Mai *et al.*, 2022). Several molecular mechanisms driving platinum-resistance have been described so far (Figure 1) (Mai *et al.*, 2022).

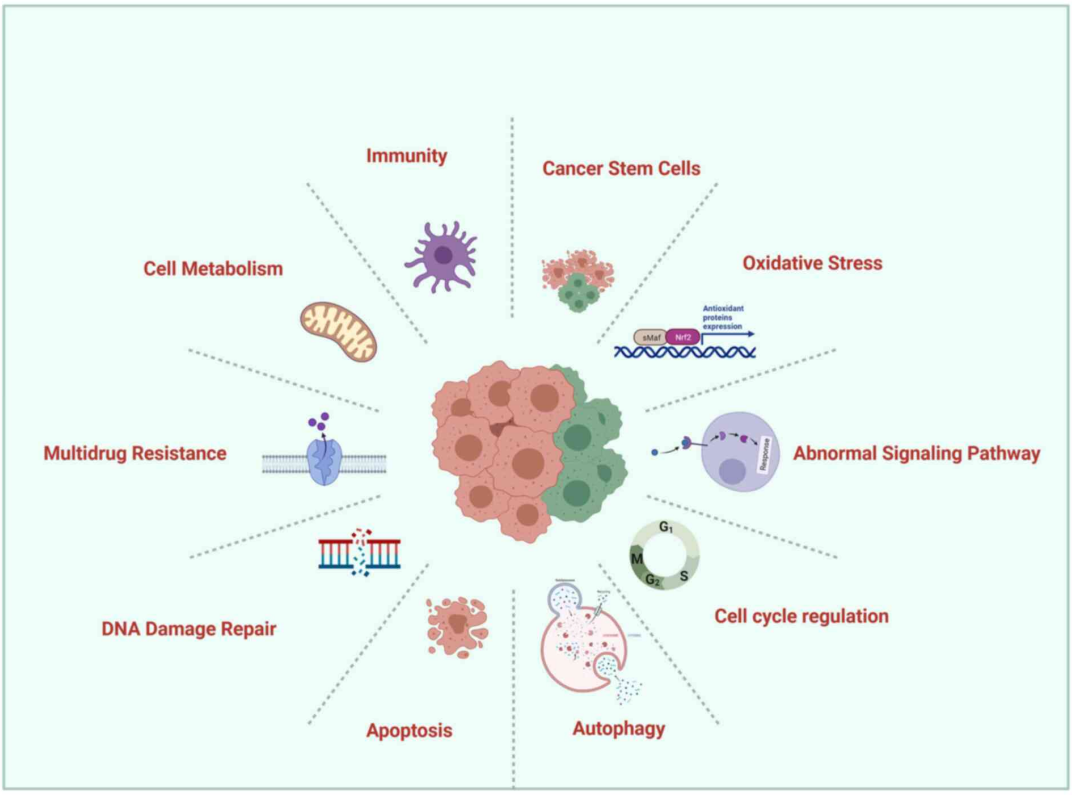


Figure 1:

Schematic overview of the mechanisms of platinum-based chemotherapy resistance in ovarian cancer (from Mai *et al.*, 2022).

DNA damage repair (DDR) is one of the most studied mechanisms of drug resistance in OC and, in this context, PARP inhibitors demonstrated outstanding activity as DNA-damaging agents in the treatment of recurrent EOC, especially for BRCA-associated HGSCs, ECs and homologous recombination (HR) deficient OCs, characterized by tumor cells unable to repair DNA double-strand breaks (Wu *et al.*, 2023). PARP inhibitors are the first FDA-approved biological agents for OC based on the individualized features of cancer, and for this reason not all women benefit from this treatment (Liu, Konstantinopoulos and Matulonis, 2014). In EU, the main three PARP

inhibitors (Olaparib, Rucaparib and Niraparib) are administrated as first-line maintenance to patients with platinum-sensitive, relapsed HGSOC (Jiang *et al.*, 2019). Unfortunately, the prolonged use of PARP inhibitors is associated to side effects and does not significantly improve overall survival. More importantly, patients tend to acquire resistance over time (Jiang *et al.*, 2019; Konstantinopoulos, Lheureux and Moore, 2020). For this reason, many efforts are focused on investigating alternative combinatorial regimens.

An active investigation area consists of combinations of PARP inhibitors and cell cycle checkpoint kinases or immune checkpoint inhibitors. Specifically, OCs are more reliant on the G2 checkpoint for DDR, as the ubiquitous loss of p53 in HGSOC causes the ineffectiveness of the S phase checkpoint. Moreover, PARP inhibitors-resistant BRCA1-deficient cells are increasingly dependent on ATR for survival (Yazinski *et al.*, 2017). ATR is involved in S and G2/M checkpoints to repair damaged DNA, and it can be inhibited by ATR inhibitors to induce cancer cell death. ATR inhibitors seem to re-sensitize OC cells to PARP inhibitors in a synergistic manner in different PDX-resistant HGSOC models with different genetic alterations like platinum-resistant BRCA mutant and $CCNE1^{AMP}$ PDX models (Kim *et al.*, 2020; Hockings and Miller, 2023). Interestingly, CCNE1, which encodes the cell cycle regulator cyclin E1, amplified in 19% of cases of HGSOC and related to resistance and decreased overall survival, impedes to benefit from PARP inhibitors because HR deficiency and CCNE1 amplification rarely co-occur (Gorski, Ueland and Kolesar, 2020). Still, this subgroup seems to be responsive to ATR inhibitors (Kim *et al.*, 2020).

The rationale for developing combinations with PARP inhibitors considers the crosstalk with other signalling pathways that can contribute to the insurgence of PARP inhibitor resistance mechanisms. Beyond the inhibitors above, some favourable results came from the combination with PI3k/AKT, MEK and RAF inhibitors, antiangiogenic agents, and immunotherapy.

Mechanisms of MDR in OC also include metabolic reprogramming and autophagy. In fact, dysregulated metabolic pathways sustain anti-apoptotic and cell survival mechanisms associated with chemoresistance. Targeting metabolic mediators that promote stress adaptation of cancer cells and disease progression is a possibility that has not been fully exploited yet.

For example, Mepron, the clinical formulation of Atavaquone approved by FDA for the treatment of malaria is an inhibitor of complexes I-III of electron transport chain able to increase intracellular oxygen radical flux and cause reduction of proliferation in OC cell lines and tumor growth in syngeneic OC mouse models (Kapur *et al.*, 2022). Promising effects obtained in NGS mice

carrying patient-derived cancer stem-like cell confirmed the importance of the inhibition of oxidative phosphorylation by atovaquone. Considering Atavaquone a candidate for OC treatment, it will be tested in combination with PARP inhibitors and antioxidant inhibitors in the next future (Kapur *et al.*, 2022).

Metformin has been tested in several clinical trials (NCT02437812, NCT02312661) in combination with paclitaxel and carboplatin in women with advanced OCs. It is well tolerated in OC patients at II, III and IV stage and causes a significant reduction of the cancer stem cell population and increased sensitivity to cisplatin (Brown *et al.*, 2020). Specifically, metformin inhibits the mitochondrial complex 1, actives AMPK, and favours the metabolism of omega-3 fAs that results in antiproliferative effects on OC cell lines and in xenograft models (Udumula *et al.*, 2022).

Thus, in addition to preferring oxidative metabolism, chemotherapy-resistant OC cells depend on lipid metabolism. Another evidence supporting the role of lipid metabolism in these settings, is the fact that blocking PCSK9, an enzyme involved in cholesterol metabolism, with either siRNA or PCSK9 specific inhibitor (PF-06446846), resulted in impaired survival in OC cell line e patient-derived OC cell cultures (Jacome Sanz *et al.*, 2021).

Although metabolic reprogramming is an interesting field to look for key mediators of OC survival, the outstanding question involves the difficulty to specifically inhibit metabolic enzymes associated with cancer. Therefore, researchers need to investigate systemic effects associated with the inhibition of metabolic targets.

Autophagy plays a crucial role in maintaining metabolic homeostasis in cancer cells during stressful conditions. Autophagy can either induce apoptosis or autophagic cell death following chemotherapy treatment or, paradoxically, represent a pro-survival mechanism to mediate the acquired resistance phenotype during chemotherapy (Sui *et al.*, 2013). As a mechanism of MDR, autophagy is a stress response involved in the acquisition of carboplatin-resistant phenotype in OC (Bowers *et al.*, 2022). In this context, Bortezomib impairs autophagic flux by blocking p62 degradation in multiple cisplatin-resistant OC cell lines without affecting the fusion of autophagosome and lysosome, which, however, is not working (Kao *et al.*, 2014). Bortezomib anti-cancer effects were also demonstrated *in vivo* (Kao *et al.*, 2014) and currently a phase II trial is verifying the combinatorial use of bortezomib and pegylated liposomal doxorubicin in women with BRCA wild-type platinum-resistant recurrent OC (Lee *et al.*, 2022). Unfortunately, the phase I

HIDRA clinical trial revealed that the safe combination of itraconazole and hydroxychloroquine, classified as a first-generation autophagy inhibitor, in platinum-resistant EOC is not showing significant antitumor activity like during preclinical phase, suggesting the necessity to go deeper into the exact mechanism of action and also improve both pharmacodynamics and pharmacokinetics (Marastoni *et al.*, 2022). The enzyme methionine synthase reductase (MTRR) involved in the folate metabolism and differentially expressed in OC tissues according to tumor grade, FIGO stage, presence of metastasis, and platinum resistance, may be a potential target in the OC therapy. MTRR silencing by RNA interference also inhibits autophagy by upregulating the ERK1/2 and AKT signaling pathway-mediated mTOR expression (Chen *et al.*, 2015). Nowadays, understanding the molecular alterations driving OCs progression could prompt the identification of new druggable targets to counteract OC growth and overcome resistance.

1.2 The complexity of the NF-κB network

Over the years, the nuclear factor κB (NF-κB) pathway has been intensely investigated as its crucial involvement in essential physiological processes, such as the coordination of immune and inflammatory responses, cell survival, cell proliferation, cell differentiation, autophagy, senescence, and metabolic reprogramming.

In mammals, the NF-κB family comprises the following five members, namely RelA/p65, RelB, c-Rel, p50/NF-κB1 (p105), and p52/NF-κB2 (p100), which can associate with each other to form homodimers or heterodimers. The NF-κB transcription factor complexes dimerize and bind the DNA by using the highly conserved 300-amino acid N-terminal region known as the Rel-homology domain (RHD) that also represents the site of interaction with IκB regulatory proteins. IκB proteins are responsible for keeping the inactive NF-κB complexes in the cytoplasm through the ankyrin repeat domain, necessary to interact with NF-κB dimers, and block their nuclear import by masking their nuclear localization signals (NLS) (Zinatizadeh *et al.*, 2021). The translocation to the nucleus follows the phosphorylation of IκB proteins on conserved serine residues by the IκB kinase (IKK) complex. In the nucleus, NF-κB complexes bind specific κB sites to regulate transcription of several genes encoding for downstream effectors of NF-κB-driven physiological function (Begalli *et al.*, 2017; Bennett *et al.*, 2018).

Two major pathways regulate the activation of NF-κB: the canonical and non-canonical pathways. The canonical or classical pathway is activated in response to inflammatory cytokines (TNFα, IL-

1β, etc.), antigens or microbial pathogens to mediate inflammatory immune response by the activation of IKK complex formed by the two catalytic subunits, IKKα and IKKβ, and the regulatory subunit, IKKγ (also known as NEMO). Specifically, the recruited TAK1 kinase on IKK complex leads to IκBs' phosphorylation and degradation, and the following nuclear translocation of NF-κB dimers (Mitchell *et al.*, 2023).

The non-canonical or alternative pathway undergoes slower kinetic activation compared to the rapid and transient activation of the canonical one. The non-canonical pathway is active to sustain immune processes, secondary lymphoid organogenesis, B-cell survival and maturation, bone morphogenesis and dendritic-cell activation. To this, the enzymatic subunits IKKβ and IKKγ of the IKK complex are not required but the activation of this pathway depends on stabilisation of the NF-κB-inducing kinase (NIK) and subsequent NIK-mediated phosphorylation of IKKα. The non-canonical pathway mainly involves the activation of the heterodimer p52/RelB. This pathway is based on the inducible processing of p100,which acts as the precursor of p52 and a RelB-specific inhibitor (Sun, 2011; Cildir, Low and Tergaonkar, 2016; Begalli *et al.*, 2017).

In addition to the several physiologic roles in host defence responses to microbial pathogens, injury, stress, and immunity, the NF-κB network, is deregulated in many diseases, including atherosclerosis, autoimmunity, chronic inflammatory diseases, neurodegenerative conditions, metabolic imbalances such as obesity or type-2 diabetes, as well as cancer.

High levels of NF-κB DNA-binding activity have been found in the nucleus of human atherosclerotic plaque cells (Monaco *et al.*, 2004). Early stage atherosclerotic lesions in both human and mice present abundant RIPK1 expression responsible of inducing NF-κB-dependent inflammation (Karunakaran *et al.*, 2021). On the other hand, it was demonstrated that the major vault protein (MVP) acts as a suppressor for NF-κB signaling in macrophages by specifically binding to TRAF6 and preventing TRAF6 oligomerization and ubiquitination, and impacting on obesity and atherosclerosis (Ben *et al.*, 2019). In fact, high fat diet-fed MVP KO mice showed defects in glucose and lipid metabolism while atherosclerotic lesions in the aorta were increased in $MVP^{KO}ApoE^{KO}$ mice in comparison with $MVP^{WT}ApoE^{KO}$ counterparts (Ben *et al.*, 2019). In autoimmune uveitis, an example of autoimmune disease in which NF-κB signaling is involved, Galectin-3 (Gal3) is the upstream regulator. The TD139-mediated Gal3 inhibition suppressed the microglial activation via interfering with the TLR4/MyD88/NF-κB signaling and p65 nucleus translocation (Liu *et al.*, 2022). In neurodegenerative conditions like Alzheimer's disease (AD) the

NF-κB pathway has been depicted as essential in mediating neuroprotection against oxidative stress. In presence of germline mutations affecting upstream modulators of NF-κB such as ADAM17, SHARPIN, HOIL, or OTULIN, the lack of NF-κB activation caused anatomical defects like thinning of the entorhinal cortex, which is one of the earliest signs of AD, and alterations in the entorhinal cortex and shrinkage of the cortex that are features of late AD. As above, loss of function of PARKIN E3 ubiquitin ligase drove Parkinson's disease by impeding TNFα/TNRF1-mediated NF-κB activation (Kaltschmidt *et al.*, 2022).

In metabolic disorders, NF-κB modulates apical components of metabolic processes like AMPK and mTOR but also metabolic hormones such as insulin and glucagon (Kracht, Müller-Ladner and Schmitz, 2020). Among others, the insulin-activated PDE3B is a negatively regulated NF-κB-effector. NF-κB promotes the cAMP/PKA pathway and the subsequent inhibition of glucagon activity through the downregulation of PDE3B transcription in hepatocytes, suggesting a new molecular mechanism in the pathogenesis of insulin resistance (Ke *et al.*, 2015).

In cancer, pathologic NF-κB signaling becomes persistent and unrestrained because of long-lasting injuries, uncontrolled environmental stimuli or genetic alterations (Verzella *et al.*, 2022). The genetic alterations can affect mainly RelA and RelB, as demonstrated in lymphoma, where increased expression of REL protein was associated to the truncated C-terminal of p100 that lacks the ANK repeat inhibitory sequence (Courtois and Gilmore, 2006; Weniger and Küppers, 2021). Moreover, loss of function mutations in IkB family proteins in lymphoma, glioblastoma, and nasopharyngeal carcinoma suggest that additional mutations can occur in the core components and regulators of the NF-κB pathway (Lake *et al.*, 2009; Bredel *et al.*, 2011; Zheng *et al.*, 2016). However, since NF-κB is a family of conserved transcription factors, the most frequent mutations that increase NF-κB signaling can be found in the downstream target genes and in the upstream regulatory molecules (Gilmore, 2021; Zhang *et al.*, 2021).

Notably, in recent times, a huge bulk of studies started to focus on the role of both upstream and downstream regulators of NF-κB in the etiopathogenesis of various human disease, as they could represent better therapeutic targets than the core NF-κB components, This goes beyond the fact that NF-κB regulates the expression of a multitude of gene sets in finely controlled processes right from the stimuli induction, as clinically, there is an urgent need to focus on downstream and upstream NF-κB effectors since the inhibition of NF-κB core pathway activity produced severe on-target (Verzella *et al.*, 2022) (Figure 2 and Figure 3).

