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Deciphering the role of the mitochondrial chaperonine MCJ in ovarian cancer

Presentata da **Stefano Miglietta**

Coordinatore Dottorato

Prof. Vincenzo Scarlato

Supervisore

Prof.ssa Anna Maria Porcelli

Co-supervisore

Prof.ssa Anna Maria Ghelli

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ABSTRACT

Ovarian cancer (OC) is the most lethal gynecological tumour due to its extremely silent invasive capacity. The high mortality of OC is often determined by a therapeutic failure caused by the development of pharmacological resistance. In this context, recent studies have shown that the condition of therapeutic resistance is influenced by the cellular metabolic state and mitochondrial bioenergetic efficiency. Interestingly, it has been reported that in OC the chemoresistance acquirement is associated with the epigenetic silencing of *DNAJC15* gene that encodes for MCJ mitochondrial co-chaperonine. The latter is reported as an endogenous negative regulator of electron transport chain (ETC), able to modify the structure and function of mitochondrial respiratory chain supercomplexes. It is known from the literature that OC growth and chemoresistance is often mediated by the activation of oncogenic pathways, such as Wnt/β-catenin axis, that can in turn be finely regulated by the mitochondrial energy state. We have therefore hypothesized that MCJ, modulating the bioenergetic profile, may lead to the re-establishment of pharmacological sensitivity of OC. In this context, our data showed an increased sensitivity to cisplatin and lower proliferative and migratory capacities of MCJ-over-expressing chemoresistant cell line. Further, by analyzing the mitochondrial bioenergetics we showed that the expression of MCJ in cisplatin-resistant cells confers a respiratory profile similar to the cisplatin-sensitive model. In addition, we observed that the MCJ over-expression was able to increase ROS production in both chemosensitive and chemoresistant cells. Since intracellular ROS are able to modulate oncogenic pathways, such as Wnt/β-catenin signaling, we investigated this molecular axis showing a decreased β-catenin expression levels associated with lower downstream multi-drug resistance proteins and epithelial-mesenchymal transition players in the MCJ-over-expressing chemoresistant cell line. Based on these data, we speculate that the over-expression of MCJ in OC chemoresistant cells may modulate the ETC activity and ROS production, inducing β-catenin degradation, which in turn leads to a reduction of both cisplatin-resistance and proliferation. The dissection of these novel molecular and metabolic-related mechanisms in which MCJ is involved, sets the bases for new insights that make such chaperonine a newsworthy factor for this silent killer disease.

INTRODUCTION

1. Ovarian cancer features

1.1 Ovarian cancer epidemiology and histopathology

Ovarina cancer (OC) is an epithelial malignant gynecological lesion that affects ovaries and nearby tissues, with variable proliferative capacity based on the istotype of the tumor. The age-changed occurrence of this disease is 12.5 per 100.000 women (Siegel RL *et al*., 2020) with a decrease of under 1%. The frequency of OC occurence and mortality rate tend to increment with age and most instances of ovarian tumor occur in women more established than 50 years. Although this type of cancer has a very low incidence (3% among all cancers), it is the fifth reason for death caused by cancer in women with five-year survival rates below 45% (Siegel RL *et al*., 2020), due to its silent and completely asystomatic invasive capacity. Indeed, when the patients present classic symptoms such as abdominal pain and discomfort, they have an extremely widespread and metastasizing advanced stage cancer in the abdominal cavity. There are several well-established risks and protective factors for epithelial ovarian cancer, most related to reproductive and hormonal factors (Friedman GD *et al*., 2005; Goff BA *et al*., 2007). Higher parity, oral contraceptive use and tubal ligation all significantly reduce the risk to develop this type of cancer; instead, family history of ovarian or breast cancer, older menopausal age, obesity, menopausal hormone therapy use, endometriosis history and smoking can all increase the risk (Thigpen T *et al*., 1993; Young RH *et al.*, 1994; Vo C *et al*., 2007). The peculiarity of ovarian tumor resides in its ability to metastasize not only through the blood circulation, disseminating metastatic sites even in organs physiologically distant from the site of origin, but also by spreading through the abdominal cavity, creating a widespread carcinosis at the level of peritoneal fluid called ascites. The fact that ovarian cancer cells are able to disseminate and progress through peritoneal interstiches such as the omentum, allows the establishment of distant metastatic niches also at the enteric, stomachs, diaphragmatic and pulmonary locations (Ozols RF *et al*., 2000) (Figure 1).

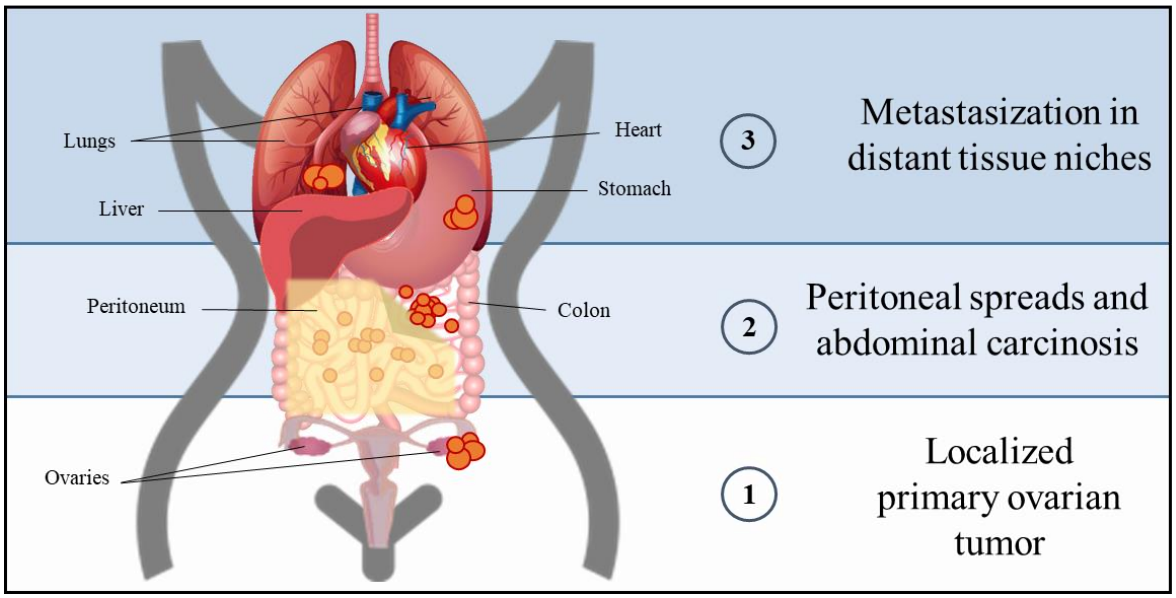


Figure 1. Scheme of the OC mechanism of primary tumor-spreading and metastasization.

The most common subtypes of ovarian cancer are serous, endometrioid, clear-cell, and mucinous carcinoma whereas transitional carcinoma and Brenner's tumors formed by transitional cells are less common (Seidman JD *et al*., 2000; Prat J, 2012). Although these subtypes were grouped together in the past and designated as epithelial OC, these types of tumors are now recognized as distinct entities with different clinical and biological behaviors. However, from a clinical point of view, present regimens, use conventional chemotherapy based on phase and grade rather than histotype of the disease (Wentzensen N *et al*., 2016). Indeed, it is now well-accepted that high- and low- grade serous carcinomas constitute distinct entities rather than a collection of the same type of tumor. Although they are similar in patients with advanced stage disease, their histological and molecular characteristics are completely different: *TP53* mutations are associated with high-grade serous carcinoma, while low-grade serous carcinomas are associated with *BRAF* and *KRAS* mutations (Leskela S *et al*., 2020; Bell D *et al*., 2011). Typically, endometrioid and clear cell carcinomas are present as early-stage diseases and related to endometriosis. Mucinous carcinomas are usually present as large unilateral mass and sometimes displays area of mucinous cysto-adenoma and mucinous borderline tumors (Wang YK *et al*., 2017). It is therefore imperative that these tumors be precisely subtyped, based primarly on morphology and immunohistochemistry studies.

1.2 Ovarian cancer care: from diagnosis to treatments

Ovarian cancer is staged by the FIGO (International Federation of Gynecology and Obstetrics) classification, which considers the degree of involvement of tissue, lymph nodes status and the extent of the metastases (Schumer ST *et al*., 2003). As a result, stage I and stage II cancers are confined to the pelvic cavity and are referred to as early stage; instead, stage III and stage IV cancers that spread into the abdominal cavity are classified as advanvced stage tumors (Prat J, 2015). Early detection of ovarian cancer provides an opportunity for successful treatment; however, early diagnosis of ovarian cancer is rare due to lack of symptoms at an early stage. Just one-fourth of patients with ovarian localized disease have a 5-year survival rate of 92%, while more than 75% shows advanced stage disease, with a 5-year survival rate ranging from 15% to 25% (Raja FA *et al*., 2012; Salani R *et al*., 2011). Since most patients are diagnosed with an advanced stage of the disorder, there is a high fatality-to-case ratio of all gynecological malignancies (Holschneider CH *et al*., 2000).