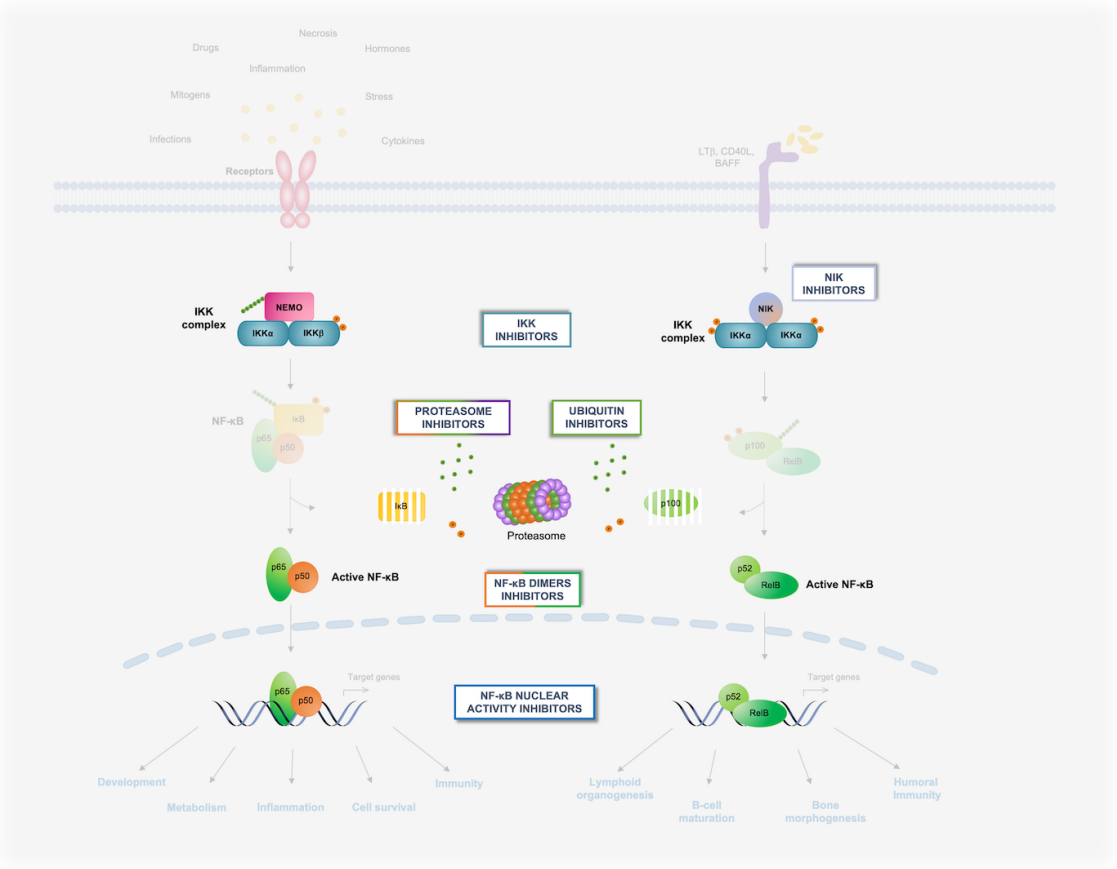


Figure 2:

NF-κB-targeted therapeutics acting by inhibiting the canonical and non-canonical NF-κB core pathway (from Verzella *et al.*, 2022)

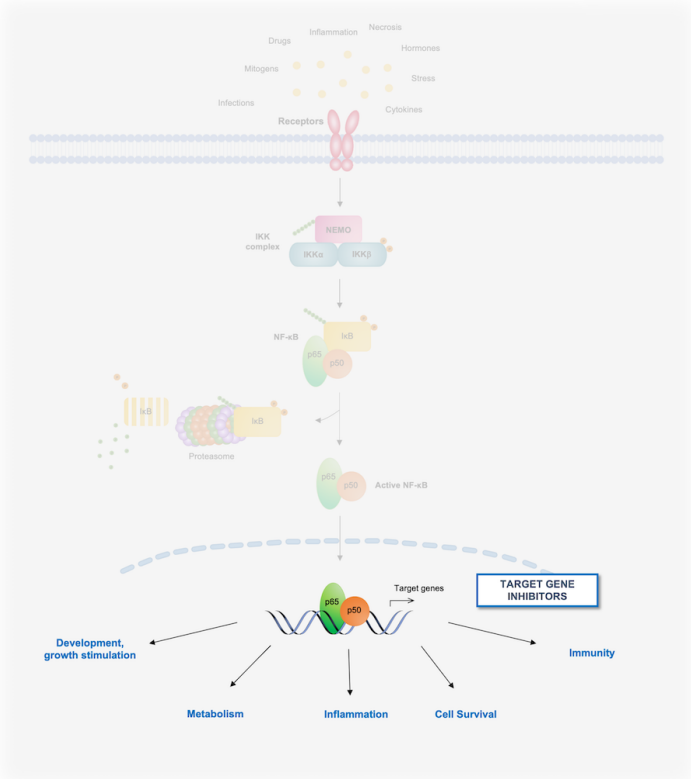


Figure 3:

NF-κB inhibitors acting by blocking downstream effectors of canonical NF-κB pathogenetic functions (from Verzella *et al.*, 2022).

Hence, the well-known IKK inhibitors or proteasome inhibitors, used to treat NF-κB-driven cancers, did not achieve cancer cell specificity due to the ubiquitous and pleiotropic physiological functions of NF-κB (Figure 2). Emerging therapeutic strategies involve the targeting of NF-kB downstream effectors (Figure 3). The growth arrest and DNA damage inducible beta (GADD45B) and the JNK kinase, mitogen-activated protein kinase kinase 7 (MKK7) complex was identified as a critical survival module able to prevent the JNK-mediated apoptosis in Multiple Myeloma (MM) cells (Tornatore *et al.*, 2014). The D-tripeptide inhibitor of this complex, DTP3, showed a high therapeutic index *in vitro* and *in vivo* without preclusive effects on normal tissues leading to the first-in-human trial and suggesting an alternative method to treat MM and other cancers whose survival is linked to NF-κB/GADD45B axis (Tornatore, et al., 2019; Tornatore, et al., 2019). In the

context of NF-κB-driven antiapoptotic genes, the overexpression of Bcl-2 and Bcl-XL is associated to drug resistance in many cancer types like breast, prostate and B cell malignancies (Li *et al.*, 2024). Among various inhibitors with different specificity able to target the Bcl-2 pro-survival proteins, Venetoclax showed a favourable safety profile but, unfortunately, it is often necessary to administer it in combination with other Bcl-2 inhibitors because of acquired Bcl-2 cancer-related mutations (Hafezi and Rahmani, 2021).

Among upstream modulators of NF-κB, tool like receptors (TLRs) contribute to abnormal NF-κB activation often because of mutations of the protein adaptor *MYD88* that frequently occur in haematological malignancies (Verzella *et al.*, 2022). The pharmacologic inhibition of the downstream protein kinases, Interleukin-1 Receptor-Associated Kinase (IRAK)1 and IRAK4 that are adaptor proteins recruited, along with MYD88, to TLR/IL1R receptors resulted in a promising therapeutic strategy against non-Hodgkin's lymphomas (NHLs), myelodysplastic syndrome (MDS), T-cell acute lymphoblastic leukemia (T-ALL), and melanoma in preclinical settings (Srivastava *et al.*, 2012; Lee *et al.*, 2015; Rhyasen and Starczynowski, 2015).

In summary, both solid and haematological malignancies show addiction to NF-κB signaling, which drives oncogenesis, disease recurrence and therapy resistance. The inhibition of aberrant NF-κB signaling is a road that has been beaten for years without much success from both academia and pharma. Instead, promising findings demonstrate that safe and cancer-selective inhibitors could be developed against NF-κB effectors able to finalise NF-κB protumorigenic functions.

1.2.1 NF-κB pathway in the metabolic reprogramming in cancer settings, including OC

The metabolic reprogramming depicted by Hanahan and Weinberg in 2011 as an emerging hallmark of cancer is now considered a crucial element in defining cancer plasticity (Hanahan and Weinberg, 2011; Hanahan, 2022).

Metabolic changes sustain the acquisition and maintenance of malignant phenotypes, in which both glycolysis and mitochondrial respiration can support tumorigenesis in a cell- and context-specific manner. The emblematic example of metabolic reprogramming is the Warburg effect or aerobic glycolysis. In the 1920s, Otto Warburg noticed that, even when oxygen was available, cancer cells preferred glycolysis to oxidative phosphorylation (OXPHOS). The increase in glycolytic flux, via upregulation of glucose transporters (GLUTs), appeared to be the strategy by which cells were able

to fulfil a higher ATP demand. Although cancer cells show a high glucose consumption rate and glycolysis plays an important role in tumorigenesis, the glycolytic pathway cannot sustain the proliferative and tumorigenic potential alone. In fact, mitochondrial functions are not necessarily compromised, and most tumours generate the majority of ATP using mitochondrial respiration (De Berardinis and Chandel, 2016; Oliveira *et al.*, 2021). In this regard, several evidence suggests that cancer cells upregulate oxidative genes at the intersection of different environmental cues to obtain metabolic benefits. For instance, Bennet and colleagues demonstrated that lung tumour cells mainly rely on the activation of genes encoding for mitochondrial ribosomal proteins and respiratory chain transporters during a specific phase of metastatic spread (Bennett *et al.*, 2022). Moreover, Martinez-Reyes and colleagues showed impairment in tumour growth when tumour cells lack the mitochondrial complex II (Martínez-Reyes *et al.*, 2020). Throughout disease progression, the necessity of cells to change their metabolic phenotypes recommends the impossibility of classifying the tumour as metabolically static. In fact, cancer is characterized by a spectrum of metabolic phenotypes in which also mitochondrial respiration supports tumorigenesis (Sheehan and Muir, 2022).

In this context, Mauro and colleagues reported a dichotomy for NF-κB in controlling the balance between the utilization of glycolysis and OXPHOS. Specifically, in physiologic conditions, NF-κB regulates OXPHOS by suppressing the Warburg effect and oncogenic transformation, while, in cancer, NF-κB mediates cancer cell metabolic adaptation and survival to hypoxic and hypoglycaemic environments by increasing OXPHOS via p53 dependent-upregulation of SCO2 (Mauro *et al.*, 2011). For the first time, *in vitro* and *in vivo* scientific results supported the bioenergetic function of NF-κB that can promote tumour progression and survival not only through the regulation of apoptosis and inflammation (Mauro *et al.*, 2011).

Over the years, the NF-κB pathway has emerged as a critical mediator in the response of cancer cells to nutrient-depleted environments by orchestrating metabolic programs that impact glycolysis, glutaminolysis, OXPHOS, and other metabolic pathways (Capece *et al.*, 2022) (Figure 4).

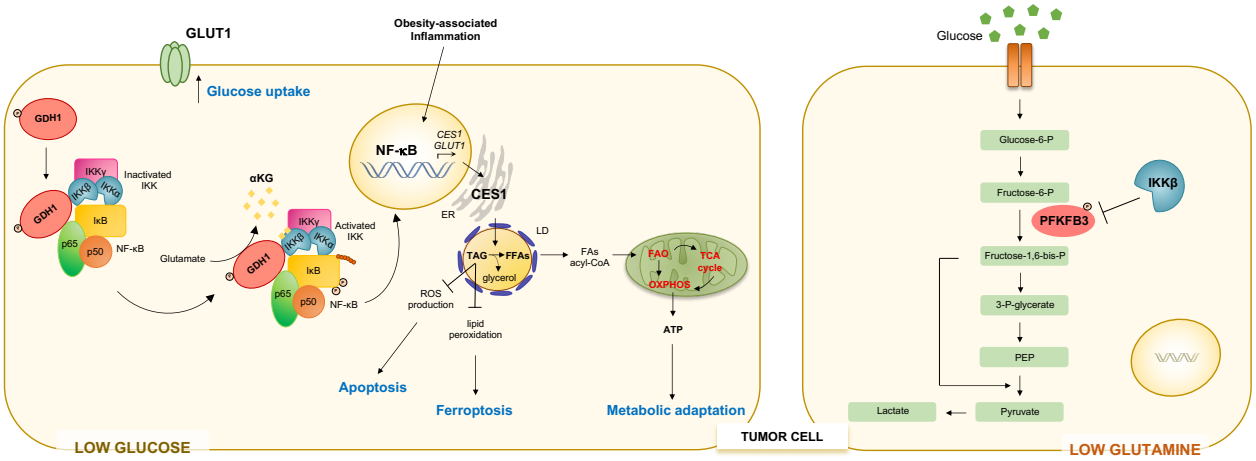


Figure 4:

NF-κB coordinates metabolic responses in cancer cells (from Capece *et al.*, 2022).

It was demonstrated in glutamine-starved human fibrosarcoma HT1080 cells that IKKβ is sensitive to glutamine fluctuations, during which IKKβ reduces aerobic glycolysis by inhibiting 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase isoform 3 (PFKFB3). The lack of IKKβ results in a strong dependence on the TCA cycle and pentose phosphate pathway from glutamine metabolism. Thus, the co-inhibition of IKKβ and glutamine metabolism via PFKFB3 produces synergistic cell death *in vitro* and *in vivo* (Reid *et al.*, 2016). The overexpression of PFKFB3 is clinically relevant in breast cancer, hepatocarcinoma, and OC because it is related to advanced stage/grade and poor outcomes (Chen *et al.*, 2019; La Belle Flynn *et al.*, 2019; Jiang *et al.*, 2022). Specifically, upregulated PFKFB3 in ascites-derived OC cells promoted clonogenicity and sphere-formation while its inhibition through 3PO inhibitor blocked lactate production and re-sensitized OC cells to cisplatin through the downregulation of inhibitor apoptosis proteins (IAPs) (Jiang *et al.*, 2022). The developed chemotherapy-induced OC recurrence and stemness is often due to the presence of OC stem cells (OCSCs) whose survival mainly depend on the NF-κB-regulated aldehyde dehydrogenase 1A1 (ALDH1A1). In this regard, the new developed 974 tool inhibits ALDH1A1 and downregulates pathways related to stemness and chemoresistance, including NF-κB (Muralikrishnan *et al.*, 2022). On the other hand, the enhancement of NF-κB signalling in OC is strictly related to that of LKB1/NUAK1 to promote the expression of antioxidant genes in response to ROS formation. The LKB1/NUAK1 activity is required to sustain metastatization in *in vivo* EOC models by enabling restoration of energy homeostasis and permitting survival during

metabolic stress. Despite this, in NUAK1-deleted HGSOC spheroid cells, the upregulated NF-κB activity is sufficiently protective against oxidative stress (Buensuceso *et al.*, 2022). Thus, NF-κB signalling is frequently activated in OC cells to confer cellular stress protection during disease progression and metastasis as also sustained by the identification of the high protein levels of Rgnef in late-stage serous OC. Rgnef supports three-dimensional ovarian spheroid formation *in vitro* and tumor growth in mice through an antioxidant gene signature including Gpx4, Nqo1, and Gsta4, common targets of the NF-kB transcription factor (Kleinschmidt *et al.*, 2019). Similarly, the activation of NF-κB pathway occurs in platinum-resistant OC cells to promote cell survival. It was demonstrated that OC cell lines resistant to carboplatin, paclitaxel and erlotinib showed significantly higher p65 activation in the nucleus while the treatment with the NF-κB inhibitor BAY 11-7082 impacted the clonogenicity, resistance and survival of OC cells in vitro by resensitizing cells to multi-drugs chemotherapy (Momeny *et al.*, 2018).

NF-κB phosphorylation is related also to the activation of lipid catabolism during energetic stress. The link between nutrient imbalance and lipid metabolism via NF-κB was discovered in Drosophila melanogaster to maintain the cellular triacylglycerol storage. NF-κB/Relish drives metabolic adaptation to fasting-induced lipolysis through specific repression of adipose triglyceride lipase (ATGL)/Brummer in adipose tissue (Molaei, Vandehoef and Karpac, 2019). On the contrary, Capece and colleagues demonstrated that NF-κB activation in human colorectal carcinoma (CRC) cell lines can increase triacylglycerol lipolysis during nutrient deprivation, thereby mobilizing endogenous free fatty acids (FFAs) from lipid droplets and fueling FAO and OXPHOS for energy provision. Specifically, the carboxylesterase 1 (CES1) acts as a NF-κB-regulated lipase that promote cancer cell survival and adaptation to nutrient limitation in aggressive human CRC (Capece *et al.*, 2021).

Collectively, these studies underlie the new important role of NF-κB pathway as a sensor of metabolic imbalances during cancer progression, including OC (Harrington and Annunziata, 2019). Further studies should consider the effectiveness of targeting NF-κB downstream effectors involved in metabolic reprogramming to overcome the toxicity associated to systemic NF-κB blockade and interfere with the preferred metabolic pathway of cancer as a cell death switch. Nowadays, although targeting cancer metabolism is an emerging field in drug discovery, studies related to energy production associated with OC are limited.

1.3 The lipid metabolic reprogramming of OC cells during disease progression paves the way of new therapeutic options

Rapidly dividing cancer cells produce significantly more fatty acids (FAs) than non-transformed ones. FAs constitute complex lipids, such as triacylglycerols, membrane phospholipids, and signaling intermediates, including diacylglycerol, phosphoinositols, sphingosine, and phosphatidic acid (Beloribi-Djefaflia, Vasseur and Guillaumond, 2016). Cancer cells usually stimulate cyclical series of degradation of FAs through a process called mitochondrial β-oxidation to support cancer progression and evade anti-tumor immune responses (Currie *et al.*, 2013).

Deregulated FA metabolism in cancer is considered relevant as FAs are not only structural components of cells but also important secondary messengers that transduce oncogenic signals to contribute to energy production during conditions of metabolic stress (Koundouros and Poulogiannis, 2020). Under oxygen and nutrient deprivation, cancer cells preferentially switch to using different FA acquisition pathways (Munir *et al.*, 2019).

For many years, researchers extensively studied glucose metabolism, but other metabolic pathways, including lipid metabolism, are currently coming into the spotlight. It has been showed that tumorigenesis in the ovaries involves lipid intermediates in ascites and fat-containing cells of the omentum that negatively affect T-cell functions (Figure 6) (Worzfeld *et al.*, 2017).

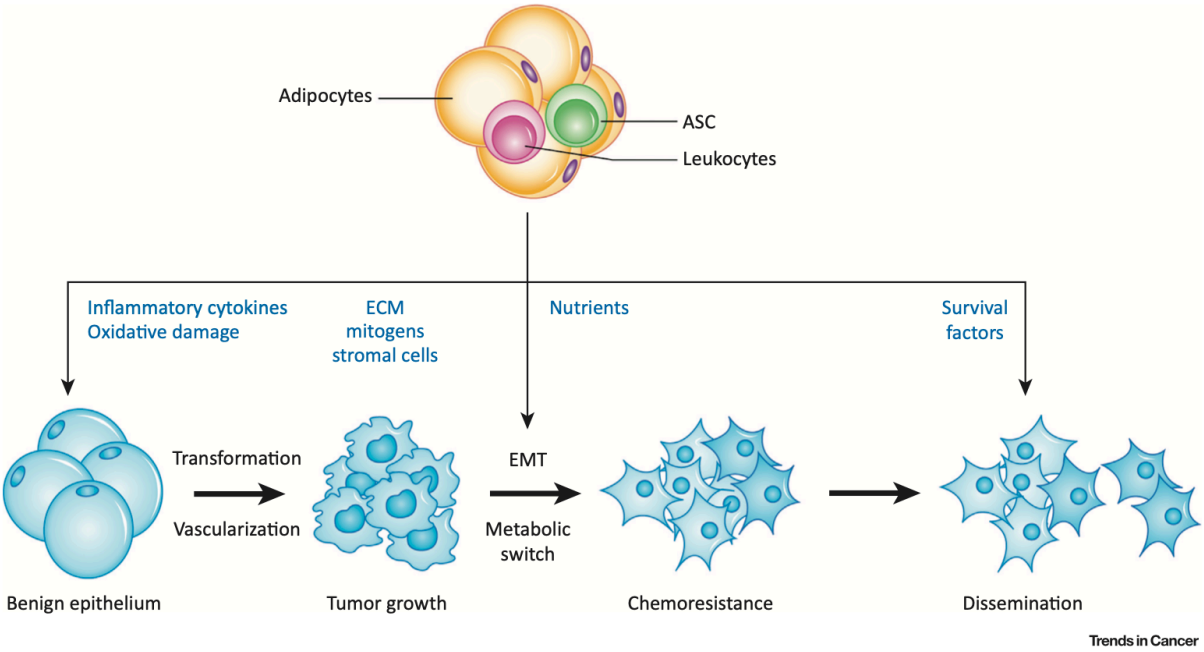


Figure 6:

Interaction between adipocytes and tumor cells during cancer initiation and progression (from Lengyel *et al.*, 2018).

Interestingly, changes in the expression and activity of key metabolic enzymes of lipid metabolism may play a unique role in OC pathogenesis and aggressiveness (Chaudhry, Thomas and Jr, 2022). Furthermore, lipid alterations in OC can be a compensatory effect in response to driver mutations involved in tumorigenesis like PTEN, TP53, and Rb1 as they impact various pathways that converge on lipid metabolism (Beloribi-Djefaflia, Vasseur and Guillaumond, 2016; Chaudhry, Thomas and Jr, 2022). Thus far, the rate-limiting enzymes associated with fatty acid synthesis (FASN, ACC, ACLY, SCD), the lipid degradation related enzymes (ATGL, MAGL, CPT, 5-LO, COX2), and the receptors related to lipid uptake (FABP4, CD36, LDLR) are depicted as new potential diagnostic and prognostic markers in OC as well as promising therapeutic targets (Ji *et al.*, 2020). For instance, the NF-κB-regulated Stearoyl-CoA Desaturase-1 (SCD1), which converts saturated FAs in MUFAs, is responsible of the dynamic balance between them that impacts on OC survival and progression (Zhao *et al.*, 2022). It was found that SCD1 is frequently upregulated in ovarian cancer stem cells (CSCs) and specifically in CSC-enriched spheroids compared to monolayer cultures of OC cell lines or primary cells (Li *et al.*, 2017). The increased lipid unsaturation mediated by SCD1 is a metabolic hallmark in ovarian CSCs that permits the protection against ferroptosis, iron-mediated oxidative damage. The inhibition of SCD1 reduces cancer stemness by blocking NF-κB activity and sphere formation and results in cell death (Li *et al.*, 2017; Tesfay *et al.*, 2019).

The receptor CD36 and the transport protein FABP4 regulate increased lipid uptake in OC cells, sealing the symbiotic relationship between OC cells and adipocytes in order to sustain widespread peritoneal and omental metastasis (Gharpure *et al.*, 2018; Ladanyi *et al.*, 2018). Knockdown of adipocyte-induced FABP4 in HGSC cells resulted in the downregulation of gene signatures associated with OC metastasis and clonogenicity, reduction of tumor burden in a syngeneic orthotopic mouse model of OC and increase of OC cell sensitivity to carboplatin both *in vitro* and *in vivo* (Mukherjee *et al.*, 2020). Specifically, the upregulation of FABP4 in omental metastasis compared to primary OCs was detected in OC cells at the adipocyte-tumor cell interface, suggesting that it exists a direct transfer of lipids from adipocytes to OC cells *in vitro* during co-cultures and *in vivo* tumor growth (Nieman *et al.*, 2011).

Given the extensive role of FAs in OC pathogenesis, there is substantial clinical interest in developing therapies that target lipid metabolic reprogramming. This interest is strictly related to the urgency to overcome the mechanisms of chemoresistance in OC, which limits the chances of success. Interestingly, resistance to various cancer treatments is often associated with changes in tumor cell lipid metabolism (Hoy, Nagarajan and Butler, 2021).

Fatty acid synthase (FASN) is the druggable dysregulated lipid enzyme that has so far attracted the most interest in cancer treatment, including OC. When upregulated, tumor intrinsic FASN activation may represent a resistance mechanism to chemo- and immune-therapies, probably by mediating a overproduction of palmitic acid (Bauerschlag *et al.*, 2015). In this context, the FASN inhibitor Cerulenin affected the growth of the syngeneic murine ID8 OC model by inhibiting immunosuppression (Jiang *et al.*, 2018) as well as the pharmacological blockade of FASN, mediated by C75 and G28UCM, impacted on OC cell growth (Veigel *et al.*, 2015). Moreover, FASN inhibition by Orlistat, using a cisplatin-resistant OC xenograft model in mice, decreased tumor fatty acid metabolism and sensitised OC cells to platinum, resulting in enhanced treatment efficacy (Papaevangelou *et al.*, 2018). The FASN inhibitor C93 also significantly increases the cytotoxicity of the chemotherapeutic drugs paclitaxel and carboplatin, as demonstrated in OC cells (Shih *et al.*, 2010; Pyragius *et al.*, 2013). More thoroughly, the knockdown of FASN together with SCD1 and Carnitine palmitoyltransferase 1 (CPT1A) restored the sensitivity of paclitaxel in paclitaxel-resistant A2780 cell line (Ma *et al.*, 2023). Notably, also CPT1A, the rate limiting enzyme of beta-oxidation, is a potential druggable target in OC. CPT1A expression is higher in omental metastases compared with primary tumors in patients with HGSOCs (Miranda *et al.*, 2016) and the inhibition of CPT1A by etomoxir resulted in reduced tumor progression in a patient-derived xenograft model of HGSOC (Sawyer *et al.*, 2020).

However, although inhibitors of lipid metabolism showed promising results when used *in vitro* and *in vivo* mouse models, they are often showing side effects on normal cells when tested in clinical trials.

Some FASN inhibitors are associated with neuronal stem cell dysfunction and serious side effects, including decreased food intake and weight loss in mice (Loftus *et al.*, 2000; Knobloch *et al.*, 2013). T cells, tumor-associated macrophages, regulatory T cells and myeloid derived stromal cells (MDSCs) are affected by lipid metabolism inhibition (Zhao, Cardenas and Matei, 2019). For instance, high doses of etomoxir inhibit the survival of T regulatory and T memory cells as well as

the polarization of macrophages via a mechanism independent of CPT1A (Divakaruni *et al.*, 2018; Raud *et al.*, 2018).

As FAs are essential to promote malignant properties in OC, lipid metabolism reprogramming can be listed among cancer MDR events. Consequently, lipid metabolism is an attractive therapeutic target in OC to overcome chemoresistance and key molecules regulating lipid metabolism at several levels have been described in OC as druggable targets. Although survival rates for patients with OC have not improved substantially over years and the systemic clinal safety of fatty acid-targeted drugs is yet a concern, the scientific community supports the idea that lipid metabolism in OC cells is a crucial metabolic process that should be further investigated to improve treatment efficacy for OC patients (Chaudhry, Thomas and Jr, 2022).

1.4 The superfamily of Carboxylesterases

Mammalian carboxylesterases (CES) belong to the serine hydrolase superfamily and are highly conserved during evolution (Wang *et al.*, 2018). The CES family is composed of six human genes, described as CES1, CES2, CES3, CES4A, CES5A and a CES1-like pseudogene CES1P1, and all are localized on chromosome 16 (Lian, Nelson and Lehner, 2018). These enzymes are soluble proteins that reside in the endoplasmatic reticulum (ER) lumen thanks to the HXEL variations of the KDEL consensus ER retrieval sequence at the C-terminal end. The release from the membrane-associated state is due to the cleavage of the C-terminal signal peptide (Satoh and Hosokawa, 2006; Wang *et al.*, 2018). CES are responsible for the ester cleavage into the corresponding alcohol and carboxylic acid. Thus, CES can hydrolyze a wide range of endo- and xenobiotic compounds, playing key roles in both endobiotic metabolism and activation and detoxification of xenobiotics (Ross and Crow, 2007). Specifically, CES metabolize several clinically important classes of drugs, such as anticoagulants, angiotensin-converting enzyme inhibitors, antihyperlipidemic agents, antivirals, chemotherapeutics, immunosuppressants, and psychoactive drugs (Casey Laizure *et al.*, 2013). Among endobiotics, cholesteryl esters (CEs), triacylglycerols (TGs) and other endogenous lipids have been objects of study for the role of CES. CES1 and CES2 are the two most extensively studied isoenzymes in human body. CES1 and CES2 are most expressed in metabolic organs, including liver, intestine, and kidney. In particular, CES1 is abundantly expressed in the liver and adipose tissue, with lesser amounts in the kidney, monocytes, lung, intestine, testis, heart, and macrophages. Instead, CES2 is expressed mainly in the small intestine and colon but is also

observed in kidney, liver, heart, brain and testis (Sanghani *et al.*, 2009; Lian, Nelson and Lehner, 2018). Both CES1 and CES2 can metabolize endogenous esters, specifically they were demonstrated to possess TG hydrolase activity, suggesting that they play a role as lipases in lipid and energy homeostasis (Dolinsky *et al.*, 2004; Lian, Nelson and Lehner, 2018). Notably, cellular energy is stored in dynamic intracellular organelles called lipid droplets (LDs) in the form of neutral lipids (TGs and CEs). The adipose tissue and the liver represent the most used tissues for lipid storage in which LDs, originating from the ER, where TAG biosynthesis takes place, accumulate in nearly all cells (Zadoorian, Du and Yang, 2023).

Interestingly, in human macrophage THP-1 cells, CES1 has been reported to be associated with cytosolic LDs upon lipid loading (Zhao *et al.*, 2005). In 2010, triacylglycerol hydrolase (TGH), the murine ortholog of CES1 also known as Ces1d or Ces3, was described as the first ER luminal lipase able to regulate the metabolism of cytosolic LDs. Specifically, it was demonstrated that TGH-deficient hepatocytes showed a decreased size and increased number of LDs, suggesting that TGH deficiency did not alter the formation of nascent LDs but altered LD morphology and delayed LD maturation (Wang *et al.*, 2010). Accordingly, several studies started to focus on the role of human CES1 and mouse Ces1d as lipases and their functions in intracellular metabolism and demonstrated the relevance of CES1/Ces1d in regulating metabolism and energy homeostasis as well as their involvement in metabolic disorders.

1.4.1 The role of CESs in the regulation of systemic metabolism and in the pathogenesis of metabolic disorders

Ces1d has a role in providing substrates necessary to assemble apolipoprotein B (apoB)-containing lipoproteins and then forming hepatic very low-density lipoproteins (VLDLs). The active role of Ces1d in mobilizing intracellular TGs for VLDL assemble was supported by several studies in mice and rats in which the expression of Ces1d correlated with increased *i)* lipolysis of TG stores, *ii)* TG secretion and *iii)* ApoB levels, while the inhibition of lipolysis by a specific Ces1d-inhibitor resulted in decreased secretion of apoB and TGs into the circulation (Lehner and Vance, 1999; Gilham *et al.*, 2003; Dolinsky *et al.*, 2004; Wei *et al.*, 2007). It was hypothesized that severe hepatic lipid accumulation could occur upon Ces1d inhibition due to the blockade of hepatic VLDL secretion. However, in *Ces1d* deficient mice, the existence of a compensatory effect to prevent hepatic lipid accumulation was shown, consisting of decreased non-esterified fatty acid (NEFA)

flux from the adipose tissue to liver and increased hepatic FAO (Wei *et al.*, 2010; Lian, Wei, *et al.*, 2012). In addition to reduced hepatic VLDL secretion, *Ces1d* deficient mice also exhibited decreased chylomicron secretion from the intestine. Overall, reduced hyperlipidaemia (Lian, Wei, *et al.*, 2012), enhanced energy expenditure, and improved insulin sensitivity were observed in the *Ces1d* knockout mice (Figure 7) (Wei *et al.*, 2010).

Over the years, Ces1d/CES1 together with other members of CES family emerged to be involved also in pathological contexts (Wang *et al.*, 2018). Dysregulated lipid metabolism commonly contributes to the development of metabolic diseases (Chalhoub *et al.*, 2021). Specifically, TG build-up in peripheral tissues is a known risk factor for insulin resistance, type 2 diabetes, and cardiovascular problems and is closely linked to obesity (Wei *et al.*, 2010). The involvement of CESs in metabolic disorders has been demonstrated in different genetic mouse models.

NAFLD is the most common form of chronic liver disease that include a range of liver disorders from steatosis to nonalcoholic steatohepatitis (NASH) and cirrhosis. Moreover, NAFLD is commonly associated with metabolic disorders like insulin resistance, type 2 diabetes, dyslipidemia and cardiovascular disease but can also occur in normolipidemic patients not affected by obesity and diabetes (Loomba, Friedman and Shulman, 2021). In this context, in addition to the abovementioned study of Wei and colleagues on Ces1d deficiency as a protective feature in chow fed *Ces1d*$^{-/-}$ mice, it was also investigated the potential protective role of Ces1d deficiency against high fat diet (HFD)-induced hepatic steatosis in a diet-induced obese mouse model (Lian *et al.*, 2016). After 16 weeks HFD feeding, *Ces1d*$^{-/-}$ mice showed a reduced both liver lipid mass and severe steatosis compared to WT mice (Lian *et al.*, 2016).

In this context, the CES1 polymorphism p.Gly143Glu that results in reduced lipolytic activity seems to provide protection against HFT-induced steatosis (Lian *et al.*, 2018). The same authors investigated the role of Ces1d in other two mouse models: hypolipidemic and insulin sensitive PEMT deficient mice and hyperlipidemic and insulin resistant LDLR deficient mice to represent different phases of NAFLD accompanied with different metabolic disorders (Lian, Quiroga, *et al.*, 2012; Lian *et al.*, 2016). Ces1d deficiency attenuated disease progression in all the models suggesting its role in ameliorating fatty liver disease and hinder the progression of NAFLD. The potentiality to target Ces1d also emerged in a recent work conducted on adipose tissue-specific *Ces1d* knockout (FKO) mouse model to study the function of lipid droplet-associated Ces1d on metabolism of obese patients with type 2 diabetes (Li *et al.*, 2022). In particular, the role of Ces1d

energy homeostasis was investigated when mice were fed with HFD. The results revealed that FKO were bigger than WT and exhibited larger fat masses upon HFD feeding. Moreover, the developing of severe liver steatosis and lipotoxicity in the peripheral organs underlined the underappreciated metabolic relevance of Ces1d in adipose tissue because neither global nor liver-specific Ces1d inactivation had resulted in severe lipotoxicity in other tissues (Li *et al.*, 2022).

Other studies demonstrated the involvement of CES1 in carbohydrate metabolism in obese and diabetic mice (Bie *et al.*, 2013; Xu *et al.*, 2014). Xu et al. deepened the role of Ces1 in carbohydrate metabolism by performing the overexpression of Ces1d in the liver of ob/ob mice and in C57BL/6 mice used as controls. In both conditions, the expression of hepatic Ces1 reduced hepatic TG levels most likely via enhanced TG hydrolysis and subsequent increase in PPARα activity and FAO, while neither lipogenesis nor VLDL secretion were altered (Xu *et al.*, 2014). As a result of the reduction of hepatic TG levels, hepatic insulin signaling was enhanced while gluconeogenesis genes expression was repressed, suggesting the intriguing role of Ces1 in mediating glucose homeostasis (Xu *et al.*, 2014). On the other hand, the loss of hepatic CES1 sustained a pro-atherogenic lipid profile by up-regulating the expression of many SREBP- or cholesterol/LXR (liver X receptor)-regulated genes (Xu *et al.*, 2014), as also demonstrated by studying the effects of Ces1d deficiency in a $Ldlr^{-/-}$ mouse model of liver-specific *Ces1d* knockout (Ces3-LKO) (Bie *et al.*, 2013). The lack of Ces1d did not affect liver function neither altered hepatic lipid accumulation and plasma cholesterol levels, but resulted in reduced cholesterol elimination from the body, leading to significant increase in atherosclerosis (Bie *et al.*, 2013). Specifically, Ces1d-deficient $Ldlr^{-/-}$ mice exhibited a decreased reverse cholesterol transport (RCT) from peripheral tissues to liver, where cholesterol is excreted in bile, after 16 weeks of western-type diet (WTD) (Bie *et al.*, 2013). This result is supported by the previous study of Zhao and collaborators in atherosclerosis-susceptible $Ldlr^{-/-}$ mice in which macrophage-specific overexpression of CE hydrolase reduced diet-induced atherosclerotic lesions by enhancing cholesterol efflux and RCT (Zhao *et al.*, 2007).