Normal treatment for advanced ovarian cancer is primary cytoreductive surgery followed by platinum-based chemotherapy (Angioli R *et al*., 2006). Cytoreductive surgery is performed to reliably diagnose, removing perfumed tissue that may cause disease and reduce the mass of tumors to improve adjuvant chemotherapy (Bristow RE *et al*., 2002; Zhu L *et al*., 2005). The utilization of cisplatin or carboplatin in the chemotherapy regimen for advanced ovarian cancer has proven to be a significant landmark among all types of OC treatments. Cisplatin is a drug that is able to bind to the nuclear DNA, resulting in interference with transcription and/or DNA replication and, ultimately, cell death induced by cell repair machinery (Fuertes MA *et al*., 2003). Platinum-based combination chemotherapy has been demonstrated better clinical response (CR) and progression-free interval (PFS) comparing to other agents (i.e. alkylating agents) alone or combinations without cisplatin (McGuire WP *et al*., 1996). Among all the combined treatments, Carboplatin-paclitaxel combination therapy had been shown to achieve a CR ranging from 50% to 81% and a median PFS ranging from 13.6 to 19.3 months. Numerous studies had shown that the combination of these two drugs is well tolerated by patients affected by advanced ovarian cancer (Ozols RF, 2000). At the end of therapy, mean global quality-of-life ratings were statistically slightly higher for the utilization of carboplatin-paclitaxel rather than singular treatments (65.25 vs 51.97) (du Bois A *et al*., 2003). Other reports have found that the quality of life has increased over time with the carboplatin and paclitaxel combined therapy relative to cisplatin and paclitaxel ones (Meier W *et al*., 1999). These groundbreaking trials have identified the mixture of carboplatin-paclitaxel as the firs-

line standard care for advanced ovarian lesions. During OC progression, environmental factors in poorly vascularized areas contribute to cells being quienscent and not responding to carboplatin and paclitaxel. Recently, in 3D OC models, it has been demonstrated that less than 40% of cancer cells are estimated to be proliferative (Riggs MJ *et al*., 2020). Moreover, a new approach namely hyperthermic intraperitoneal chemotherapy (HIPEC), has been used to kill poorly vascularized tumors with some success. A Multicenter, Phase 3 open-label study on patients with stage III disease of ovarian lesion showed that the use of HIPEC contributed to longer recurrence free survival and overall survival than surgery alone. This suggested that HIPEC could be used as a part of first line care and second line therapy for chronic conditions in the treatment of ovarian cancer (Riggs MJ *et al*., 2020).

Furthermore, new-targeted therapies were developed in the last 10 years. For example, Poly [ADP-ribose] polymerase (PARP) inhibitors trials were focused on patients with mutations in *BRCA* gene (Breast cancer type 1 susceptibility protein, involved in homologous recombination repair pathway). *BRCA* mutations occur in approximately 15% of patients affected by OC (Neff RT *et al*., 2017). A seminal Phase II study presented clinical proof of olaparib (as PARP inhibitor) activity in patients that have and not have *BRCA* mutations (Gelmon KA *et al*., 2011). The pharmaceutical approval was based on a Phase II evaluation of the patients with germline *BRCA* mutations of that population with a response rate of 46% in platinum-sensitive patients and 30% in platinum-resistant patients (Gelmon KA *et al*., 2011). In a randomized, double-blind, Phase II study in patients affected by highe-grade platinum-sensitive OC, who had undergone at least two prior platinum regimens and currently in the lasting response to platinum-based therapy, olaparib maintenance treatment decreased the risk of cancer progression by 65% and almost doubled the average PFS time (Ledermann J *et al*., 2012). Despite the efficacy of the treatment, all PARP-inhibitors showed gastrointestinal and hematologic toxicity, which can be problematic especially for patients on maintenance therapy, who have already been treated with a myelosuppressive regimen (LaFargue CJ *et al*., 2019). In 2011, was demonstrated by Burger RA *et al*. that the use of bevacizumab (Avastin), a humanized anti–vascular endothelial growth factor (VEGF) monoclonal antibody, in patients with advanced epithelial ovarian cancer for up to 10 months after carboplatin and paclitaxel chemotherapy, prolongs the median PFS for about 4 months. In the EU, bevacizumab, in combination with conventional carboplatin and paclitaxel, is indicated as the front-line treatment of patients with advanced (stages IIIB, IIIC and IV) epithelial ovarian, fallopian tube, or primary peritoneal cancer (Marchetti C *et al*., 2019). In 2020, the Food and

Drug Administration (FDA), extended the inclusion of olaparib, as well as bevacizumab, in combination as first-line chemotherapy of epithelial OC, fallopian or primary peritoneal cancer, in patients completely or partially reacting to first-line platinum-based chemotherapy and who have cancer associated with homologous recombination deficits (Walsh CS, 2020).

1.3 Ovarian cancer chemotherapy resistance and relapses

First-line carboplatin and paclitaxel chemotherapy produces enhanced CR; however, recurrence occurs in 25%of patients with early-stage disease and in more than 80% of patients with advanced disease (O'Toole O'Leary SJ, 2011). The majority of advanced ovarian cancer patients undergo relapse within 2 years of initial combination chemotherapy (Gottesman MM, 2002). Chemo-resistance to conventional chemotherapy regimens has now arisen as a significant challenge. Drugs resistance is one of the most important causes for failure of chemotherapy in advanced ovarian tumours. Resistance to chemotherapy can be classified in two subtypes: the innate chemo-resistance, where cancer cells are naturally drug-resistant, and adaptative chemo-resistance, which can be acquired during the therapeutical treatments (Rubin SC *et al*., 1999). The acquired chemo-resistance can occur due to genetic and epigenetic alternations that can enhance cancer cells response to chemotherapy-mediated effects such as oxidative stress, DNA damage and apoptosis (Armstrong DK, 2002). Therefore, chemo-resistance results as a multifactorial phenomenon that needs to be investigated properly, with the final goal of deciphering chemo-resistance pathways and defyning new targeted-drugs in order to re-estabilish therapeutical sensitivity (Lippert TH *et al*, 2011; Zahreddine H *et al*., 2013). Despite the initial response to optimal surgery and combined carboplatin and paclitaxel chemotherapy, around 70-80% of patients develop chemo-resistant recurrences. Nodules recurrence was observed in 38% of all recurrences, usually in tandem with peritoneal recurrence and abdominal diffuse carcinosis. Isolated remote metastases are an unusual form of recurrence (8%) (Amate P. *et al*., 2013). The biggest challenge remains the difficult detection of patients pre-disposed to chemo-resistance, as there are no available tests to direct clinicians to make decisions to adjust the course of care prior to chemotherapy. Further, while existing treatment regimens are set linear procedures, cancer biology is a highly dynamic and complex field. Adapting a preventive technique using a system-based approach to temporal biology and spatial differences of tumors is a futuristic goal in oncology (Gatenby RA *et al*., 2009).

1.4 Main molecular pathways involved in ovarian cancer chemoresistance

The delineation of molecular signatures in the field of ovarian lesions has paved the way for biomarker discovery. Over the last decade, omics approaches applied to the clinic have led to the detection of biomarkers in advanced ovarian cancer that helps scientific researchers to elucidate cellular molecular pathways that dictate drug resistance, to develop novel clinical methods to resolve resistance to therapy and to develop the right chemotherapy approach and improve patient management. Further, help to forecast the tumour response to the chemotherapy regimen, allowing treatments to be given to patients who would benefit and avoid the adverse effects of chemotherapy (Sehrawat U *et al*., 2016). In the field of cancer biology, a lot of genes, mRNA and proteins have been identified as involved in mechanisms underlying therapeutical resistance. In particular, according to the knowledge in literature, the molecular mechanisms of chemoresistance includes transporter pumps, oncogenes, tumor suppressor genes, mitochondrial alterations, DNA repair systems, autophagy, epithelial-mesenchymal transition (EMT) players, cancer stemness, and exosomes (Brasseur K *et al*., 2017; Lu C *et al*., 2008).

In most cases, drug resistance has the characteristics of multiple drug resistance (MDR), namely the insensitivity of cancer cells not only to drugs previously used, but also to many other drugs with various chemical structures and mechanisms of action (Lu C *et al*., 2008). Many of the compounds used in chemotherapy act as cytotoxic agents rather then as cytostatic. While cancer cells evolve different mechanisms for resistance to cytotoxic drugs, the main players involved in MDR are the ATP-binding cassette (ABC) drugs proteins. The latters, use ATP hydrolysis energy to actively transport drugs from the cytosol of cance cell to the extracellular environment (Altenberg GA, 2004). The most important drug carrier is glycoprotein P (P-gp) encoded by the multidrug resistance protein 1 gene (*MDR1, ABCB1*) (Choi CH, 2005). Expression of this protein has been observed in more than 50% of MDR-phenotype cancers and may be inherent or induced by chemotherapy (Moitra K *et al*., 2011). The second most significant drug carrier is the breast cancer resistant protein (BCRP) encoded by the *ABCG2* gene, first cloned from the MCF-7 breast cancer cell line (Doyle LA *et al*., 1998). Highly regulated expression of BCRP has been observed in many cancers, including breast cancer (Zhang F *et al*., 2011) and ovarian cancer (Maliepaard M *et al*., 1999), and it is known to be regulated, together with P-gp, by oncogene-related pathway such as Wnt/β-catenin, that is known as able to increases the defense of cancer cells from a lot of pharmacological compounds (Chikazawa N. *et al*., 2010). Others essential ABC transporters

involved in cancers MDR include MRP1 and MRP2 (MDR1-related protein 1 and MDR-related protein 2) encoded by *ABCC1* and *ABCC2* genes, respectively (Leonard GD *et al*., 2003; Cole SP *et al*., 1992; Fardel O *et al.,* 2005). Substrates used by MRP1 are similar to those used by P-gp, with the exception of taxanes (Ozben T, 2006). Instead, cisplatin and/or carboplatin is the main substrate of MRP2 (Ozben T, 2006; Robey RW *et al*., 2007).