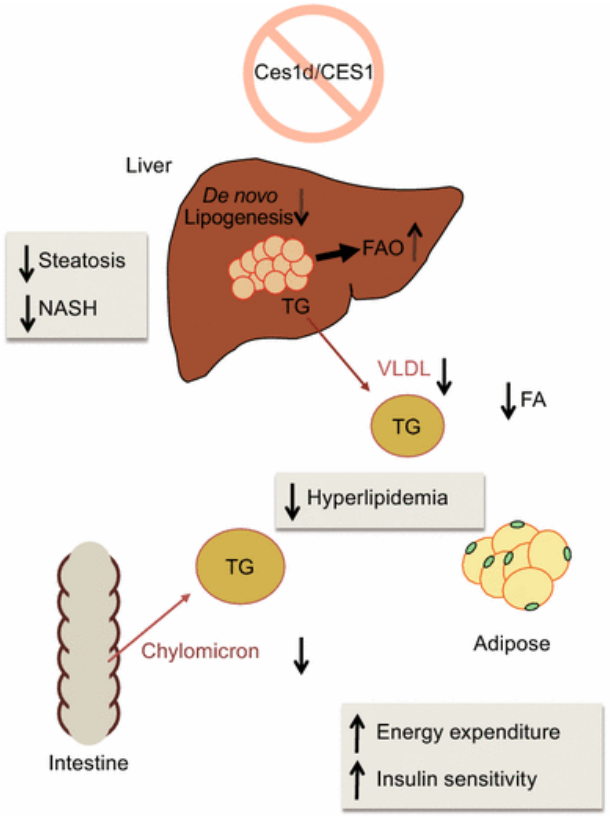


Figure 7:

Effects of Ces1d/CES1 deficiency on lipid and energy homeostasis (from Lian, Nelson and Lehner, 2018).

In 2021, while on one hand, Lian et al. did not support the proposed function of Ces1d/CES1 as a cholesteryl ester (CE) hydrolase as in their model of liver-specific Ces1d-deficient mice it was not found a reduction in RCT flux neither additional liver CE accumulation after high-fat, high-cholesterol WTD feeding (Lian *et al.*, 2021), on the other hand in the same year, Capece et al demonstrated how Ces1d knockdown impacted on neutral lipid accumulation in the context of CRC. Ces1d-depleted CT-26 colorectal carcinoma cells showed a significant accumulation of TAGs and CEs when cultured under basal and glucose limitation conditions (Capece *et al.*, 2021). In humans, Dominguez et al. measured the activity of hCES1 in adipose tissue biopsy of lean, obese and type 2 diabetic individuals. They found an increase in hCES1 activity in obese individuals and patients with type 2 diabetes compared to lean subjects. In fact, enhanced hCES1 activity and an increased number of fatty acids in the circulation are features involved in the pathogenesis of obesity and diabetes (Dominguez *et al.*, 2014). A DNA microarray analysis showed

that CES1 expression was associated with body fat and adipocyte fat content and can be reduced in subcutaneous adipose tissue after 8 and 16 weeks of very low calory diet (CD) (Jernås *et al.*, 2009). hCES1 in humans and Ces1d in mice, that were found involved in basal lipolysis and not in hormonally induced lipolysis, can be blocked more safely than molecules that hamper hormone-induced lipolysis. Accordingly, the treatment with Ces1d inhibitor WWL113 elicited therapeutic benefits in *in vivo* models of genetic and diet-induced obesity, insulin resistance and diabetes (Dominguez *et al.*, 2014).

By screening the role of other serine hydrolases in influencing obesity, CES2 was found to decrease in the liver of obese humans and multiple mouse models (obese mice because of high-fat diet, ob/ob mice with mutation of leptin, or db/db mice with mutation of the leptin receptor). The expression of CES2 activity was evaluated in liver biopsy of lean and obese subjects not affected by NAFLD, identifying CES2 reduction as a causative factor in obesity-induced metabolic diseases mechanistically related to an impairment of glucose storage and lipid oxidation (Ruby *et al.*, 2017). Strategies restoring CES2 activity may prove effective in the treatment of obesity-associated metabolic diseases like NAFLD. It was demonstrated that the knockdown of hepatic Ces2c reduced fatty acid oxidation and stimulated lipogenesis by inducing ER stress and causing liver steatosis and damage in HFD-fed mice. Moreover, the hepatocyte nuclear factor 4α (HNF4α), whose expression was markedly reduced in nonalcoholic steatohepatitis (NASH), diabetes and obesity, regulated hepatic Ces2c expression in pathological contexts suggesting that HNF4a/Ces2c axis plays a protective role in NAFLD progression (Li *et al.*, 2016). Findings on human CES2 expression by Ruby et al. are in agreement with the results of Li et al. on mouse Ces2c reporting that in the presence of CES2 TAG activity, adiposity decreased, glucose tolerance improved, and steatosis was prevented.

Quiroga and colleagues were the first to identify Ces1g as a new player in energy metabolism (Quiroga *et al.*, 2012). Specifically, unlike Ces1d, *Ces1g* KO mice showed increased hepatic lipogenesis, over secretion of apoB-containing VLDLs responsible for hyperlipidemia, and increased lipid deposition in peripheral tissues. Spontaneously, *Ces1g* KO mice became obese and developed hepatic steatosis, hyperinsulinemia, and insulin resistance (Figure 8) (Quiroga *et al.*, 2012).

In these mice, despite a standard chow diet, obesity probably occurred because of the persistence of impaired energy utilization, while the increment of hepatic lipogenesis in *Ces1g*$^{-/-}$ mice was due

to the activation of SREBP-1c and -2, master transcription factors respectively controlling lipogenesis and sterol biosynthesis. From a mechanistic point of view, the authors demonstrated that the absence of Ces1g impeded the release of PUFA from intracellular TG stores and the PUFA-mediated inactivation of SREBP-1c. The nutritional supplementation of fish oil reverted the lipogenic phenotype in *Ces1g*$^{-/-}$ mice, suggesting the crucial role of Ces1g in lipid biosynthesis, secretion, and deposition (Quiroga *et al.*, 2012).

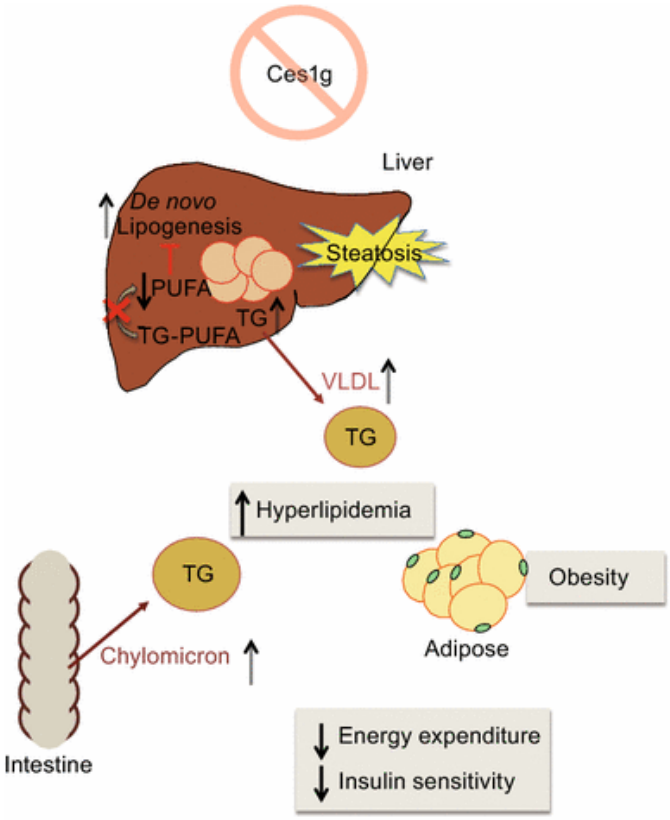


Figure 8:

Effects of Ces1g deficiency on lipid and energy homeostasis (from Lian, Nelson and Lehner, 2018).

All these studies unveil the importance of Ces1d/CES1-mediated metabolic flexibility in response to metabolic cues as well as of other carboxylesterase isoenzymes in different, even conflicting ways. The liver and adipose tissue are the two metabolically active organs in which Ces1d/CES1 exerts tissue-specific function related to its intracellular localization. In the liver, Ces1d/CES1 localizes on ER, while in adipose tissue, significant levels of Ces1d/CES1 are detected in the lipid

droplets. However, Ces1d translocates onto lipid droplets in both adipose tissue and liver upon HFD feeding, suggesting its potential role in lipid catabolism under nutritional stress conditions (Li *et al.*, 2022). The demonstrated relevance of Ces1d/CES1 in metabolic disorders indicates the potentiality to pharmacologically target Ces1d/CES1 to treat metabolic disorders and obesity-related conditions. However, other isoenzymes of the CES family appear to be potential pharmacological targets that cannot be disregarded from developing specific inhibitors.

1.4.2 Ces1d/CES1 in the metabolic adaptation of cancer cells

Obesity is a well-known risk factor linked to cancer incidence, recurrence, and progression. Certainly, rapidly proliferating tumour cells may depend on lipid metabolism to generate free FAs and cholesterol. In this context, enzymes involved in fatty acid metabolism are emerging as cancer biomarkers (Du *et al.*, 2023). Huang and colleagues demonstrated that in *Ces1d* deficient mice the associated phenotype of attenuated VLDL production reversed tumor-induced hyperlipidemia and consequently suppressed tumor growth, supporting the hypothesis that VLDL-derived lipids had a role in tumorigenesis. The study also found that Ces1d deficiency was able to alleviate tumor-induced liver enlargement which could be explained by a reduction of hepatic PCSK9-mediated degradation of LDLR (Huang *et al.*, 2016). The bottom line is that Ces1d deficiency might protect mice against tumor-triggered liver dysfunction and consequently aberrant lipoprotein homeostasis. Liver diseases associated to lipid disorders, like NASH and NAFLD, have been directly linked to the development of HCC, in which abnormal lipid metabolism is recognized as a pivotal factor during cancer progression (Pei *et al.*, 2020). In a recent work, CES1 levels were measured in an array of human liver cancer samples (n=120). CES1 protein was detected in 110 HCC samples but the levels were lower or equal than normal livers. In contrast, the evaluation of CES1 expression on available datasets showed an upregulation of *CES1* mRNA levels in malignant liver tissues (Li *et al.*, 2023). Moreover, a more extensive analysis based on the correlation between the protein levels of CES1 and survival rate in different cancers found that CES1 could play distinct roles depending on the tumor (Li *et al.*, 2023). The curiosity to evaluate the role of CES1 in HCC was also related to the necessity to find new druggable targets to overcome resistance to chemotherapeutic agents like cisplatin and sorafenib. The results sustained that interfering with lipid metabolism by blocking CES1-PPARα/γ-SCD axis could be a promising strategy to treat HCC and sensitize it to standard chemotherapy. Specifically, the pharmacological blockade of CES1

impeded *i)* the release of free FAs provided by CES1 *ii)* the activation of PPARα/γ by free FAs that act as signaling molecules and *iii)* the following upregulation of SCD mediated by PPARα/γ. Therefore, the enhanced ER stress, the impairment of mitochondrial functions and ATP production as well as the increase of ROS production measured in absence of CES1 induced cancer cell death (Figure 9) (Li *et al.*, 2023).

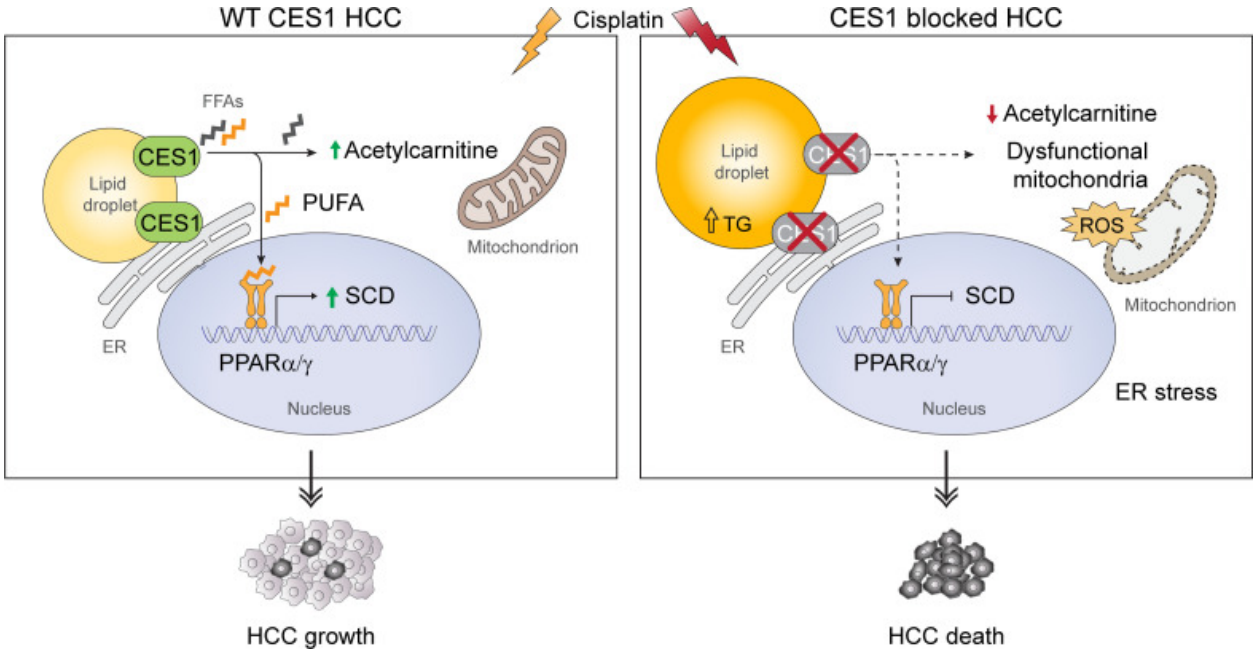


Figure 9:

The proposed working model of CES1 regulation and function in lipid metabolism and tumor growth in HCC (from Li *et al.*, 2023).

The metabolic relevance of CES1 during cancer progression was investigated for the first time in colorectal cancer (CRC) by Capece and colleagues. It was demonstrated that CES1 expression, regulated by NF-κB transcription factor, was augmented in metastasis-prone CRC consensus molecular subtype (CMS) 4, associated with obesity, epithelial-to-mesenchymal transition (EMT), inflammation, and worse clinical outcomes in CRC patients (Capece *et al.*, 2021).

Specifically, the authors found that Ces1d/CES1 promoted CRC cell survival by two cell-autonomous mechanisms (Capece *et al.*, 2021; Capece and Franzoso, 2022): 1) Ces1d/CES1 increased TAG and CE breakdown to provide free FAs to sustain FAO-mediated ATP production during GL; and at the same time 2) it prevented the toxic build-up of neutral lipids triggering

apoptosis and ferroptosis because of ROS production and phospholipid peroxidation. Accordingly, CES1 inhibition by genetic knockdown or commercially available CES1 inhibitors resulted in CRC cell death upon GL *in vitro* and suppression of CRC growth in mouse xenograft models *in vivo* (Figure 10) (Capece *et al.*, 2021).

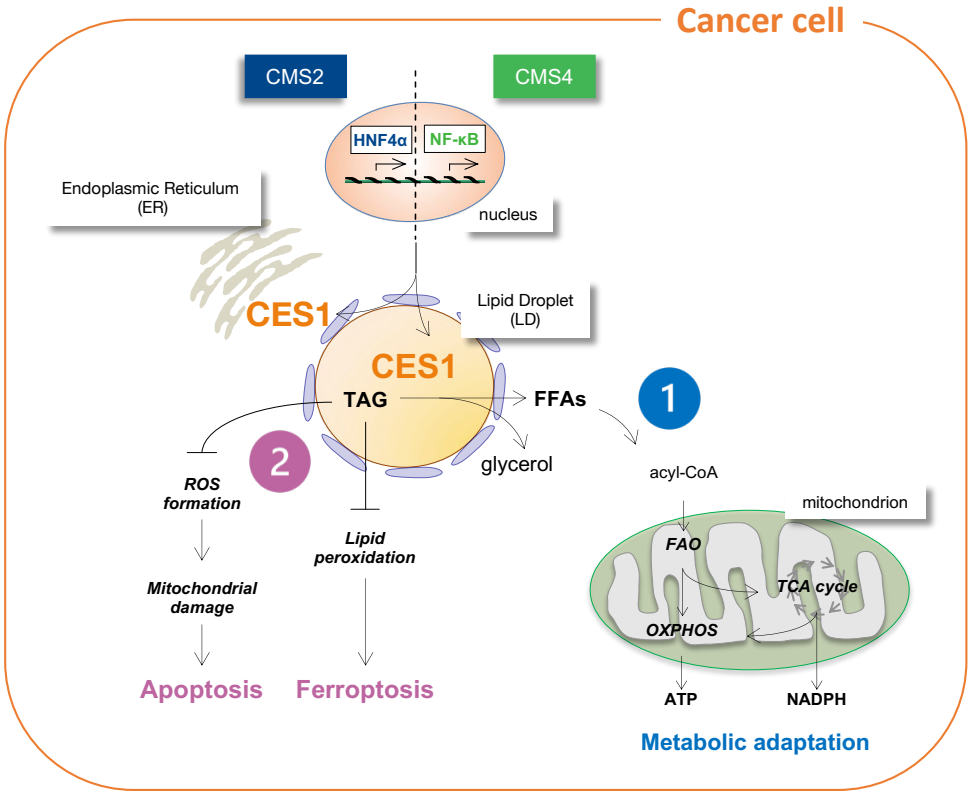


Figure 10:

The proposed working model of CES1 regulation and function in lipid metabolism and tumor growth in CRC.