For what concern the activation of oncogenes and/or inactivation or tumor suppressor genes during chemotherapy, *TP53* oncosuppressor gene play a key role in OC progression and chemoresistance as its genetic alteration is defined as predominant (Zhang Y *et al*., 2016). The encoded p53 protein is a nuclear transcription regulator involving in several cellular processes (Mantovani F *et al*., 2018). By binding to DNA, p53 regulates hundreds of target genes to preserve homeostasis and genome integrity. p53 is able to activate DNA repair proteins when DNA has sustained damage, stop cell growth by keeping the G1/S cell cycle, allow DNA repair, and initiate apoptosis if the DNA damage is irreparable (Brosh R *et al*., 2009). Numerous stimuli have been shown to trigger p53, including UV or gamma-induced DNA damage, inappropriate proto-oncogenic activation, mitogenic signaling, cellular oxidative stress and hypoxia (James A *et al*., 2014; Brooks CL *et al.,* 2010). Once triggered, p53 can induce cell cycle arrest, senescence, apoptosis or ferroptosis, depending on the cell context, by promoting the expression of a variety of genes crucial to the previous mentioned cellular functions (Jiang L *et al*., 2015; Chen J, 2016). Moreover, p53 induces the expression of p21, responsible for the cell cycle arrest on G1 phase and senescence (el-Deiry WS *et al*., 1993). In this context, the role of *TP53* mutations in OC occurrence and progression was also demonstrated *in vivo* showing that ovaries and falopian tubes, harbouring *TP53* mutations, tend to acquire a high-grade serous more aggressive phenotype (Kim J *et al*., 2015). For instance, another recent study, by using an ultra-deep sequencing, showed a higher amount of *TP53* mutants cells in the peritoneal ascitic fluid of women with high-grade serous OC (Krimmel JD *et al*., 2016). Therefore, it is now recognized that p53 oncosuppressor must be identified as a biomarker for cancer risk, prognosis and response to current therapy.

Furthermore, hypoxia is a typical feature of a significant number of malignant tumors and increased hypoxia-inducible factor-1α (HIF-1α) expression, predicts a weak ovarian cancer prognosis. In particular, it has been reported a strong relationship between hypoxia condition and cancer stem-like properties in human ovarian cancer cell lines (Qin J *et al*., 2017). Interestingly, by performing the knockdown of endogenous HIF-1α, significantly alleviates the chemotherapeutic resistance and promotes G1/S cell cycle transition resulting in an

increased sensitivity of OC cells to conventional therapies under hypoxic condition (Huang L *et al*., 2010).

A small subset of "tumor initiating cells" has been detected in ovarian cancer as previously observed in other tumor types (Abubaker K *et al*., 2013; Alwosaibai K *et al*., 2017; Bapat SA *et al*., 2005). These cells exhibit mesenchymal and stem-like characteristics and are intended to drive the initiation of the tumor (Pattabiraman DR *et al*., 2014). EMT's contribution to triggering ovarian cancer has been indicated by the discovery that EMT induction caused the repression of pairbox protein 2 (PAX2), a transcription factor that maintains the oviductal epithelial cell differentiation status (Alwosaibai K *et al*., 2017). Different stimuli like TGF-β, a strong EMT inducer in follicular fluid released during ovulation, could trigger EMT process and subsequent production of high-grade OC precursor lesions (Alwosaibai K *et al*., 2017; Newsted D *et al*., 2018). A lot of *in vitro* and *in vivo* studies had shown that cells that are resistant to carboplatin and/or paclitaxel, tend to acquire a mesenchymal phenotype that lead to the envision of the EMT process as a therapeutic resistance engine (Baribeau S *et al*., 2014; Chowanadisai W *et al*., 2016; Haslehurst AM *et al*., 2012). Growing evidence shows that PI-3K/AKT/NF-kB- and JAK/STAT-pathways can cause and be involved in resistance to chemotherapy. In particular, PI-3K/AKT pathway is frequently activated in OC resistant cells and triggers EMT by inducing the β-catenin pathway that flow on downstream EMT players such as SNAI1 and SNAI2 (SLUG), TWIST1/2, as well as the already mentioned ABC drug transporters (Shayesteh L *et al*., 1999). Additionally, resistin that is a macrophage-related cytokine, showed the importance of the JAK/STAT pathway in EMT-mediated chemoresistance. This cytokine mediates the *in vitro* and *in vivo* induction of platinum resistance, EMT, and stemming by binding Toll-like receptor 4 (TLR4), as well as stimulating the NF-kB-STAT3 pathway (Bugide S *et al*., 2017; Teeuwssen M *et al*., 2019). In addition, the inverse mesenchymal-epithelial transition (MET) process is required to form a macroscopic metastasis and EMT transcription factors are often down-regulated (Brabletz T, 2012). Therefore, the leading factors that govern this phenomenon, such as Zeb1, Zeb2, Snail, Slug, and Twist1/2, are all able to induce EMT but also have different non-redundant tissue- and context-specific roles. They may also affect the regulation of each other, leading to a complex regulatory network (Stemmler MP *et al*., 2019). A deeper understanding of how EMT affects the development and therapy resistance of ovarian cancer clearly warrants more study.

2. Mitochondria and Cancer

It is well known that most tumors are capable to increase the uptake and oxidation of glucose producing energy even in the presence of oxygen (aerobic glycolysis). This peculiar feature led Otto Warburg to propose that mitochondrial respiration deficiencies are the fundamental basis for aerobic glycolysis occurrence and tumour energetic support (Warburg O, 1956). Indeed, the "Warburg effect" is nowdays the basis for FDG- positron emission tomography (PET) tumor imaging, which is commonly used by clinicians for the diagnosis of the metastasis and aggressiveness of tumors (Gallamini A *et al*., 2014). However, during the last 15 years with the development and applications of the omics approaches in the oncological field, it clearly emerged that not all tumors possess the metabolic feature of aerobic glycolysis (Danhier P *et al*., 2017). In particular, cancer cells acquire a metabolic plasticity in the use of oxidative phosphorylation (OXPHOS) or glycolysis to adapt to the adverse microenvironment (Obre E *et al*., 2015; Zong WX *et al*., 2016; Vander Heiden MG and DeBerardinis RJ, 2017; Faubert B *et al*., 2020). Indeed, metabolic reprogramming of tumor cells is thought to be a result of metabolic adaptation involving mutations in oncogenes, dysregulating the expression of metabolic enzymes and the flows of metabolic pathways and thus influencing the whole cellular metabolism. In this framework, the metabolic rewiring it has been stated as an hallmark of cancer since uncontrolled proliferation that is the peculiarity of neoplastic disease is based on both alterated control of cell divison and adjustments of energy metabolism for supporting building blocks production (Hanahan D and Weinberg RA, 2011; Faubert B *et al*., 2020; Lee NCW *et al*., 2018; Boroughs LK *et al*., 2015). It is interesting to note that several studies highlighted the contribution of metabolic reprogramming also in chemotherapy resistance occurence (Cardoso MR *et al*., 2018; Rahman M *et al*., 2015; Maria RM *et al*., 2017). Further, the concept of metabolic rewiring is not only limited to an increased glucose uptake and use (Shukla SK *et al*., 2017), but also to the oxidation of other substrates such as glutamine, serine, glycine or acetate (Mashimo T *et al*., 2014; Jain M *et al*., 2012; Maddocks OD *et al*., 2016; Metallo CM *et al*., 2011). These data clearly underline that cancer cells are able to adapt to the use of different substrates due to their flexibility in the utilization of glycolysis, OXPHOS and FAO as resource of energy depending on the microenvironment influences (Corbet C *et al*., 2016). In the scenario of metabolic plasticity and rewiring, mitochondria are a pivotal hub since these organelles regulate the cellular profile in terms of bioenergetic and biosynthesis both in physiology and pathophysiology context (Jia D *et al*., 2018). Mitochondria are parental highly conserved cytoplasmic organelles derived from

symbiotic bacteria (Wallace DC, 2012). They co-evolved with their host, so that most mitochondrial proteins were nuclear encoded. Mitochondria, however, retains a small 16 Kb DNA genome (mtDNA) that encodes tRNAs and rRNAs and 13 proteins of respiratory complexes that constitute the OXPHOS system. As bioenergetic and biosynthetic organelles, they are responsible for many biochemical pathways as fatty acid oxidation (FAO), tricarboxylic acid (TCA) cycle, oxidative phosphorylation, and are involved in the synthesis of amino acids, lipids, nucleotides, heme and iron sulfur clusters, as well as NADPH for their own antioxidant protection. NADH and $FADH_2$ formed by TCA cycle activity are oxidized by the electron transport chain (ETC), generating across the inner mitochondrial membrane the electrochemical gradient that is used by FoF_1-ATP synthase complex for ATP production (Murphy E *et al*., 2016). In particular, the chemiosmotic coupling of the ETC to OXPHOS includes the activities of four multisubunit enzyme complexes: complex I (CI; NADH-ubiquinone oxidoreductase); complex II (CII; succinate-quinone oxidoreductase) complex III (CIII; cytochrome bc1 complex); complex IV (CIV; cytochrome c oxidase); and ATP synthase (complex V [CV]) as the site of OXPHOS, various assembly proteins and two electron carriers (ubiquinone and cytochrome c) (Sirey TM *et al*., 2016). The five enzyme complexes contain about 100 different proteins in mammals. They are unique since their protein component originated from two distinct genomes (mitochondria and nucleus). In particular, the mitochondrial genome codes for 13 subunit proteins (7 CI, 1 CIII, 3 CIV and 2 CV), but the majority of ETC proteins are encoded by the nuclear genome, translated in the cytoplasm and introduced into the mitochondria (Sirey TM *et al*., 2016). Due to the presence of hundreds of mitochondria in a cell and thus a variable number of mtDNA (polyploidy), with the onset of a mtDNA mutation these organelles can exhibit a condition known as heteroplasmy, in whose mutant genomes coexist with wild-type genomes. Depending on the degree of heteroplasmy, cells can show different effects on mitochondrial functions. In addition, mtDNA mutations can occur in both somatic and germline inducing defect in specific cells, tissues or in the whole organism and leading pathologies such as cancer and neurodegeneration (Nunnari J and Suomalainen A, 2012; Yan C *et al*., 2019; Perrone AM *et al*., 2018; Gasparre G *et al*., 2011). Nonetheless, there is debate about the pathological importance of mtDNA mutations in cancer cells (Frezza C and Gottlieb E, 2009). A study on 1675 tumors revealed that mtDNA mutations are not permissive for cancer development and invasions showing that severe mtDNA mutations, such as nonsense substitutions and frameshift indels, may cause a severe energetic impairment and thus are negatively selected in cancer cells. These data also suggest that a certian degree of respiraratory chian function may

be necessary for carcinogenesis and tumor progression (Ju YS *et al*., 2014; Iommarini L *et al*., 2013). It is interesting to note that oncocytic tumors are the exception to this rule since they are characterized by the accumulation of mtDNA. However, a clear correlation between pathogenic mtDNA mutations occurrence and mitochondrial energy impairment has been clearly demonstrated in oncocytic tumors (Bonora E *et al*., 2006; Porcelli AM *et al*., 2010). For instance, a statistically significant prevalence of disruptive mutations in mitochondrial genes coding for the ETC complex I has been reported as frequent in thyroid oncocytic tumors (Gasparre G *et al*., 2007). Moreover, in 2018 Perrone AM *et al*. provide an update on the potential use of mtDNA sequencing to distinguish specific somatic mutations in synchronous primary tumors, such as ovarian and endometrial cancers, and thus identify clonality within the two distinct lesions.

Once it has been established that mitochondrial mutations could have a linkage with cancer, it is interesting to unravel the mechanisms by which the mutations can arise. For instance, mtDNA somatic mutations can occur due to the reactive oxygen species (ROS) production, as result of the leakage of electrons from respiratory complex I (CI) and III (CIII), the main mitochondrial production sites of these signals. In particular, intracellular ROS can increase the occurrence of mtDNA mutations that affect respiratory complexes subunits. Moreover, ROS can in turn be enhanced by the occurrence of mtDNA mutation as in a vicious cycle. These ROS are able to modify mitochondrial phisiology by several mechanisms such as the direct alterations of respiratory complexes (as CI) and mitochondrial phospholipids peroxidation, in particular cardiolipin (Paradies G *et al*. 2010; Petrosillo G *et al*., 2009), that is necessary for proper assembly of mitochondrial supercomplexes and their functionality. In this context, it is important to introduce the supramolecular organization of ETC complexes. It is known that CI is able to form a supercomplex with CIII dimer and CIV (SC I+III_2+IV, known as the respirasome), as well as with $CIII_2$ alone (SC I+III_2). $CIII_2$ can forms a supercomplex with CIV (SC III_2+IV) and CV usually can forms dimers (CV_2) (Lobo-Jarne T and Ugalde C, 2018; Letts JA and Sazanov LA, 2017). These structures have been reported as functionally fundamental as they lead to the maintenance of the stability of complexes, the reduction of ROS production and the efficiency of electron channelling (Letts JA and Sazanov LA, 2017; Genova ML and Lenaz G, 2013). It is well known from the literature that mitochondria can adapt their physiology in terms of membranes distributions and super-assembly of ETC complexes, in order to prevent the mitochondrial electron leakage and ROS production (Maranzana E *et al*., 2013). However, the importance of mitochondrial

supercomplexes in pathologies is still debated in the literature. Nonetheless, it has been interestingly reported that the respiratory supercomplexes disruption, associated with the mitochondrial network fragmengtation, leads to the increases of mitochondrial superoxide production that is tightly linked to the tamoxifen-resistant phenotype of breast cancer cells (Tomková V *et al*., 2019). Moreover, it is know that the ROS generation is increasingly lost during ageing and their production is irreversibly improved, leading to the occurrence of alterated signalling and structural damage, as in a vicious cycle (Genova ML *et al*., 2015). Hence, the supramolecular organization of ETC complexes may play a key role in maintaining cellular redox homeostasis and thus exhert an influence in stress-related pathologies such as neurodegeneration, aging and cancer (Lenaz G *et al*., 2012; Genova ML *et al*., 2015).

Changes in cellular redox states have long been studied in various diseases and their role in tumorigenesis have been of considerable interest (Cross CE *et al*., 1987; Mugge A, 1998; Paravicini TM, 2006). Contradictory evidence regarding the role of mitochondrial ROS in tumorigenesis has emerged over the past decade. Elevated ROS are thought to contribute to tumorigenesis by acting in part as a direct DNA mutagen (Ogrunc M *et al*., 2014) or by induced genomic instability when topoisomerase II is triggered (Shibutani S *et al*., 1991). These findings support the fact that mutagenesis induced by ROS is a crucial driver for initiation and growth of the tumor. In this context, it has been demonstrated that ROS are able to promote cell migration by activating the k-Ras induced anchorage-independent growth in lung cancer cells (Weinberg F *et al*., 2010). Further, it has been demonstrated that ROS levels are able to drive mitogenic signaling cascades such as the PI3K/AKT/mTOR and MAPK/ERK axes (Roberts PJ and Der CJ, 2007). In this framework, emerging evidences indicate also that intracellular ROS levels can induce the stabilization and thus activation of HIF-1α onco-protein, one of the hallmarks of tumorigenesis (Klimova T and Chandel NS, 2008; Bell EL *et al*., 2007). Moreover, Funato Y *et al*. in 2006 have demonstrated that ROS can active the signaling of the Wnt/β-catenin pathway with a mechanism that remain poorly understood. In particular, it has been demonstrated that mitochondrial ROS can firstly induce the stabilization of β-catenin, which is in turn functionally active and migrates into the nucleus transcribing for EMT, MDR and proliferation genes (Shin SY *et al*., 2004). Nonetheless, when ROS are persistent in the cell, they lead to the inactivation of the β-catenin pathway (with a mechanism that needs to be elucidates), reducing its nuclear concentration and leading to its proteasomal degradation (Shin SY *et al*., 2004). By contrast, high

concentrations of ROS were also shown to be toxic for the cell, since it was demonstrated that are able to induce the cell cycle alteration, senescence and death by triggering the ASK1/JNK and ASK1/p38 signaling pathways in human fibroblasts and cancer cells (Ichijo H *et al*., 1997; Moon DO *et al*., 2010). Overall, there are many demonstrating evidences for either a supportive or a suppressive role of ROS during tumorigenesis.

Therefore, understanding in depth mitochondrial bioenergetics, metabolism and physiology of cancer cells, may open up a new horizon for the diagnosis and care of most malignancies, including ovarian cancer.

2.1 Ovarian cancer metabolic adaptations

The topic of metabolic rewiring in ovarian cancer is much debated in literature. It has been demostrated that OC cell lines with high glycolysis rate also showed high OXPHOS profile and increased metabolic-related gene expression, indicating that most of the ovarian cancer cell lines favor a highly metabolic phenotype (Dier U, 2014; Fabian C *et al*., 2012). Moreover, migration of ovarian cancer cells has been demonstrated to be induced by pyruvate, involving mitochondrial activity (Caneba CA *et al*., 2012). Other studies have shown that certain invasive OC cells tend to fuel TCA increasing the utilization of glutamine, which suggests a functional involvement of OXPHOS (Yang L *et al*., 2014).

In particular, metabolic reprogramming acts as a key regulator of progression and chemoresistance processes in OC. There are several schools of thought in considering high- or low-OXPHOS the bioenergetic profile of OCs when become chemoresistant to therapies. First, it has been demonstrated that OC cell lines adapt their mitochondrial metabolism overcoming the "Warburg effect", by favoring oxidative phosporylation when becomes chemoresistant to conventional chemotherapies (Dar S *et al*., 2017; Zampieri LX *et al*., 2020). This suggests that, chemoresistant cell lines need a higly metabolic profile in order to progress and increase their aggressiveness. On the other hand, it has been recently demonstrated that high-OXPHOS OC cells and patient-derived xenografts (PDXs) are better responder to therapies rather than low-OXPHOS ones, that hence resulted as chemoresistant to therapies (Gentric G *et al*., 2019). In the latter, while low-OXPHOS OCs exhibit a glycolytic preference, high-OXPHOS ovarian lesions rely on oxidative phosphorylation, supported by glutamine and fatty acid oxidation with chronic ROS production and oxidative stress. In addition to the glucose and glutamine metabolism, many studies have documented

alterations in lipid metabolism, which collectively suggest that tumours often exhibit a lipogenic phenotype (Menendez JA *et al*., 2017). For instance, it has been demonstrated that high lipid synthesis rates, that reflect increased lipogenic enzyme expression such as fatty acid synthase (FASN), and high fatty acids (FA) uptake through FA binding proteins (FABPs) and CD36 channel is strongly linked to cancer progression (Menendez JA *et al*., 2017; Zaidi N *et al*., 2013; Bensaad K *et al*., 2014; Ackerman D *et al*., 2014). Moreover, the inhibition of lipid storage by FABP4 or FABP7 knockdown decreased OC cells survival under hypoxia-reoxygenation and impaired tumorigenesis *in vivo* (Bensaad K *et al*., 2014). In fact, FABP4 can be a reliable molecular predictor of residual disease in high-grade serous ovarian cancer (Tucker SL *et al*., 2014). Several studies have indicated that insaturated fatty acids play a key role in tumor growth pathways by stimulating the β-catenin pathway, downregulating PTEN or increasing the adherence of OC cells (Kim H *et al*., 2015; Vinciguerra M *et al*., 2009). Metastases in breast and ovarian cancer models have been shown as tightly dependent on FA β-oxidation (Nieman KM *et al*., 2011; Park JH *et al*., 2016). Apart from metastatic ovarian cancer, altered lipid metabolism was also observed in Lee *et al*. as a crucial metabolic feature of lymph node tumor metastasis (Lee CK *et al*., 2019). The authors used subcutaneous xenograft models and compared cancer cells isolated from the primary tumor with the micrometastatic ones and macrometastatic lymph nodes. RNA-sequencing showed that top regulated gene sets were positively associated with adipogenesis, metabolism of fatty acids, homeostasis of cholesterol, and oxidative phosphorylation in the micro- and macro metastatic tumors. In a more recent study, patient-derived models of cisplatin-resistant OC were used and it was demostrated that the intraperitoneal injection of orlistat (FASN inhibitor) along with cisplatin, significantly decreased the tumor growth and tumor burden (Chen RR *et al*., 2019). Based on the research state, previous studies have shown that adipocytes may potentially be associated with chemoresistance development in ovarian cancer (Nowicka A *et al*., 2013). Adipocyte co-culture with ovarian cancer cells can trigger multiple signal transduction pathways, such as MyD88/NF-kB/Bcl-xL, but it has not extensively investigated the mechanism with which adipocytes are involved in regulating this effect (Cardenas C *et al*., 2017). More strickly to the OC progression in patients, ovarian cancer cells isolated from ascites or omental microenvironments showed an enhanced lipid metabolism during metastatic progression via lipogenesis mediated by AMPK/ACC/FASN and cascades signaled by AMPK/TAK1/NF-kB. Targeting these pathways with the combined cocktail of low-toxic compounds can also be an alternative therapeutic technique to avoid peritoneal metastases of ovarian cancer (Chen RR *et al*., 2019).