In summary, the extensive versatility of lipid metabolism confers on tumor cells the ability to adapt under nutrient-depleted conditions. In this context, CES1 upregulation emerges as a strategy that cancer cells adopt to meet increased energy demand required by tumor growth. Thus far, the actionability to target CES1 has been successfully demonstrated in *in vitro* and *in vivo* models of HCC and CRC underpinning the importance to identify and target tumor cell metabolic vulnerabilities.

2. AIM OF THE PROJECT

OC is the deadliest gynaecological malignancy worldwide due to the frequent failure of first-line platinum-based chemotherapy. Increasing evidence indicates that drug resistance is often associated with metabolic reprogramming, a fundamental property of cancer cells, which can adapt to harsh microenvironments. Understanding how metabolism is rewired in tumour cells and which are the molecular mediators underneath could pave the way for developing new target therapies to use in combination with conventional chemotherapy. Notably, several evidence support the extensive role of FAO and OXPHOS in OC pathogenesis, as changes in the expression and activity of key metabolic enzymes of lipid metabolism have been identified in OC. Therefore, there is substantial clinical interest in developing therapies that target lipid metabolic reprogramming, although the difficulty of specifically inhibiting metabolic enzymes associated with cancer.

The NF-κB pathway is implicated in OC advanced disease, where it drives chemoresistance, stemness, metastasis and immune evasion. Moreover, NF-κB has emerged as a critical mediator in the response of cancer cells to nutrient-depleted environments by regulating several metabolic programs including lipid metabolism. However, inhibiting NF-κB core pathway activity produces severe systemic toxicity. To overcome this barrier, both academia and pharma have been focusing on characterising NF-κB effectors.

Recently, CES1, known as a major mediator of endobiotic and xenobiotic metabolism, emerged to be also involved in pathological contexts, first in metabolic disorders and only recently in tumor settings where it was depicted as an essential NF-κB-regulated lipase able to promote the survival of CRC cells by increasing TAG breakdown to fuel β-oxidation.

In this context, considering 1) the crucial role of both lipid metabolism and NF-κB in driving both CRC and OC progression; and 2) the fact that both tumors share the tendency to preferentially metastasize to the peritoneal cavity and infiltrate the omentum, a fat-rich organ, we aimed to investigate whether CES1 could be crucial also in the metabolic adaptation of OC cells representing an alternative druggable target, especially in the chemorefractory subsets.

3. MATERIAL & METHODS

3.1 Cell cultures and reagents

Human OC cell lines OV-56 and OVCA-429 were cultured in RPMI 1640 (Roswell Park Memorial Institute) and supplemented with 10% FBS (Fetal Bovine Serum) (GIBCO, Carlsbad, CA, USA); EFO-21 and ES-2 cell lines were cultured in DMEM (Dulbecco's Modified Eagle's Medium) with 10% FBS. All media were supplemented with L-glutamine 2mM (Euroclone, Milan, MI, Italy), Penicillin-StreptoMycin 50U/mL (Euroclone, Milan, MI, Italy).

Cells were cultured in a humidified incubator in 5% CO2 at 37°C. All cell lines were routinely tested using the Mycoplasma Detection Kit (#ABM G238). Cell line authentication (STR) was carried out for all OC cell lines. For experiments under energy stress (ES), OV-56 and OVCA-429 cell lines were cultured in RPMI-1640 medium without glucose, supplemented with 5% FBS, antibiotics, 2mM L-glutamine, and 1 mM D-glucose (G8769 Sigma-Aldrich, St.Louis, USA). EFO-21 was cultured in DMEM without glucose, supplemented with 2% FBS, antibiotics, 2 mM L-glutamine, and 1 mM D-glucose. ES-2 was cultured in DMEM without glucose, supplemented with 5% FBS, antibiotics, 2 mM L-glutamine, and 0,5 mM D-glucose.

The following reagents were used for cell treatments: GR-147286X (10 μM) from Chemgood LLC, Tween 80 (2%, #P8192) from sigma Aldrich, Oleic acid-BSA solution (50 μM, #29557) from Cayman Chemical, Carboplatin (10-100 μM, #C2538) from Sigma Aldrich, Bafilomycin A1 (50 nM, #B1793) from Sigma Aldrich.

3.2 RNA Extraction and Quantitative Real-Time Polymerase-Chain Reaction (Q-PCR) analysis

Total RNA was extracted using Quick-RNA™ MiniPrep kit (Zymo Research, Irvine, USA) according to the manufacturer's specifications. The isolated RNA was spectrophotometrically quantified and 1μg of RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Inc Foster City, CA, USA). Q-PCRs were carried out using Taq Man Universal Master Mix and ABI 7500 Fast real-time PCR machine (Life Technologies) and the following predesigned TaqMan® Gene Expression Assays: Actin Human Hs01060665_g1 FAM, CES1 Human Hs00275607_m1 FAM. Ct values were normalized to Actin gene expression.

3.3 Western blot analysis

Total cell extracts were prepared in Lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM Na_3VO_4, 50 mM NaF) containing complete mini EDTA-free protease inhibitors (Roche Molecular Biochemicals, Basilea Svizzera) and protein concentration was assessed by the BCA assay kit (Pierce, Thermo Fisher Scientific Carlsbad, CA, USA). Primary antibodies used were: CES1 (ab45957 Abcam), LC3B (D11) XP (3868 Cell Signaling), cleaved caspase 3 (Asp175) (5A1E) (9664 Cell Signaling), PARP (9542 Cell Signaling), GPX4 (ab125066 Abcam), Actin (C-11) (sc-1615 Santa Cruz Biotechnology). Western blot detection was performed using ECL (Amersham).

3.4 Cell viability assays

Cell viability assays were carried out using CellTiter-Glo® 3D Cell Viability Assay reagent (G9681 Promega) according to the manufacturer's protocol. Counting was performed using Cyto Smart cell counter (Corning). For cell treatments, cells were counted and plated in 96 well plates a day before treatment. Next day cells were treated daily for 96 hours. At the endpoint, cells were lysed in CellTiter-Glo® 3D reagent and luminescence was determined with Infinite® 200 PRO, TECAN, Männendorf, Switzerland.

3.5 Flow cytometry

Flow cytometry analysis was conducted with OC cells in presence and absence of GR-148672X for the indicated times under ES. Cyto-ID® Autophagy Detection Kit 2.0 (ENZ-KIT175-0050 Enzo Life Sciences) was used for selectively quantification of accumulated autophagic vacuoles according to the manufacturer’s specification. Nucleosomal DNA fragmentation was assessed by using propidium iodide (PI, #550825, BD Biosciences) nuclear staining assays and processed according to the manufacturer’s specification. Data were acquired using a LSR II flow cytometer (BD, Biosciences) and analyzed using FlowJo software.

3.6 Oxygen consumption rate (OCR) analysis

For bioenergetic profiling, OC cells were seeded onto Seahorse 96-well plates. Mitostress, ATP rate and Mito fuel stress tests were conducted using a XF-96 Extracellular Flux Analyzer (Agilent

Seahorse), and oxygen consumption rates (OCR) was measured in XF Assay Medium (#102365, Agilent Seahorse) containing 1 mM pyruvate, 2 mM glutamine, and 10 mM glucose. In the Mitostress test, OCR was measured following injection of 10 μM GR-147286X or vehicle (DMSO) in combination with 50 mM 2-deoxy-D-glucose (#D8375, Sigma Aldrich); 1,5 μM oligomycin A (#75351, Sigma Aldrich); 75 μM 2,4- Dinitrophenol (2,4-DNP; #D198501, Sigma Aldrich); 0,5 μM rotenone (#R8875, Sigma Aldrich); and 0.5 μM antimycin A (#A8674, Sigma Aldrich). Basal respiration, ATP production and proton leak were calculated in accordance to the manufacturer's specifications as follows: basal respiration (*i.e.*, the last measurement recorded prior to oligomycin A injection after subtracting the non-mitochondrial respiration rate), ATP production (*i.e.*, the last measurement before oligomycin A injection after subtracting the minimun rate measurement after oligomycin A injection), and proton leak (*i.e.*, the minimun rate measurement after oligomycin A injection after subtracting non-mitochondrial respiration). Cell pre-treatment with oleic acid-BSA solution and GR-148672X was conducted where indicated.

ATP production rates were measured using the XF Real-Time ATP Rate Assay Kit (103592-100, Agilent Seahorse) following the injection of GR-148672X where indicated. The detected mitochondrial OCR and glycolytic Extra-Cellular Acidification Rates (ECAR) were then transformed into mitochondrial (mito-ATP) and glycolytic (glyco-ATP) ATP production rates, using validated algorithms provided in the Seahorse Agilent software.

The metabolic dependency of cells for glutamine and long chain fatty acids was measured by using XF Mito Fuel Flex Test Kit (103260-100, Agilent Seahorse) detecting basal state mitochondrial fuel oxidation in live cells by injecting the following inhibitors: BPTES (3 μM), Etomoxir (4 μM), and UK5099 (2 μM) according to the manufacturer's specification. All values were normalized on a per-cell basis.

3.7 Fluorescence imaging

BODIPY™ 493/503 (#D3922, Thermofisher) was used to stain neutral lipids at the final concentration of 3 μM. LysoTracker™ Red DND-99 (#L7528, ThermoFisher) was used to stain lysosomes at the final concentration of 50 nM. Cells were incubated with the dyes for 30 minutes and then slides were mounted. Slides were imaged using an EVOS FL Auto imaging system at 40x objective magnification. Corrected total cell fluorescence (CTCF) was calculated as follows: CTCF = Integrated Density – (Area of selected cell * Mean fluorescence of background readings).

3.8 Primary OC cells

Primary OC cells were kindly provided by Dr. Ugo Cavallaro from the European Institute of Oncology (IEO, Milan) and were obtained upon informed consent from patients undergoing surgery. Sample collection was approved by the Ethics Committee of the IEO.

3.9 Statistical Analyses

For the analysis of biological data, the statistical significance for two-sample comparisons was calculated using two-tailed Student's t-test. The statistical significance for multiple-sample comparisons was calculated using one-way ANOVA. Differences were considered to be statistically significant if the p value, after correction in the case of multiple tests, was lower than 0.05. Survival curves of overall survival (OS) and Disease Specific Survival (DSS) were derived from the publicy available TCGA dataset and were estimated using the Kaplan-Meier method. Statistical differences were tested using the log- rank test. p values <0.05 were considered to be statistically significant.

4. RESULTS

4.1 Metabolic reprogramming of OC cells toward OXPHOS and FAO during nutrient deprivation-induced energy stress is associated with increased carboplatin resistance

Lipid metabolism sustains tumor progression in the nutrient-depleted TME, especially when glucose runs low (Cai, Ying and Wu, 2021). To this end, we metabolically profiled a panel of four OC cell lines. Three of them (*i.e.*, OVCA-429, OV-56, and ES-2) showed a marked glycolytic metabolism when cultured in basal medium (Figure 11A), but energetically switch when cultured under ES conditions. Specifically, when culturing OVCA-429, OV-56, and ES-2 in restricted glucose and serum conditions, thus mimicking low-glucose concentrations present in the TME, they all reprogram their metabolism toward the preferential use of OXPHOS (Figure 11B). Instead, EFO-21 mainly relied on oxidative metabolism, showing that the majority of ATP produced comes from mitochondrial respiration (mito-ATP) in both culture conditions (Figure 11B).

Moreover, during ES, the β-oxidation of free FAs was upregulated in all four OC lines analysed (Figure 11C), while glutaminolytic pathway usage was reduced (Figure 11D), suggesting that the increased dependency on FAO sustained cell viability against nutrient fluctuations.

Interestingly, ES conditions also impacted the sensibility of OC cells to treatment with carboplatin, the first-line chemotherapeutic drug used to treat OC patients. Hence, OC cells cultured under ES showed a more resistant phenotype to increasing doses of the drug (Figure 11E), underlining how the nutrient scarcity in the TME could negatively affect therapy response. Notably, the EFO-21 cell line, the only one featuring an oxidative metabolic profile in all culture conditions, showed resistance to carboplatin even in basal medium, in accordance with data in the literature suggesting that increased OXPHOS/FAO is linked with platinum-resistance (Huang *et al.*, 2021).

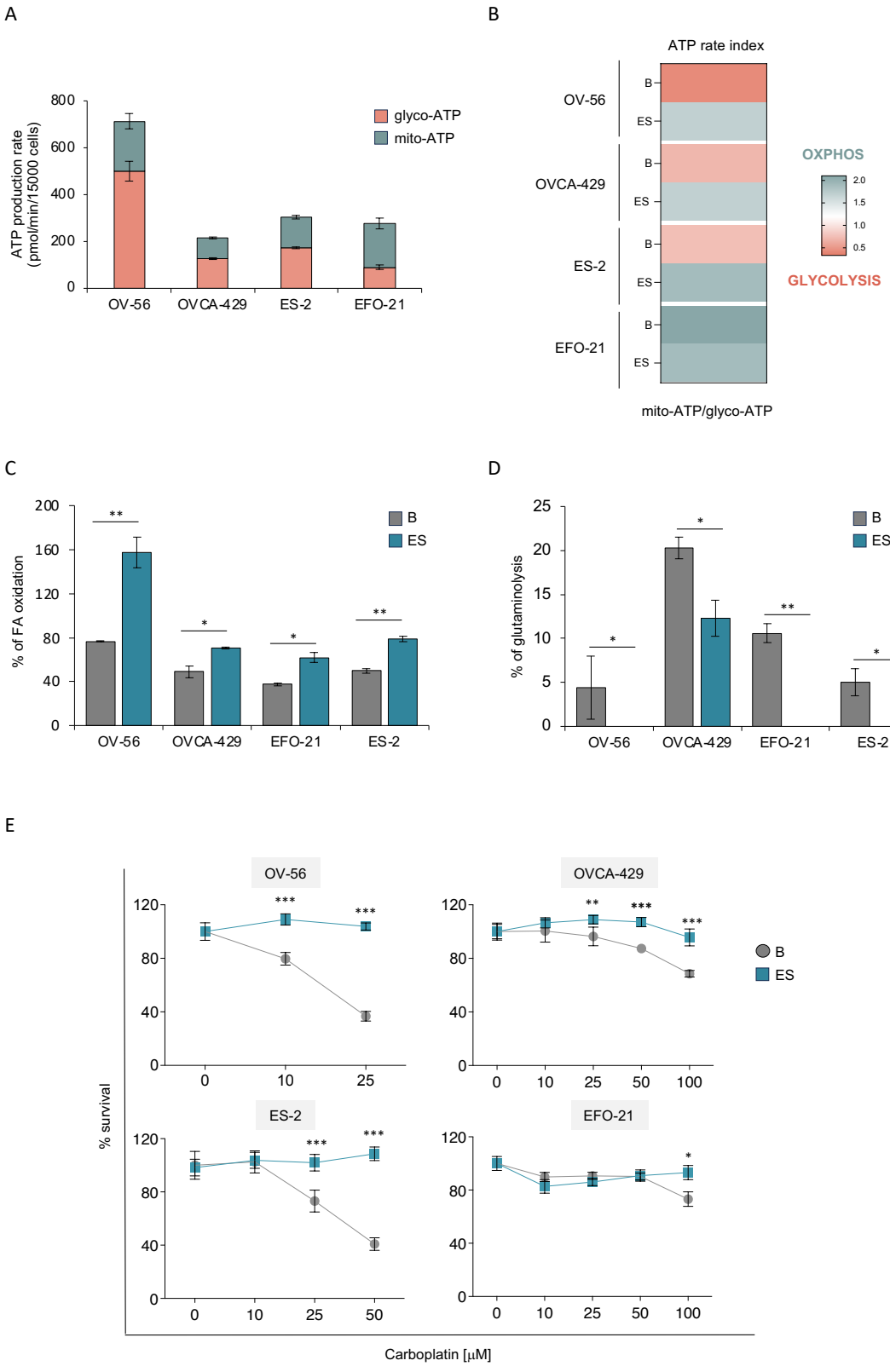
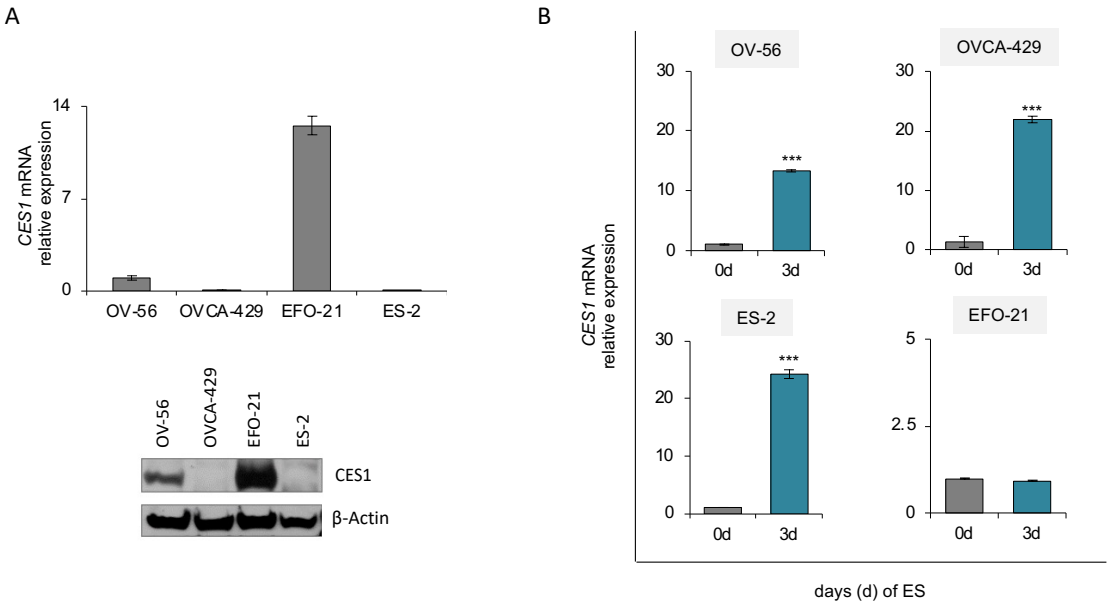
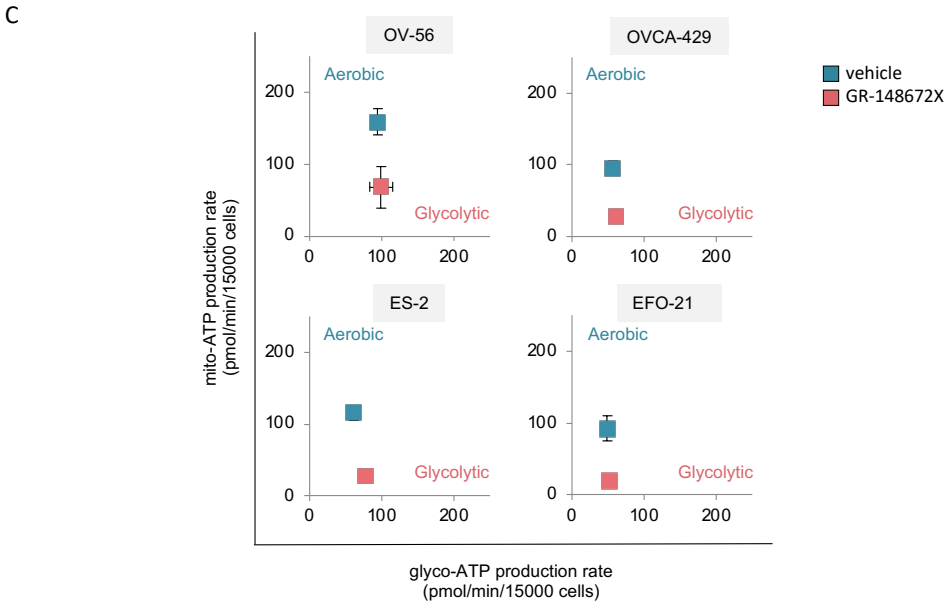