In addition to the OXPHOS modification, genetic and biochemical evidence has suggests that deregulations of mitochondrial biogenesis, morphology, dynamics (fusion/fusion) and apoptosis are the main aspects of carcinogenesis (Iommarini L *et al*., 2017; Srinivasan S *et al*., 2017). However, further investigation needs to be performed to define the role of these mitochondrial physiologic processes in OC. In this regard, mitochondrial biogenesis is the process with which cells increase their mitochondrial mass, and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) is one of the master regulators of this process (Wu Z *et al*., 1999). As a transcription factor, PGC-1α can bind on targets as PPARα, PPARβ/δ and PPARγ, which coordinate the expression of mitochondrial genes and indirectly lead to the transportation and use of FA (Lin J *et al*., 2005). In addition, PGC-1α upregulates multiple gene expression of enzymes of the tricarboxylic acid cycle (Hatazawa Y *et al*., 2015) and of FA oxidation in mitochondria (Calvo JA *et al*., 2008). PGC-1α also controls the expression of nuclear and mitochondrial genes that encodes for electron transport and OXPHOS components via nuclear respiratory factors 1 and 2 (NRF-1 and NRF-2) and the co-activation of the estrogen-related receptor α (ERRα) (Dillon LM *et al*., 2012). Furthermore, the lipid distribution is enhanced by PGC-1α that acts upregulating CD36 and FABP proteins (Supruniuk E *et al*., 2017). In this context, it has been demonstrated that CI-KO xenografts displayed an increased expression of PGC-1α (Kurelac I. et al., 2019) and that CI-deficient tumors harbor swollen mitochondria with disorganized cristae, recapitulating oncocytic lesions (De Luise M. *et al*., 2017), supporting that exist a tight and direct linkage between mitochondrial physiology alterations and PGC-1α expression levels.

Recent articles showed altered expression of PGC-1α in several tumors and metastasis (Mastropasqua F *et al*., 2018). Moreover in human serous and mucinous OC tissues was seen an increased mitochondrial biogenesis through increased protein levels of PGC1α and mitochondrial transcription factor (TFAM), as well as mtDNA content (Signorile A *et al*., 2019). In addition, analysis of ETC complexes activity showed a sharp drop in complex I functionality. Furthermore, activation of the cAMP/PKA pathway and an increased amount of SIRT3 protein (Sirtuin also involved in mitochondrial biogenesis mechanisms) was observed (Signorile A *et al*., 2019). Moreover, in Gentric G. *et al*. 2019, several *in vivo* and *in vitro* data highlighted a stress-mediated PML-PGC-1α-dependent mechanism that is able to promotes OXPHOS metabolism and restores chemosensitivity in ovarian cancer. Collectively, all these observation culminate in the fact that exist an urgent need in unravel molecular and

metabolic features of OC, useful to outline its mechanisms of neoplastic progression in order to make the treatments as efficient and targeted as possible.

2.2 Role of mitochondrial chaperonines in cancer

It has been shown that chaperonines (heat shock proteins, HSPs) are involved in malignant processes and molecular mechanisms underlying drug resistance in cancer. HSPs are considered highly conserved stress-inducible molecules of prokaryotic and eukaryotic organisms range from bacteria to humans (Richter K *et al*., 2010). These molecules are renowned for their molecular activities such as protein folding, anti-aggregation and trafficking (Hartl FU, 1996; Calderwood SK *et al*., 2006). The expression of HSPs is either constitutive or caused by a range of factors such as physiological, environmental and pathological pressures, hypoxia, inflammation, toxic agents, heavy metals exposure and cancer (Chatterjee S *et al*., 2017). HSPs are historically classified, according to their molecular weight, into six major families. These includes small HSPs (sHSPs), HSP40 (DNAJ chaperones), HSP60, HSP70, HSP90 and the large HSPs (HSP110 and GRP170) (Kampinga HH *et al*., 2009). Many cancers had been reported with a high expression of HSPs, including breast, head and neck, gall bladder, colorectal, skin, liver, colon, kidney, prostate and ovarian cancer (Chatterjee S *et al*., 2017; Jego G *et al*., 2013). In apoptosis, HSPs play a dual and complex function by triggering or counteracting cell death. For example, HSPs have been shown to activate apoptotic mediators such as pro-casapase 3 (Samali A *et al*., 1999; Xanthoudakis S *et al*., 1999), and to bind and inhibit multiple molecules in the apoptotic pathways at different levels (Kennedy D *et al*., 2014). Moreover, some oncogenes, including mutant p53, have been found to be chaperoned by HSPs in order to prevent its degradation and thereby evading classical apoptotic pathways and favoring the cell survival in cancer (Alexandrova EM *et al*., 2015; Wawrzynow B *et al*., 2018). In several cancers, including prostate (Hoter A *et al*., 2019), liver (Wang C *et al*., 2016), lung (Hendriks LE *et al*., 2017), head and neck (Yin X *et al*., 2005) and ovarian (Stope M *et al*., 2016), increased levels of some HSPs have been reported as involved in drug resistance.

In addition, others studies suggest that high HSP90 levels of expression are associated with tumour aggressiveness, metastasis and chemotherapy resistance (Ciocca DR *et al*., 2005). HSP90 has been identified among the tumor antigen proteins in OC by advanced serological approaches (Luo LY *et al*., 2002). In this regard, the mitochondrial homologue of HSP90,

tumor necrosis factor receptor-associated protein 1 (TRAP1), is significantly involved in many cancers including ovarian cancer. In tumor cells such as breast, colon, pancreas, and lung, TRAP1 has been strongly expressed while basal expression has been found in corresponding healthy cells (Amoroso MR *et al*., 2014; Matassa DS *et al*., 2018). Interestingly, recent large-scale studies have shown that TRAP1 levels of expression in ovarian cancer has been found consistent with the poor prognosis (Lettini G *et al*., 2017; Amoroso MR *et al*., 2016). In addition, TRAP1 expression was found to correlate inversely with tumor grade or stage and directly correlate with overall survival (Matassa DS *et al*., 2016). These findings are consistent with studies in which a better response to chemotherapeutics has been found in patients with higher levels of expression of TRAP1 leading to the inference that TRAP1 may acts as tumor suppressor (Aust S *et al*., 2012). Furthermore, Amoroso et al., 2016 suggested that the decrease in TRAP1 expression in ovarian cancer might be due to genetic deletion or gene-level copy number variations (CNVs) particularly in late stages of high-grade serous OC (Amoroso MR *et al*., 2016).

Recent insights on OC have provided TRAP1 major role in the growth, platinum response and inflammatory activation of the disease (Amoroso MR *et al*., 2017). TRAP1 can inhibit the respiratory mitochondrial chain activity by its direct interaction with the mitochondrial subunit B of succinate dehydrogenase (SDHB). This effect that supports a mainly glycolytic metabolic phenotype of cancer cells, leads to envision TRAP1 as factor that may be considered a pro-oncogene, depending on the metabolic characteristics of the tumor tissue in certain types of cancer (Matassa DS *et al*., 2018). In addition to these interesting observations on the various function played by TRAP1, it was reported that TRAP1 is located in a molecular complex with the cytosolic homolog HSP90 and cyclophilin D, which suppress apoptosis by controlling transitional mitochondrial pores opening (Kang BH *et al*., 2007). Further, OC cells PE01-CDDP, which have relatively high TRAP1 levels, were twenty times much resistant to cisplatin compared to the parental PE01 cell line that was less resistant to cisplatin (Landriscina M *et al*., 2010). For what concern the HSP70 family, aroused a high interest mitochondrial Mortalin or HSPA9, which has been reported as involved in ovarian carcinogenesis and malignancy of the tumor (Kaul SC *et al*., 2007). Microarray analysis of OC tissue have shown that, relative to early stage of ovarian carcinomas and normal ovarian tissues, mortaline is commonly expressed at advanced stages (Kaul SC *et al*., 2007). Mortalin over-expression and its ability to trigger malignancy is likely induced by its cytoplasmic p53 binding (Kaul SC *et al*., 2005). Hu *et al*. have demonstrated the oncogenic function of