A
ATP production rate (pmol/min/15000 cells)
glyco-ATP
mito-ATP
OV-56
OVCA-429
ES-2
EFO-21
B
ATP rate index
OXPHOS
GLYCOLYSIS
mito-ATP/glyco-ATP
C
% of FA oxidation
B
ES
D
% of glutaminolysis
E
% survival
Carboplatin [μM]

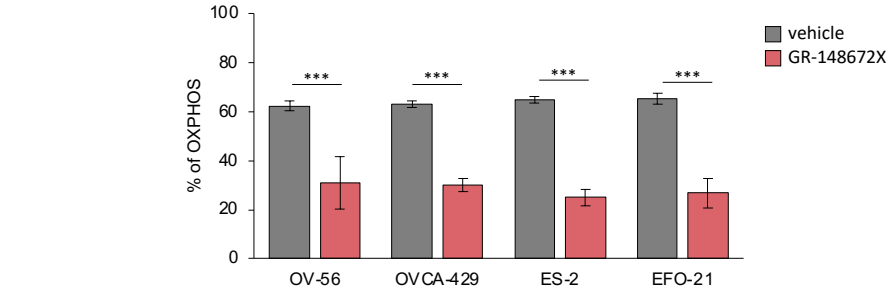
Figure 11:
A] ATP production rate of OC cells cultured in basal conditions measured by Seahorse XFe96 showing the amount of mito-ATP and glyco-ATP for each cell line. Seahorse ATP rate injection strategy was as follows: *i)* Oligomycin, 1.5µM and *ii)* Rotenone/Antimycin A, 0.5µM.
B] Heatmap showing the ATP rate index in the indicated OC cell lines cultured under basal conditions (B) or energy stress (ES) for 3 days.
C-D] Percentage of fatty acid (FA) oxidation (C) and glutaminolysis (D) dependency in OC lines under B or ES as indicated measured by Seahorse XFe96. Injection strategies was as follows: *1)* Etomoxir, 4µM and BPTES 3µM /UK 5099, 2µM (FA Oxidation); *2)* BPTES 3µM and Etomoxir, 4µM/ UK 5099, 2µM (glutaminolysis). Values are shown as mean ± SEM. *, $p<0.05$; **, $p<0.01$.
E] Percentage of OC cell survival measured by CellTiter-Glo® 2.0 assay after a 3-day treatment with different concentration of carboplatin under B or ES as indicated. Values are shown as mean ± SD. *, $p<0.05$; **, $p<0.01$; ***, $p< 0.001$.

4.2 CES1 is expressed during ES and mediates OC cell metabolic switch

Since the carboxylesterase CES1 has been depicted as a lipase involved in cancer cell metabolic adaptation, and targeting CES1 was able to counteract CRC growth and sensitize hepatocellular carcinoma to platinum-based chemotherapy *in vivo* (Capece *et al.*, 2021, Li *et al.*, 2023), we evaluated the expression of CES1 in OC cells both in basal and ES conditions. We found an upregulation of CES1 expression in the glycolytic cells OVCA-429, OV-56, and ES-2 under carboplatin resistance-associated ES, while the oxidative cell line EFO-21 maintained a constant high expression of CES1 both in ES and basal settings (Figure 12A-B). Notably, the upregulation of CES1 in OC cells when cultured in ES suggests that, despite the lack of CES1 expression in basal condition, this lipase could be relevant for their adaptation to harsh metabolic environments. Accordingly, the pharmacological blockade of CES1 by using the commercially available CES1 inhibitor GR-148672X induced a significant reduction of mito-ATP as a result of a significant reduction of OXPHOS percentage used by OC cells to adapt to ES conditions (Figure 12C-D). Hence, the inhibition of CES1 impairs the bioenergetic parameters of mitochondria by mainly reducing basal respiration and ATP production, while increasing proton leak (Figure 12E). These results underlie the link existing between CES1 inhibition and the occurrence of oxidative stress damage, and the importance of CES1 in mediating the metabolic reprogramming of OC cells during ES.


A
CES1 mRNA relative expression
14
7
0
OV-56
OVCA-429
EFO-21
ES-2
OV-56
OVCA-429
EFO-21
ES-2
CES1
β-Actin
B
CES1 mRNA relative expression
OV-56
OVCA-429
ES-2
EFO-21
30
20
10
0
5
2.5
***
0d
3d
days (d) of ES


C
OV-56
OVCA-429
ES-2
EFO-21
Aerobic
Glycolytic
200
100
0
vehicle
GR-148672X
mito-ATP production rate
(pmol/min/15000 cells)
glyco-ATP production rate
(pmol/min/15000 cells)


D
% of OXPHOS
100
80
60
40
20
0
***
OV-56
OVCA-429
ES-2
EFO-21
vehicle
GR-148672X

E

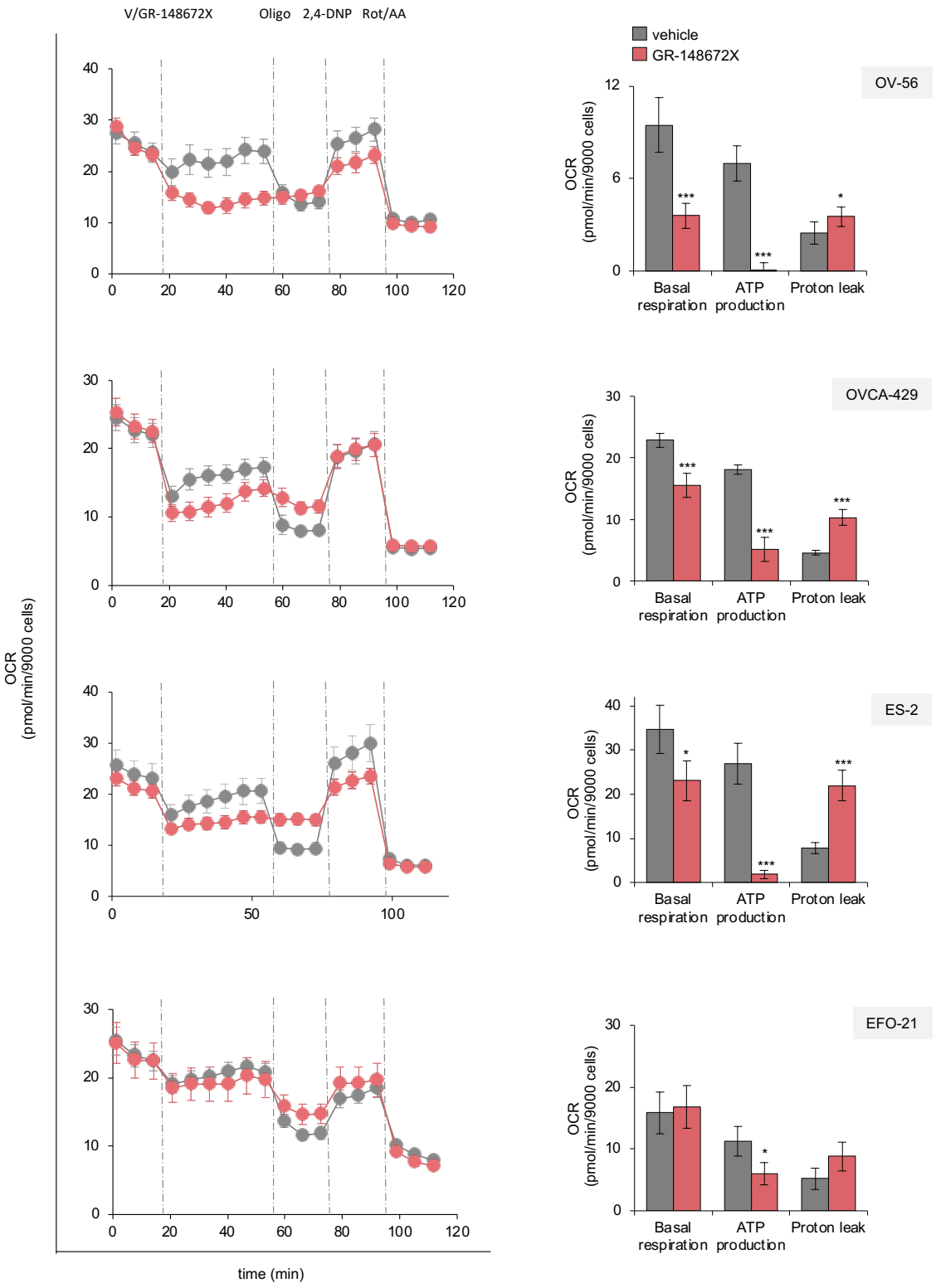

V/GR-148672X
Oligo
2,4-DNP
Rot/AA
OCR (pmol/min/9000 cells)
time (min)
vehicle
GR-148672X
OV-56
OVCA-429
ES-2
EFO-21
OCR (pmol/min/9000 cells)
Basal respiration
ATP production
Proton leak

Figure 12:
A] qRT-PCR showing CES1 mRNA levels in the indicated OC cell lines cultured under basal conditions (top). Western blot showing CES1 and β-actin protein levels in OC cells as indicated (bottom).
B] qRT-PCR showing CES1 mRNA levels in the indicated OC cell lines cultured under basal condition (0d) or 3 days of ES. Values are shown as mean ± SD. ***, $p< 0.001$.
C] ATP production rate of OC cells treated with vehicle or 10μM GR-148672X after 3 days of ES measured by Seahorse XFe96. Shown is the amount of mito-ATP and glyco-ATP for each cell line. Seahorse ATP rate injection strategy was as follows: *i)* vehicle/GR-148672X 10μM + 2-Deoxy-D-glucose 50mM, *ii)* Oligomycin, 1.5μM and *iii)* Rotenone/Antimycin A, 0.5μM.
D] Percentage of OXPHOS after 3 days of ES in OC cells treated with vehicle or 10μM GR-148672X measured by Seahorse XFe96 Seahorse ATP rate injection strategy was as follows: *i)* vehicle/GR-148672X 10μM + 2-Deoxy-D-glucose 50mM, *ii)* Oligomycin, 1.5μM and *iii)* Rotenone/Antimycin A, 0.5μM. Values are shown as mean ± SEM. ***, $p< 0.001$.
E] Oxygen consumption rate (OCR) in OC cells treated with vehicle or 10uM GR-148672X after 3 days of ES (left). Shown are the following bioenergetic parameters: *i)* Basal respiration; *ii)* ATP production-coupled respiration; *iii)* Proton leak (right). Seahorse Mitostress injection strategy was follows: Oligomycin (Oligo), 1.5μM; 2,4-Dinitrophenol (2,4-DNP), 75μM; Rotenone/Antimycin A (Rot/AA), 0.5μM. Values are shown as mean ± SD. *, $p<0.05$; ***, $p< 0.001$.

4.3 CES1 blockade impairs chemoresistance-associated autophagy

Autophagy is a well-known mechanism supporting cancer metabolic adaptation and therapy resistance in OC (Sandeep *et al.*, 2022). Specifically, under ES conditions, nutrient depletion requires the mobilization of free FAs to supply energy, which connects the functions of lipid metabolism and autophagy (Zhang *et al.*, 2022). Accordingly, we evaluated the relevance of CES1 in this scenario. by monitoring autophagic flux in presence or absence of CES1 inhibitor GR-148672X. Interestingly, we found that the expression of the lipidated and active form of the typical autophagosomal biomarker LC3B-II increased under ES, as already shown in the literature (Huang *et al.*, 2015), but decreased in presence of CES1 inhibitor, suggesting that CES1 blockade impairs autophagy in OC cells cultured under ES conditions (Figure 13A). The effects of CES1 inhibition on lipidation of LC3B propose the involvement of CES1 in the autophagic machinery. Accordingly, the combinatorial treatment of Bafilomycin (BafA1) and GR-148672X resulted in an intermediate phenotype where LC3BII levels were lower than those observed following Baf1A-mediated LC3B accumulation and higher than those detected upon GR-148672X-induced LC3B reduction (Figure 13B). The autophagy impairment caused by CES1 pharmacological inhibition was confirmed by FACS analysis, showing that GR-148672X treatment dramatically decreased the accumulation of autophagic vesicles (Figure 13C).

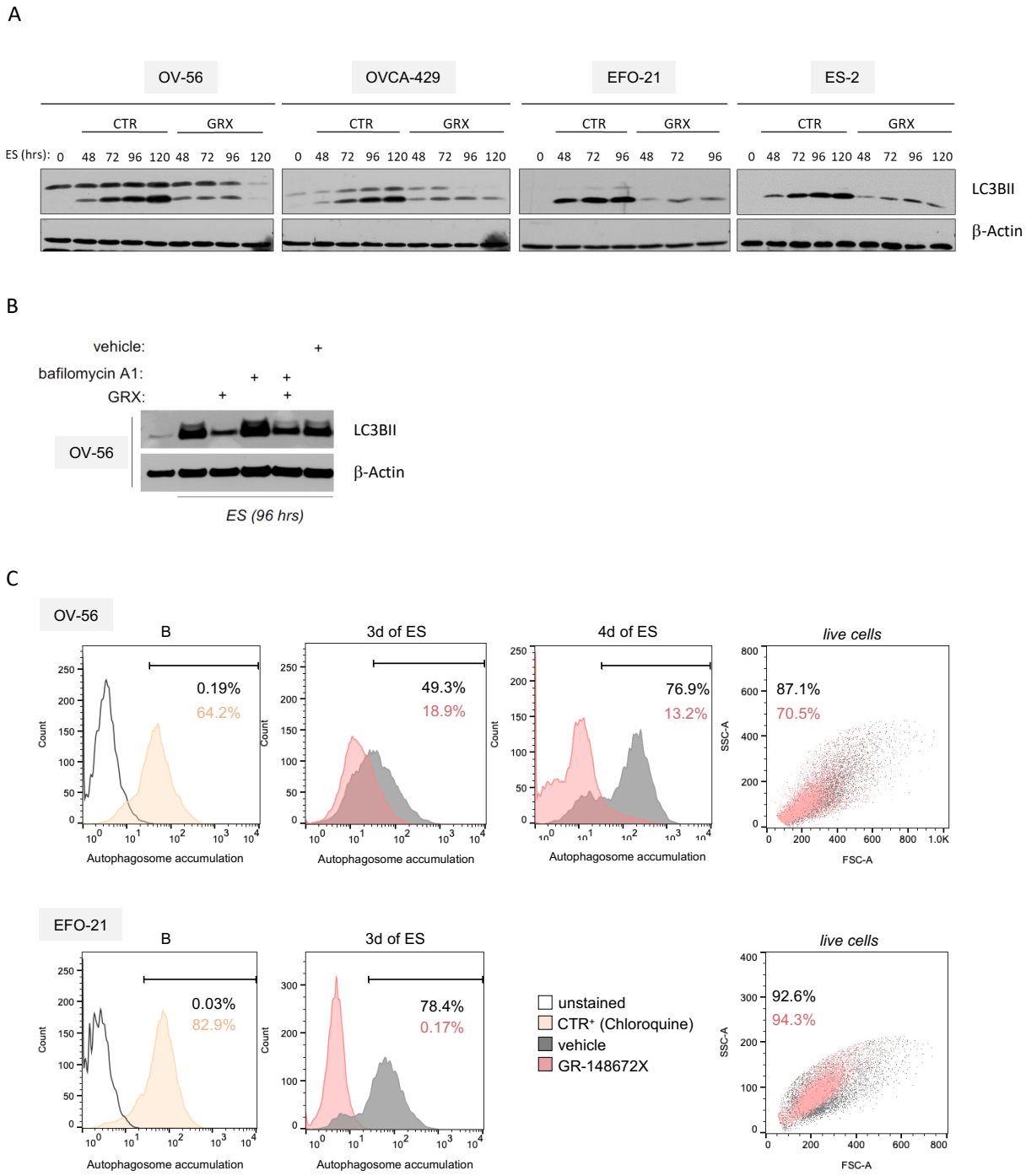


Figure 13:

A] Western blots showing the protein levels of activated-LC3B (LC3B-II) in OC cells treated with vehicle or GR-148672X (10μM) at the indicated time points under ES. β-actin is shown as loading control.