mortalin in ovarian cancer by promoting the tumor growth and migration/invasion through crucial pathways, including cell cycles and MAPK–ERK signaling pathways (Hu Y *et al*., 2016). Other studies have shown that the inhibition of mortalin might suppress serous ovarian cell proliferation, cell motility, and EMT through the inhibition of the signaling pathway of Wnt/β-catenin (Xu M *et al*., 2019). In addition, recent studies have shown that mortalin expression in OC cells is regulated by the NF-kB p65 promoter specific association (Li S *et al*., 2019). In this context, it has also been studied the important mitochondrial import coiled-coil helix coiled-coil helix domain protein 4 (CHCHD4) that is able to regulates the ETC activity and the consumption of oxygen and involved in the *in vivo* tumor occurrence (Yang J *et al*., 2012; Thomas LW, Stephen JM *et al*., 2019). In the same framework, Thomas LW *et al*. 2019, it has been demonstrated that the tumor cell growth mediated by CHCHD4 is related to the CI-regulated mTORC1 signaling pathway and amino acid metabolism. It was reported that the expression of CHCHD4 in tumors is inversely linked to gene expression associated with EMT and that increased expression of CHCHD4 modulates EMT-related phenotypes in tumour cells. Furthermore, in human 41 DNAJ/HSP40 chaperone proteins were identified. Three classes of J proteins can be distinguished by their domain structure: DNAJA, DNAJB and DNAJC (Kampinga HH *et al*. 2009). J proteins belonging to the DNAJA class are largely homologous to DnaJ and are characterized by an N-terminal J domain, a G/F-rich region, a C-rich region (cysteine), and their C-terminus is variable. J proteins belonging to the DNAJB class contain the N-terminal J domain and the G/F (glycine/phenylalanine) rich region. Class J DNAJC proteins are characterized by containing a J domain which does not necessarily have to be located at the N-terminus of the protein (Kampinga HH *et al*. 2009). In this regard, a lot of DNAJ proteins are reported as adept at influencing the metabolic and molecular features of cancers. For example, the Hsp40 member C12 (DNAJC12) has been significantly linked with lymphatic involvement, infiltrative type progression, lymph node metastasis in advanced stage of gastric cancer and has been established in multivariable analysis as independent prognostic factor for overall survival (Uno Y *et al*., 2019). Moreover, DNAJB4 was found to be downregulated in breast cancer and could contribute to this downregulation in epigenomic events such as methylation of CpG and histone deacetylation. It has been concluded that DNAJB4 is a candidate as tumor suppressor and a potential biomarker for breast cancer (Acun T *et al*., 2017). It has been also demonstrated that the over-expression of another chaperonine, DNAJA1, can inhibits the stress response capabilities of c-jun, the oncogenic transcription factor, and reduces the cell survival of pancreatic cancer cells. DNAJA1 probably activates the DNAK protein by forming a complex that suppresses the JNK

signaling pathway, c-Jun phosphorylation and anti-apoptosis (Stark JL *et al*., 2014). Despite the fact that the role of DNAJs chaperonine is largely investigated in many types of tumors, very low is the knowledge on the role of these chaperons in OC. Nevertheless, an interesting observation by Shridhar *et al*. has led to the identification of a novel gene that is in homologous to the DNAJ domain, which exists in a several proteins including HSP40 family (Shridhar V *et al*., 2001). The identified gene has been designated as *DNAJC15* and the codified protein as methylation-controlled J protein (MCJ). Strikingly, loss of MCJ expression had been reported as frequent in OC cell lines. This aspect and the roles of MCJ in cancer will be fully discussed in the next paragraph.

2.3 Roles of MCJ/*DNAJC15* chaperonine

MCJ is a mitochondrial chaperone protein of the inner mitochondrial membrane (IMM) encoded by *DNAJC15* gene. In Figure 2 is showed the co-immunofluorence of MCJ and cytocrome c, which confirms that MCJ resides in mitochondria. It belongs to the J-protein family and is the homolog of yeast TIM-14. MCJ anchors with its transmembrane domain in the IMM, interfacing in the intermembrane space with the N-terminal and in the mitochondrial matrix with its J-domain. (Kampinga HH *et al*. 2009).

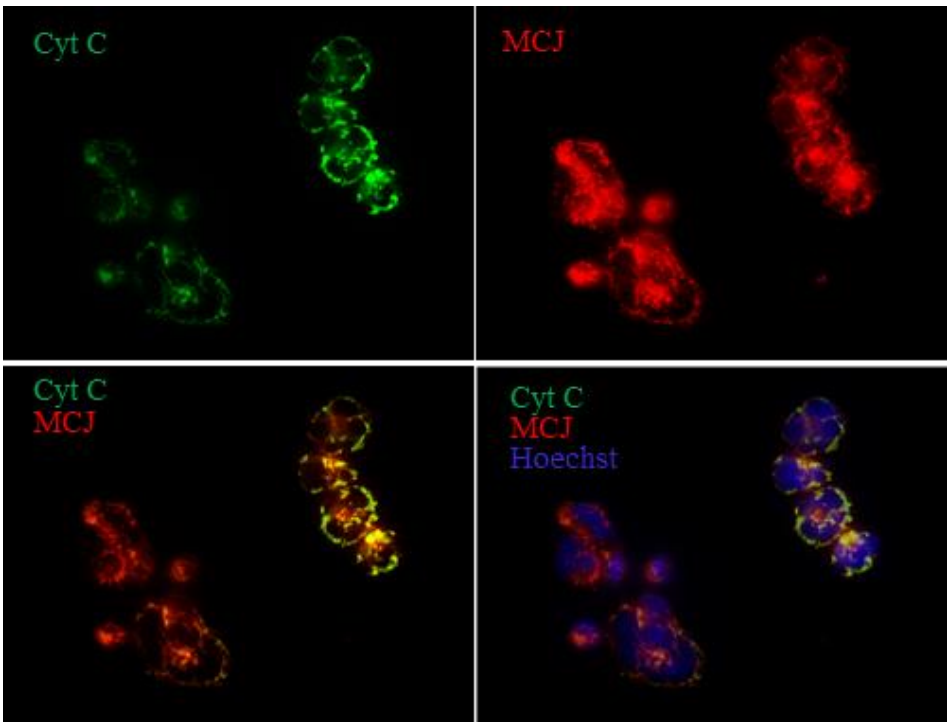


Figure 2. Co-immunofluorescence of MCJ and Cytocrome C in A2780 OC cell line, performed by epifluorescence digital microscopy (60x objective, Nikon).

It has been investigated the first time in cancers when the reasearchers discovered the *DNAJC15* loss of expression as the result of the methylation of the gene and, therefore, it was attributed the name of Methylation Controlled J protein (Shridhar V *et al*., 2001). MCJ, residing in the IMM, acts as co-importer chaperone protein involved in the mitochondrial translocation mechanisms associated with the TIM-23 complex (Figure 3). MCJ interacts with MAGMAS, a component of the translocation system, forming a stable complex with its soluble domains. When MCJ is missing, TIM-23-mediated protein translocation at the mitochondrial inner membrane undergoes structural alterations and functions loss (Schusdziarra C *et al*., 2013). MCJ contains an active and functional J domain capable of stimulating the ATPase activity of mtHSP70, favoring the folding of imported proteins. This functional interaction appears to be countered by MAGMAS (Schusdziarra C *et al*., 2013).

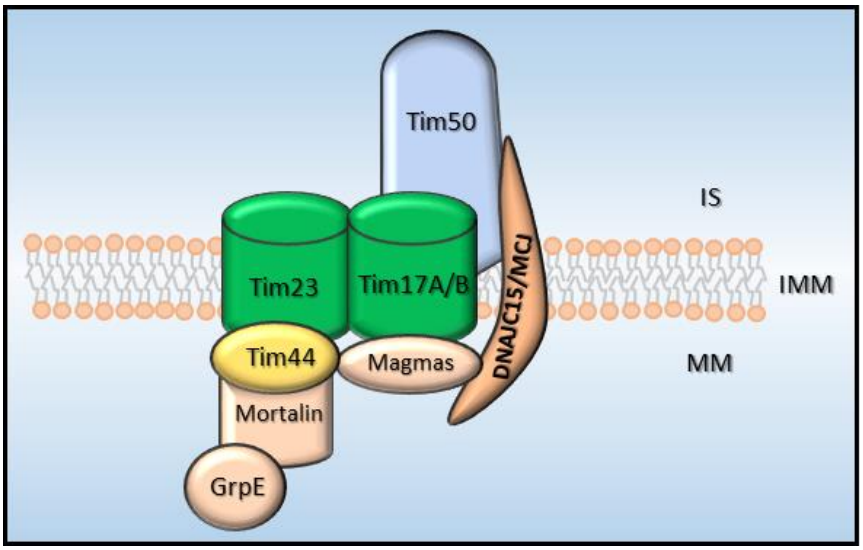


Figure 3. Schematic representation of MCJ localization in the Tim-23 import machinery. Intermembrane space (IS); inner mitochondrial membrane (IMM); mitochondrial matrix (MM).

It was later described in detail that the expression of MCJ is tissue specific and depends on the methylation state of the CpG islands associated with its gene promoter and close to the first coding exon. MCJ loss of expression was observed in different types of cancer and correlated with increased chemoresistance of cancer cell lines (Strathdee G *et al*., 2004; Fernández-Cabezudo MJ *et al*., 2016). MCJ deficient cells are reported as less sensitive to apoptosis processes. It was suggested that MCJ could be able to allow the translocation of