B] LC3BII expression levels of OV-56 cells treated as indicated after 96 hours of ES. β-actin is shown as loading control. Bafilomycin A1, 50nM; GR-148672X, 10μM.

C] Cyto-ID FACS analysis showing autophagosome accumulation in OV-56 (top) and EFO-21 (bottom) cells treated as indicated in basal condition (B) and after 3 and 4 days of ES. Chloroquine, 10μM; GR-148672X, 10μM. Graphs showing the percentage of live cells are depicted (right).

4.4 CES1 blockade kills OC cells as single agent by triggering either apoptosis or ferroptosis

Protective autophagy can inhibit tumor apoptosis induced by anti-cancer drugs (Xie, Liu and Li, 2020). Moreover, recent evidence showed that not only autophagy but also increased FAO are linked with platinum-resistance in OC cells (Huang *et al.*, 2021). In our context, increased autophagy and FAO occur in ES conditions when CES1 is upregulated. Crucially, pharmacological blockade of CES1 using the specific CES1 inhibitor GR-148672X caused cell death in ES-induced carboplatin-resistant OC lines, thus identifying CES1 as an alternative druggable target to overcome the limitations of standard chemotherapy in advanced disease (Figure 14A).

Interestingly, treatment with GR-148672X demonstrated a contextual specificity related to an increased reliance on oxidative metabolism. Thus, while the oxidative EFO-21 cells showed responsiveness to CES1 inhibition also in basal condition, although at a lesser extent compared to ES, the glycolytic OV-56 cells, even if they basally express CES1, do not respond to GR-148672X (Figure 14B).

In presence of CES1 inhibition, apoptosis and ferroptosis are the cell-death mechanisms alternatively activated in OC cells, as previously shown for CRC cells (Capece *et al.*, 2021). The activation of apoptosis pathway was demonstrated by the cleavage of caspase-3 and PARP proteins and by propidium iodide staining showing apoptotic DNA fragmentation (Figure 14C-D), while the reduction of glutathione peroxidase 4 (GPX4) is a hallmark of ferroptosis (Figure 14C).

Furthermore, the cytotoxic effect of CES1 depletion was recapitulated by the autophagy inhibitor BafA1, suggesting a role for autophagy in the pro-survival function of CES1 under ES. Specifically, we tested OC cell response to autophagy inhibitor Bafilomycin A1 at nanomolar concentrations to block autophagic flux, demonstrating that BafA1 suppressed OC cell growth in the same ES conditions in which carboplatin is ineffective (Figure 14E), underlying the intrinsic and inducible protective role of autophagy that also OC cells exploit to normalize cellular processes in presence of extrinsic perturbations like nutrient-depleted culture conditions. Notably, ES-2 cell line did not show sensitivity to the treatment with BafA1 suggesting that additional cell line autonomous mechanism could mediate the survival under ES.

Taken together, these results sustain the importance of CES1-dependent oxidative metabolism to support the adaptation of OC cells in harsh environments via increasing FAO/OXPHOS and

autophagy and identify this lipase as druggable cancer cell liability to counteract OC advanced disease.

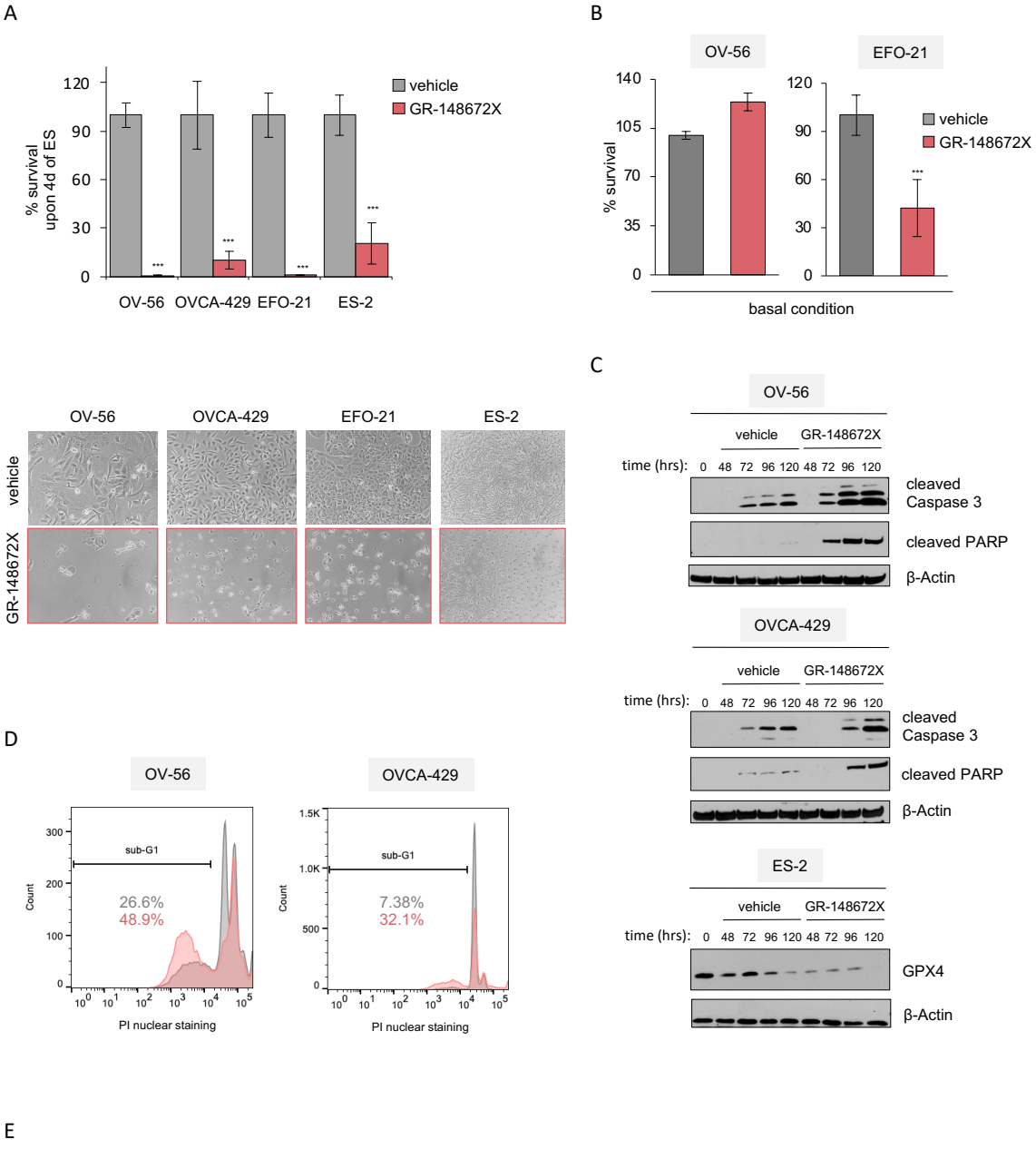
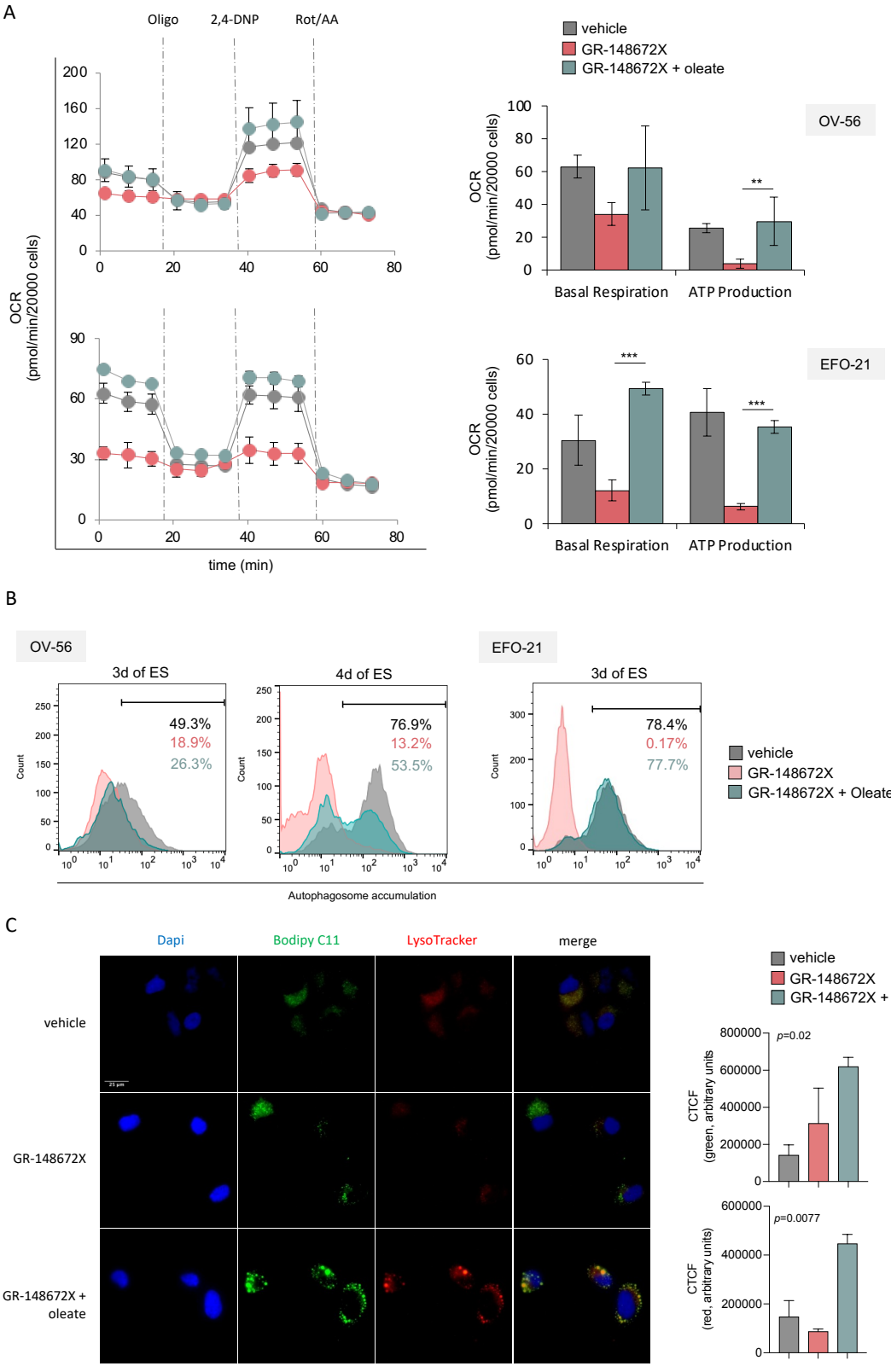


Figure 14:
A] CellTiter-Glo® viability analysis of OC cells after a 4-day treatment with vehicle, or GR-148672X (10μM) under ES (top). Values are shown as mean ± SD. ***, p< 0.001. Representative images of OC cell lines treated as indicated at the endpoint (4d, bottom).
B] CellTiter-Glo® viability analysis of OV-56 and EFO-21 cells after a 4-day treatment with vehicle, or GR-148672X (10μM) under B. Values are shown as mean ± SD. ***, p< 0.001.
C] Western blots showing the protein levels of apoptotic (cleaved caspase 3, cleaved PARP), and ferroptotic (GPX4) markers in cells treated as in A. β-actin is shown as loading control.
D] Propidium iodide staining showing apoptotic DNA fragmentation in OV-56 and OVCA-429 treated as in A after 3 days of ES.
E] CellTiter-Glo® viability analysis of OC cells after 4 days of ES and treated with vehicle, or Bafilomycin A1 50nM. Values are shown as mean ± SD. ***, p< 0.001.

4.5 Oleate exogenous supplementation rescues autophagic flux, ATP production and survival

We demonstrated that CES1 sustains OC survival during ES by enhancing autophagy and enabling them to to meet their energy demand by providing free fatty acids for FAO and OXPHOS.
Accordingly, oleate exogenous supplementation was able to rescue the bioenergetic profile and ATP production (Figure 15A) and restore accumulation of autophagic vesicles in CES1 activity-depleted OC cells (Figure 15B).
Moreover, CES1 inhibition caused changes in lipid storage by reducing LD destruction. When we treated OV-56 cells with GR147286X, we observed an increase of LD accumulation (green) in parallel with a reduction of lysosomes vesicles (red), confirming the blockade of the autophagic process (Figure 15C). Notably, oleate supplementation resulted on one hand in the stimulation of lipid droplets biogenesis through esterification, and on the other hand rescued the autophagolysosome formation. In fact, oleate was able to reactivate the autophagic machinery as demonstrated by the enhancement of LC3B lipidation (LC3BII) in OC cells treated with a combination of GR-148672X and oleate (Figure 15D). Accordingly, oleate-restored autophagy and OXPHOS were involved in rescuing OC cell survival from GR-148672X-mediated killing (Figure 15E).


A
Oligo
2,4-DNP
Rot/AA
OCR (pmol/min/20000 cells)
time (min)
vehicle
GR-148672X
GR-148672X + oleate
OV-56
Basal Respiration
ATP Production
EFO-21
B
OV-56
EFO-21
3d of ES
4d of ES
49.3%
18.9%
26.3%
76.9%
13.2%
53.5%
78.4%
0.17%
77.7%
Count
Autophagosome accumulation
vehicle
GR-148672X
GR-148672X + Oleate
C
Dapi
Bodipy C11
LysoTracker
merge
vehicle
GR-148672X
GR-148672X + oleate
CTCF (green, arbitrary units)
p=0.02
CTCF (red, arbitrary units)
p=0.0077

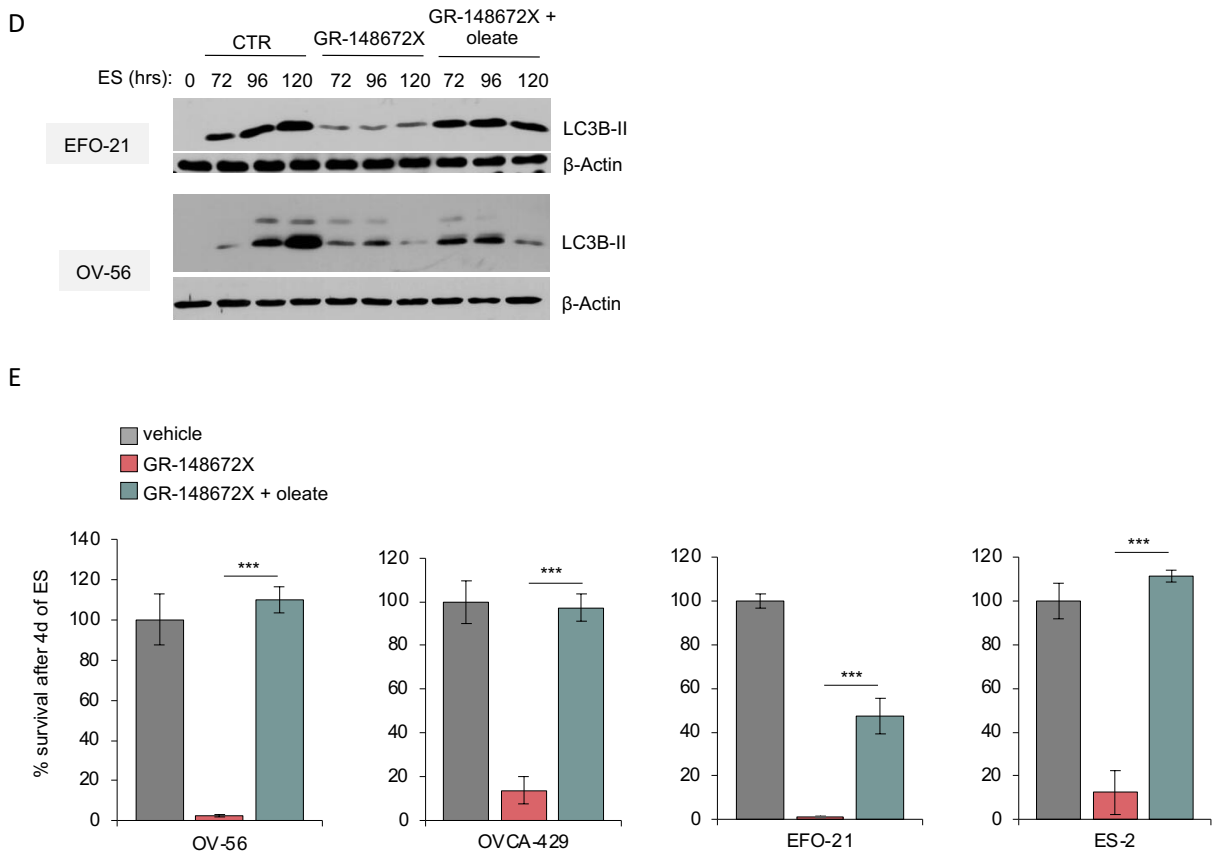


Figure 15:

A] OCR in OC cells treated with Vehicle (V), or 10μM GR-148672X or a combination of GR-148672X and 50mM Oleate after 3 days of ES (left). Shown are the following bioenergetic parameters: i) Basal respiration; ii) ATP production-coupled respiration; iii) Proton leak (right). Seahorse Mitostress injection strategy was as follows: Oligomycin (Oligo), 1.5μM; 2,4-Dinitrophenol (2,4-DNP), 75μM; Rotenone/Antimycin A (Rot/AA), 0.5μM. Values are shown as mean ± SD. *, $p<0.05$; **, $p<0.01$; ***, $p< 0.001$.