pro-apoptotic factors into the mitochondrial matrix (Sinha D *et al*., 2014) or to sequester anti-apoptotic factors, which it then releases into the matrix when celld undergo apoptosis (Sinha D et *al*., 2014). Finally, beyond this role, it has been also shown that MCJ is able to recruits cyclophilin D to the transitional mitochondrial permeability pore and promotes its opening (Sinha D *et al*., 2014). Furthermore, MCJ is also reported as negative endogenous regulator of the respiratory chain by physically interacting with subunit NDUFV1 of respiratory complex I (Hatle KM *et al*., 2013). In the latter, by using nude mice KO for *DNAJC15,* authors had demostrated that the lack of MCJ expression leads to an increased activity of complex I and organization of mitochondrial supercomplexes, and higher mitochondrial membrane potential and ATP production. They had also observed that the enhanced mitochondrial respiration in the absence of MCJ seems to prevent the pathological accumulation of lipids in hepatocytes (higher lipid catabolism), in response to a diet rich in cholesterol. More, In CD8+ T-lymphocytes, carrying higher levels of MCJ expression, the oxidative metabolism that is crucial for T-mediated memory, was reduced (Champagne DP *et al*., 2016). Recently, it has been reported a tight association between glycolytic phenotype, MCJ expression and active caspase-3, all aiming to prevent the accumulation of highly proliferative CD8+ T-cells and trigger their timely death (Secinaro MA *et al*., 2019). Moreover, in 2020 Barbier-Torres L *et al.* have demonstrated that the reduction in MCJ expression improves hepatocyte capacity to mediates fatty acid β-oxidation and minimizes lipid accumulation, resulting in less hepatocyte harm and fibrosis. In addition, MCJ levels in patients affected by Nonalcoholic fatty liver disease (NAFLD) were higher compared to healthy subjects. Hence, the inhibition of MCJ appears as an alternative approach to NAFLD treatment. Even more, MCJ has been reported as crucial in the control of the mitochondrial metabolic state of macrophages and able to regulates their response to inflammatory stimuli (Navasa N *et al*., 2015). A novel mechanism of gene regulation of MCJ, different from CpG island methylation, has been demonstrated: IKAROS, a transcriptional factor, appears to bind the promoter of the MCJ gene in a Casein-kinase II-dependent manner, mediating its transcriptional repression. IKAROS is a transcription factor stimulated, for example, by IFN-γ, suggesting a dynamic metabolic adaptation following external stimuli that occurs in inflammation processes (Navasa N *et al*., 2015). Thus, despite its location within mitochondria, MCJ is able to exert, directly or indirectly, an influence in key molecular pathways in the cell biology.

Despite the studies above mentioned, the knowledge about the role of MCJ in cancer and, in particular in OC, is very missing nowadays. Recent studies have showed that the

hypermethylation of *DNAJC15* gene is associated with cancer aggressivenes and stages in malignant paediatric brain tumors (Lindsey JC *et al*., 2006), breast cancer (Fernández-Cabezudo MJ *et al*., 2016) and ovarian cancer (Shridhar V *et al*., 2001). In the latter, it was reported that MCJ resulted inactivated in over 50% of ovarian cancer cell lines and primary tumours by epigenetic silencing. This observation was supported later in 2004, by Strathdee G *et al*., which corroborate that the loss of MCJ is typical of drug-resistant ovarian cancer cell lines and depends on methylation of a CpG island within first exon of the gene but independent of methylation within the promoter region. Furthermore, this observation was supported by the demonstration that high levels of *DNAJC15* methylation in ovarian cancer patients are associated with both poor response to conventional chemotherapy and overall survival (Strathdee G *et al*., 2005).

Thus, all these findings highlight MCJ as a putative crucial factor in OC chemoresistance and progression mechanisms, leading to the hypothesis that this chaperonine may be a novel player in in the OC field.

AIMS OF THE STUDY

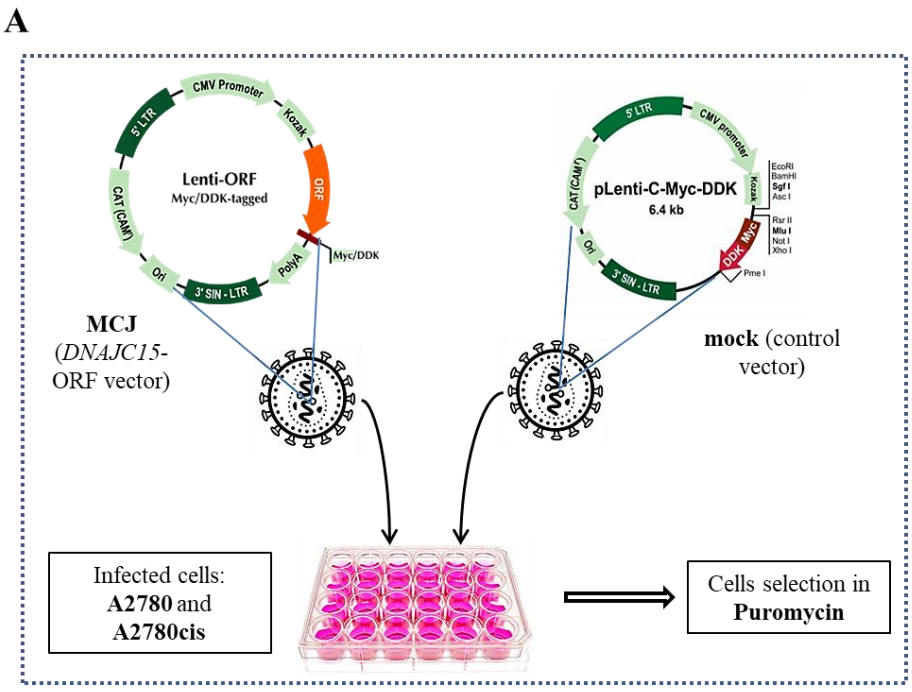
Ovarian cancer (OC) is the most lethal gynecological tumor due to its extremely silent invasive capacity. The high mortality of OC is often determined by a therapeutic failure due to the development of pharmacological chemoresistance associated with changes in cellular metabolic state and mitochondrial bioenergetic efficiency. Moreover, it has been reported that ovarian lesions chemoresistance is associated with the epigenetic silencing of *DNAJC15* gene that encods for the mitochondrial co-chaperonine MCJ. The lack of this is able to modify the structure and function of mitochondrial respiratory chain complexes. The findings that mitochondrial functions are a pivotal hub for OC progression and chemoresistance and that MCJ co-chaperonine is generally downregulated when these lesions becomes more aggressive and pharmacologically resistant, lead to envision this chaperonine as a crucial player in the metabolic adaptation of OC.

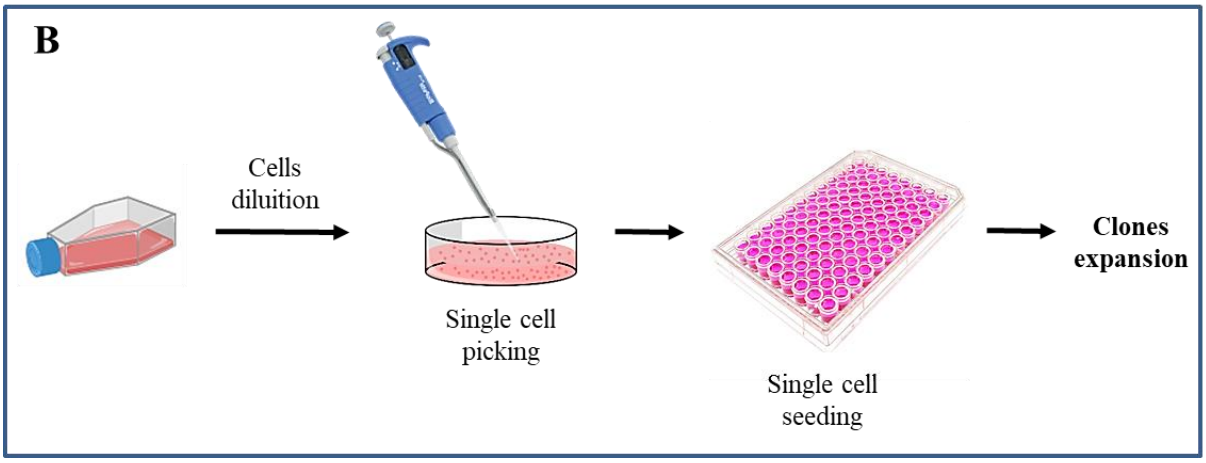
Hence, the main aim of this project is to assess whether the expression of MCJ mitochondrial chaperone can modulates the OC tumorigenesis and the multi-drug resistance occurrence, making this tumour more sensitive to chemotherapy. To achieve this aim we have first generated chemosensitive and chemoresistant OC cell lines in which MCJ was over-expressed. Then, these OC models have been used to elucidate the impact of MCJ expression on resistance to therapies and *in vitro* migratory and proliferative capacities. Further, the role of MCJ as molecular player in mitochondrial metabolism and extra-mitocondrial pathways was investigated, with particular focus on mitochondrial bioenergetics and chemoresistance-related signaling pathways. The results reported in this study will allow increasing the knowledge of MCJ biology in OC, particularly with new insights in molecular contexts such as the mechanisms of metabolic adaptation, resistance to therapies and proliferation of this aggressive tumor.

EXPERIMENTAL PROCEDURES

Cell cultures

A2780 and A2780cis cell lines from ovarian-endometrioid carcinoma, respectively chemosensitive and chemoresistant to cisplatin, were purchased from American Type Culture Collection (ATCC, Manassas, VA). In order to maintain the state of chemoresistance, the A2780cis cell line underwent a treatment with 1µM of cisplatin every eight passages in culture. Both cell lines were cultured in RPMI-1640 medium (ThermoFisher Scientific) containing 11mM of glucose and 2 mM of L-glutamine, supplemented with 10% of fetal bovine serum (FBS), 100µg/mL of penicillin-streptomycin at 37°C, in humidified atmosphere and 5% of CO_2. The stable MCJ-over-expressing cell lines were generated through Lentivirus-mediated infection (Origene), by using lentivirus containing both the over-expression construct and the empty-vector (mock) which was used as a control. The infection was performed in both cell lines, which subsequently underwent a selection in puromycin antibiotic (0.75µg/mL), as the lentiviral constructs contained the selection gene that conferred resistance to this antibiotic (Figure 4A). Once the over-expression was assessed by qRT-PCR analysis (refer to the specific procedure in next paragraphs), clonal selection was performed by diluiting cells suspensions and picking and isolating single over-expressing clones which were then grown in culture, pooled together and these pools were used for all the further





Figures 4. [A] Work-flow procedure for the generation of the A2780 and A2780cis MCJ-over-expressing cell lines, using cell lines infected with the mock vector as control. **[B]** Schematic experimental procedure of the performed clonal selection in both A2780 and A2780cis, after the infection with MCJ- or mock- expressing vector and subsequently the selection of cell lines in puromycin.

experiments in this work (Figure 4B). We isolated nine clones for each cell line and then three clones (shown in the Results section), with higher MCJ over-expression evaluated by Western blot analysis, where pooled together. Moreover, three clones were also pooled for the mock cell lines. The obtained cell lines ($A2780^{mock}$, $A2780^{MCJ}$, $A2780cis^{mock}$, $A2780cis^{MCJ}$) were then constantly grown in RPMI medium as described above, with the addition of a maintenance concentration of 125ng/mL of puromycin.