B] Cyto-ID FACS analysis showing autophagosome accumulation in OV-56 (left) and EFO-21 (right) cells treated as indicated after 3 and 4 days of ES. GR-148672X, 10μM; oleate, 50mM.

C] Representative fluorescence images (magnification 40X) of OV-56 cells stained with Bodipy 493/503 (neutral lipids in LDs, green), 2μM, LysoTracker DND 99 (lysosome, red), 50nM and DAPI (nuclei, blue) after 2 days of ES (left). Corrected total cell fluorescence (CTCF) for green (top) and red fluorescence (bottom) across the three experimental groups is shown (right). Statistical significance was calculated by One way ANOVA.

D] Western blots showing the protein levels of activated-LC3B (LC3B-II) in OV-56 and EFO-21 cells treated with vehicle, GR-148672X (10μM) or a combination of GR-148672X and oleate (50mM) at the indicated time points under ES. β-actin is shown as loading control.

E] CellTiter-Glo® viability analysis of OC cells after 4 days of ES and treated with vehicle, GR-148672X (10μM) or a combination of GR-148672X and oleate (50mM). Values are shown as mean ± SD. ***, $p< 0.001$.

In light of these results, we hypothesized that CES1 could be depicted as a neutral lipase able to mediate LD breakdown to provide free FA for fueling FAO and sustain energy demand and, at the same time, promoting the expansion of phagophore membrane by localized phospholipid synthesis. Hence, lipid metabolism is involved in regulating a variety of cellular processes beyond energetic provision, including autophagy and the formation of autophagy-related membrane structures (Zhang *et al.*, 2022). Recently, acyl-CoA synthetase Faa1 was described as accumulated on phagophores and able to locally channel FAs into phospholipid (PL) synthesis and promote their assembly into autophagic membranes (Schütter *et al.*, 2020). We hypothesize that CES1 and Faa1 could co-work to channel FAs into PLs in nascent autophagosomes and trigger autophagy (Figure 16). Specifically, CES1 would mobilize FAs from LDs to provide lipid building blocks to use in the Faa1-mediated PL genesis. For this reason, the lack of CES1 caused LD accumulation and reduced autophagosome biogenesis, as neutral lipids cannot be converted in free FAs by CES1-mediated lipolysis to fuel autophagic machinery. On the other hand, the exogenous supplementation of oleate rescued the availability of free FAs that can be directed to the mitochondria to participate in the β-oxidation pathway and to autophagosome production process.

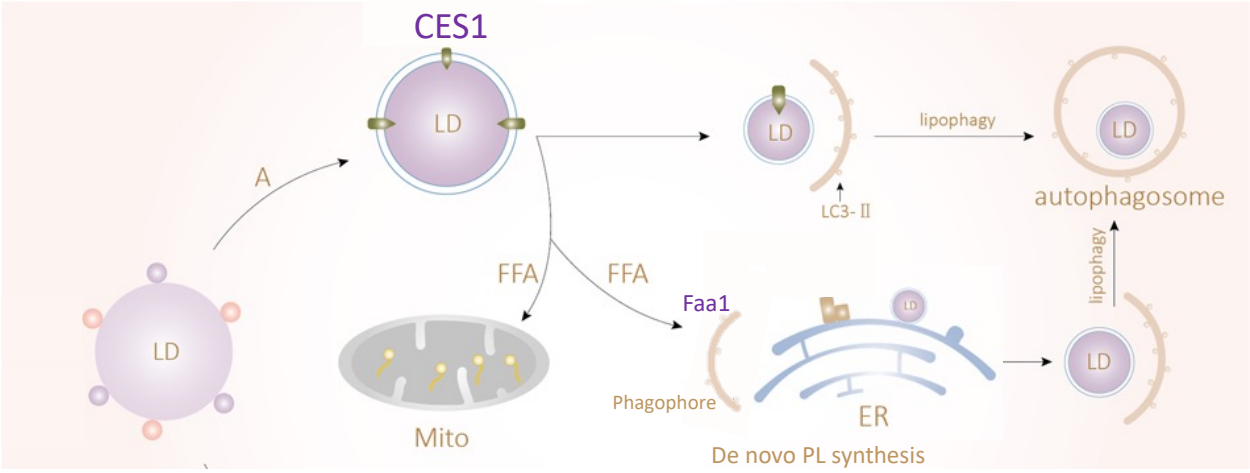


Figure 16:

Schematic representation of the CES1 proposed mechanism to sustain survival of OC cells. Depicted is the CES1-mediated metabolic mechanism increasing lipid droplet (LD) breakdown and free fatty acid (FFA) availability to fuel 1) FAO and OXPHOS and 2) phospholipid (PL) synthesis for their Faa1-mediated assembly into autophagic membranes. Mito, mitochondrion; ER, endoplasmic reticulum.

4.6 Targeting CES1 kills primary OC cells derived from ascites and omental metastasis

The relevance of CES1 as a critical mediator of metabolic adaptation of OC cells was confirmed by analyzing primary cells derived from ascites and omental metastases of women affected by OC. CES1 expression was measured in basal condition and up to three days of limiting glucose-induced ES. We found an upregulation of CES1 in those samples lacking CES1 basal expression without any specificity to ascites or omental metastasis (Figure 6A-B). To this date, the survival of OC matched cells derived from ascites and omental metastasis of one patient was tested in the presence of CES1 inhibitor and we found that GR-148672X reduced their survival in both in basal and ES conditions, suggesting that these metastatic cells could have already switched toward oxidative metabolism. In the future, we will analyze the metabolic phenotype of these OC primary cells in basal and under ES to test our hypothesis, according to which the responsiveness to the treatment with CES1 inhibitor is strictly related to the OC cell reliance on oxidative metabolism in which CES1 has a role. Nevertheless, the clinical relevance of CES1 has been demonstrated by analysing the publicly available TCGA-OVCA, dataset including 425 OC patients. We found that the expression of CES1 is associated to worse overall survival (OS) and disease specific survival (DSS), reinforcing the potential of exploiting CES1 inhibition to treat oxidative and chemoresistant OC tumors.

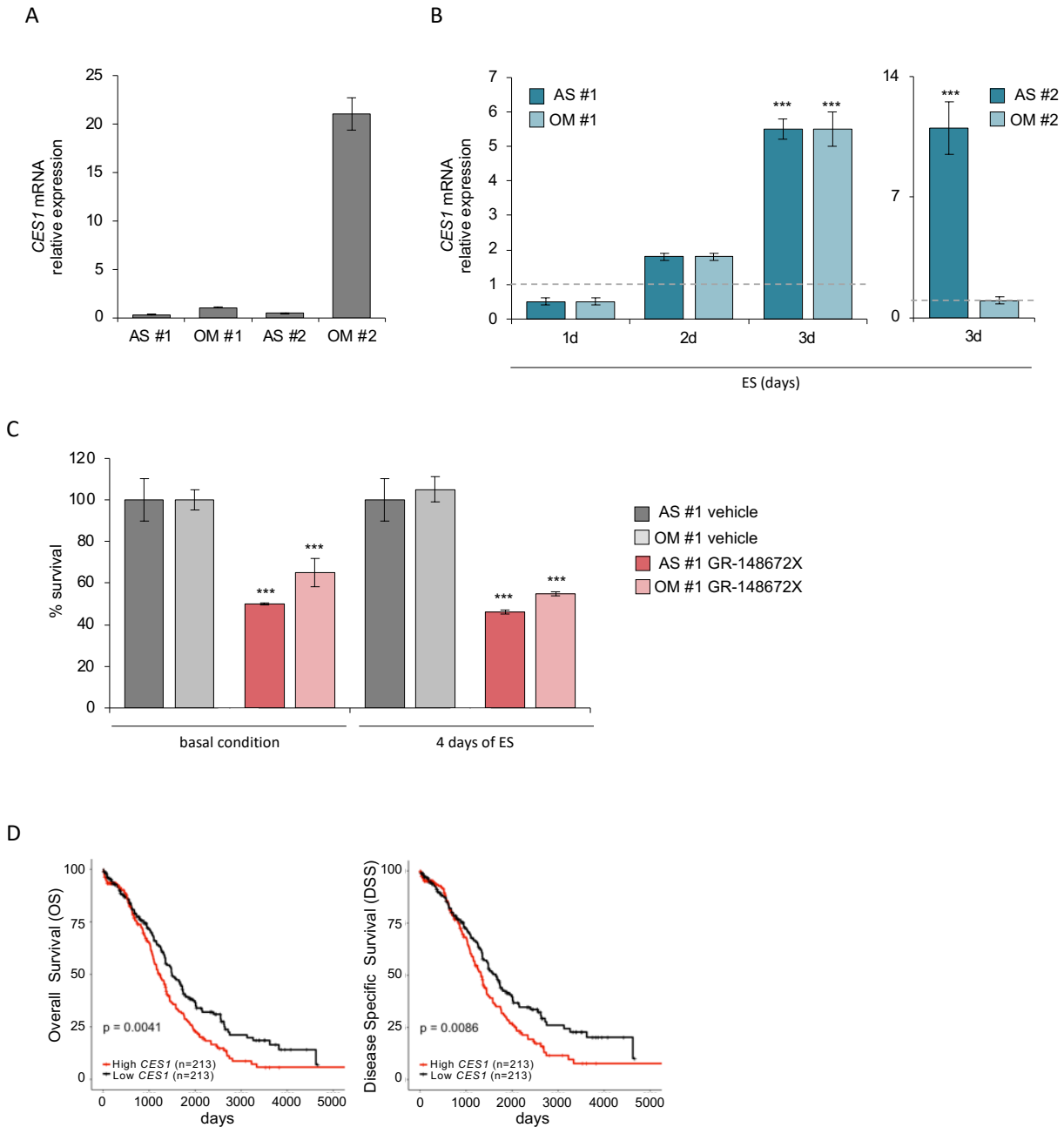


Figure 17:

A] qRT-PCR showing CES1 mRNA levels in two OC patients. Cancer cells were isolated from ascites (AS) and omental metastasis (OM) derived from the same patient and cultured under basal conditions.

B] qRT-PCR showing CES1 mRNA levels in matched AS- and OM- derived OC cells cultured under ES as indicated. Values are shown as mean ± SD. ***, $p < 0.001$.

C] CellTiter-Glo® viability analysis of matched AS- and OM- derived OC cells after 4 days of ES or basal condition and treated with vehicle or GR-148672X (10µM). Values are shown as mean ± SD. ***, $p < 0.001$.

D] Overall survival (OS, left) and disease-specific survival (DSS, right) in OC patients from the TCGA dataset stratified based on tumour-associated CES1 expression. Statistical significance for Kaplan-Meyer curves was calculated using the log-rank test.

5. DISCUSSION

In this work, we demonstrated the relevance of CES1-driven fatty acid catabolism in promoting the metabolic adaptation and survival of OC cells, thus enhancing their aggressiveness. We found that CES1 is associated with worse OS in OC patients and is induced during ES in both OC cell lines and primary cancer where it sustains autophagic flux and energetic needs.

Thoroughly, CES1 expression is higher when OC cells - facing nutrient scarcity - metabolically switch to an oxidative phenotype and become platinum-resistant. Accordingly, metabolic reprogramming in ES conditions is hampered upon CES1 pharmacological inhibition, which also resulted in an impairment of bioenergetic parameters, inhibition of autophagic flux and autophagic vesicle formation, and massive cell death. The crucial role of CES1 in providing essential free FAs to fuel FAO and autophagy is confirmed in the OC context by the rescued survival of GR-147286X-treated cells upon oleate supplementation. Specifically, oleate was shown to restore bionergetic parameters, ATP production, autophagosome accumulation and autophagy, suggesting that it could be used to fuel FAO/OXPHOS and PL synthesis, as well as be re-esterificated back to TG and packaged into new LDs ready for lipophagy.

These results reveal that CES1 seems crucial in sustaining metabolic adaptation by refurnishing autophagic machinery. Recent studies underlie how pharmacological strategies targeting the regulation of autophagy offer promising avenues to enhance cisplatin sensitivity in cancer therapy (B. Yang et al., 2020; Y. Yang et al., 2024). In this context, it has been well documented that autophagy-deficient tumors show some tumor-suppressing features like dysfunctional mitochondria, ineffective FAO, reduced carbon flux through the TCA cycle, and lipid accumulation (Miller & Thorburn, 2021). Specifically, down-regulating the expression of essential autophagy proteins in Ras-expressing cell lines where high levels of basal autophagy are required to maintain functional mitochondria and support cancer growth in starvation resulted in defective autophagosome formation or cargo delivery causing accumulation of abnormal mitochondria and reduced oxygen consumption (Guo *et al.*, 2011). Therefore, considering that OC cells featuring inactive CES1 show a phenotype resembling the one of autophagy-deficient tumors, CES1 could be depicted as an autophagy inhibitor, at least in this tumor setting.

Notably, for all these reasons, CES1 represents a promising metabolic target in OC chemorefractory subsets. Over the years, other metabolic targets emerged as major players in cancer therapy. For instance, transcriptomic data found CPT1A, FASN, and SCD overexpressed in seven paclitaxel-resistant OC cell lines and targeting these lipid metabolism-related enzymes reduced cell viability and migration (Huang *et al.*, 2021; Ma *et al.*, 2023). In this regard, a key obstacle in targeting cancer metabolism in clinical setting is the difficulty of minimizing off-target effects in normal cells, given the sharing of many metabolic pathways between tumour and normal cells. For instance, the electron transport chain complex I selective inhibitor IACS-010759 in the phase I clinical trial resulted in premature termination due to dose-limiting toxicities (Machado *et al.*, 2023; Yap *et al.*, 2023). In this regard, our proposal of targeting CES1 appears to be clinically safe, given its contextual specificity for the oxidative switch of cancer cells in ES conditions, which occurs in TME but not often in physiological contexts. Crucially, as systemic pharmacologic inhibition, genetic CES1 deletion appears to be tolerated *in vivo* since *Ces1d*-knockout mice are viable, lean and die of old age, suggesting the redundancy of CES1 in physiological contexts (Wei *et al.*, 2010). Accordingly, the identification of healthy individuals carrying homozygous *CES1* null mutations unequivocally demonstrates the clinical safety of our CES1 targeting approach (Zhu *et al.*, 2008; Varfolomeev *et al.*, 2016).

Numerous drugs widely used in oncology, including chemotherapeutics targeted-agents are subject to clinically significant drug-drug interactions (Yu, Wang and Ragueneau-Majlessi, 2022). CES1 hydrolyses a wide range of xenobiotics, including prodrugs, raising the concern that also CES1 inhibitors could trigger drug/herb-drug interactions (DDIs/HDIs), especially when these inhibitors are co-administrated with CES substrate-drugs with very narrow therapeutic windows (Y. Song *et al.*, 2021). Evidence indicates that the risks of DDIs/HDIs via inhibiting CES1 are relatively low. For instance, while irinotecan can be metabolised by CES1, its main activator is CES2, essentially eliminating any risk of interaction (Wang *et al.*, 2018). Similarly, capecitabine, another anti-cancer prodrug, is activated by both CES1/CES2, mitigating the risk of drug-drug interactions via CES1 (Her and Zhu, 2020; Y. Song *et al.*, 2021). Other CES1 substrates include recreational drugs, antiviral agents, methylphenidate (ADHD-medication), mycophenolate (immunosuppressant) and clopidogrel (anticoagulant) (Her & Zhu, 2020; Y. Q. Song et al., 2021) which either present low/no clinical-significance in OC or can be replaced by comparably effective non CES1-dependent

alternatives (Di, 2018; Her and Zhu, 2020; Y. Song *et al.*, 2021). In this regard, it should also be noticed that inhibition of CES1 could be also beneficial to treat hyperlipidemia and obesity-associated metabolic diseases (Dominguez et al., 2014; Lian, Nelson, et al., 2018).

However, although further *in vivo* studies are needed to explore the actionability of CES1 inhibition in OC cells, this lipase seems to be a promising druggable target in oxidative and chemoresistant OC tumors, supporting the development of drug-discovery strategies to generate useful clinical CES1 inhibitors to target cancer cell metabolic vulnerabilities in the era of precision medicine.

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