Total lysates preparation

Cells were seeded in 25 cm^2 flasks until reaching 80% of confluence. Thus, cells were trypsinized and the pellet was washed once with PBS and centrifugated at 1800 rpm for 10 minutes at 4°C. Then, the pellet was resuspended in RIPA buffer (50mM TrisHCl pH7.4, 150mM NaCl, 1% SDS, 1% Triton, 1mM EDTA pH 7.6) supplemented with protein and phosphatase inhibitors and incubated for 15 minutes in ice. The lysate was frozen and thawed twice and centrifugated at 13000 rpm for 15 minutes at 4°C. The supernatant was recovered, and the total protein content was quantified by Bradford's method (Bradford MM, 1976).

SDS-PAGE and Western Blot

To the total lysates, obtained as described, was added Laemmli sample buffer 5X (300mM Tris-HCl pH 6.8, 25% glycerol, 10% SDS, 12.5% 2-mercaptoethanol, 0.125% bromophenol blue) and samples were boiled for 5 minutes in order to induce a complete denaturation. Samples were loaded in SDS-denaturing gels in order to have 50µg of protein concentration. Denaturing SDS-PAGE Electrophoresis was performed at 100V at room temperature in 1X Tris/Glycine/SDS running buffer (Bio-Rad; 25mM Tris pH 8.3, 192mM glycine, 0,1% SDS). At the end of the run, proteins were transferred onto a nitrocellulose membrane (Bio-Rad) 0.45mm in full-wet system in 1X Tris/Glycine western transfer buffer (25mM Tris pH 8,3, 1.92M glycine, adding 20% methanol) at 100V for 1 hour at room temperature using Bio-Rad Mini Trans-Blot Cell system. After, the membrane was incubated in powdered milk solved in TBS 1X-Tween 0.05% (25mM Tris-HCl pH 7.4, 137mM NaCl, 0,05% Tween) for 1 hour at room temperature and incubated with primary antibodies, diluted in 5% milk-1X TBS-0.05% Tween solution or 3% bovine serum albumin (BSA)-1X TBS-0.05% Tween solution, using the following dilutions/conditions: anti-DDK-TAG (Origene #TA50011-100) 1:1000, o/n at 4°C; anti-Calreticulin (Sigma-Aldrich #C4606-.2ML) 1:5000, 1h at room temperature; anti-GAPDH (Sigma-Aldrich #G8795-100UL) 1:10000, 2h at room temperature; anti-NDUFB6 (Abcam #ab110244) 1:1000, o/n at 4°C; anti-SDHA (Abcam #ab14715) 1:2000, 2h at room temperature; anti-UQCRC2 (Abcam #ab14745) 1:1000, o/n at 4°C; anti-COX5A (Abcam #ab110262) 1:1000, o/n at 4°C; anti-ATP5A (Abcam #ab14748) 1:1000, o/n at 4°C; anti-Catalase (Sigma-Aldrich #C0979-.2ML) 1:2000, 2h at room temperature; anti-PRX3 (Abfrontier #LF-PA0030) 1:1000, o/n at 4°C; anti-HSP60 (Santa Cruz Biotechnology #sc-13966) 1:1000, o/n at 4°C; anti-citrate synthase (Abcam #ab96600) 1:1000, o/n at 4°C; anti-VDAC/porin (Abcam #ab154856) 1:2000, 2h at room temperature; anti-β-catenin (GeneTex #GTX633010) 1:1000, o/n at 4°C; anti-GSK3β (Cell signaling #12456) 1:1500, o/n at 4°C; anti-phospho(S9)-GSK3β (Cell signaling #9323) 1:1000, o/n at 4°C; anti-SNAI1 (GeneTex #GTX125918) 1:1000, o/n at 4°C; anti-N-cadherin (GeneTex #GTX127345) 1:1000, o/n at 4°C. Membranes were washed 3 times with TBS 1X-Tween 0.05% and incubated with the proper secondary antibody from Jackson ImmunoResearch Laboratories anti-Mouse (#115035146) and Anti-Rabbit [#111035144], diluted 1:5000 in 5% milk-1X TBS-0.05%Tween solution and incubated 1 hour at room temperature. Further, 3 wash in TBS 1X-Tween 0.05% were performed and chemiluminescence was obtained by incubating the membrane with Clarity Western ECL (Bio-Rad). Images were acquired by using Gel Logic

1500 Imaging System (Kodak, Ronchester, NY, USA). The densitometric analysis was performed using ImageJ software.

Enriched crude mitochondria fractions preparation

Enriched mitochondrial fractions were obtained from cells seeded into 10 cm dish. Cells were trypsinized and the pellet was washed once with PBS and centrifugated at 1800 rpm for 10 minutes at 4°C. Then, the pellets were resuspended in isotonic Saccarose/Mannitol buffer (70mM Saccarose, 200mM Mannitol, 10 mM HEPES, 1mM EGTA pH 7.5) and cells disruption was performed mechanically by using a glass/Teflon potter. The mechanic procedure was repeated until at least 90% of cells were permeabilized, as detected at the optical microscope using Eritrosine B. After that, cells were centrifuged at 3000 rpm for 10 minutes at 4°C and the obtained supernatants centrifuged at 13000 rpm for 20 minutes at 4°C. Thus, pellets of crude mitochondria obtained from the last centrifugation were stored at -80°C. At the moment of their utilisation, mitochondria were resuspended in Saccarose/Mannitol buffer and mitochondrial protein content was quantified according to Bradford MM, 1976.

Blue Native (BN)-PAGE

The mitochondria in saccarose/mannitol buffer were centrifuged at 13000 rpm for 20 minutes at 4°C. Then, were suspended in Mitochondrial Solubilization Buffer (750mM 6-aminocaproic acid, 50mM Bis-Tris/HCl pH 7.0) in order to have a protein concentration of 5µg/µL. Then was added the right amount of 10% digitonin to have it concentrated to 2%. Samples were kept in ice for 10 minutes and then centrifuged at 14000 rpm for 30 minutes at 4°C. The supernatant was recovered, and mitochondrial proteins quantified using Bradford's method. Supernatants were then resuspended in the appropriate calculated final 1X volume of the 10X BN-Sample Buffer (750mM aminocaproic acid, 50mM BisTris /HCl pH 7, 0.5M EDTA, 5% Serva Blue G-250). Samples were loaded in order to have 70 µg of protein onto a native gel constituted by a gradient of polyacrylamide from 3% to 12%, made with a gradient building machinery and a peristaltic pump (Delta-Pump). The electrophoresis was performed at 80V using Anode Buffer (25mM Imidazole, pH 7) and Blue-Cathode buffer (50mM Tricine, 7.5mM Imidazole, 0.02% Serva Blue G-250 pH 7). At midway of the native electrophoretic run, the blue cathode was changed with the cathode devoid of the serva blue G-250 and electrophoresis then performed at 120V. At the end of the electrophoretic run, the

gel was used either to perform the CI-in-gel activity assay (described subsequently) or the western blot protocol was followed, by transferring native proteins onto methanol-activated polyvinylidene fluoride (PVDF) membrane at constant 300mA for 75 minutes at 4°C.

Complex I-In Gel Activity

After electrophoresis, native gel was incubated in a solution containing 2mM Tris-HCl, 0.5% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 0.02% NADH for 15 minutes at room temperature, in the dark. The detection of dark bands on the gel revealed the enzymatic function of CI in a single complex or in supercomplexes and the colorimetric reaction was due to reduction of MTT to tetrazolium salts by NADH oxidation through CI. Images were acquired by using Gel Logic 1500 Imaging System (Kodak, Ronchester, NY, USA).

Sulforhodamine B (SRB) viability assay

Cells were seeded in 24-wells plates ($15x10^3$ cells/well for all the cell lines). For additional treatments with cisplatin or paclitaxel as shown in the Results, cells were incubated 72 hours after seeding with fresh medium with different increasing concentrations of cisplatin (indicated in legends of figures). Cells seeded without treatments and grown for 72 hours were used as viability controls. At the end of incubations time, cells were fixed to the well with 50% trichloroacetic acid dissolved in the grow medium for 1 hour at 4° C, washed 5 times with water and dried at room temperature. Attached cells were incubated for 30 minutes with SRB 0.4% diluted in 1% acetic acid at room temperature. SRB is an anionic dye, amino-xanthene, which forms electrostatic complexes with basic protein residues, in acid conditions. Subsequently, wells were washed 4 times with 1% acetic acid in order to remove the excess of dye. The dye bound to the cells was finally solubilized with 10mM Tris (pH 10.5) and the relative absorbance of SRB was detected using plate reader VICTOR^3 (1420 Multilabel Counter-PerkinElmer, Turku, Finland) at the wavelength of 560 nm. The IC_{50} of cisplatin and paclitaxel reported in the results were both calculated at 72h of treatments in according to Girolimetti G *et al*., 2017.

Clonogenic assay

The ability of single cancer cell to grow and form colony was evaluated by seeding 300 cells/well for all the cell lines in a 6-well plate in their growth medium. After seeding, cells were incubated at 37° C in a humidified 5% CO_2 atmosphere. To assess the effect of cisplatin

in the ability to form colonies, treatments (2µM for chemosensitive cell lines; 7µM for chemoresistant cell lines) were performed 24 hours after the seeding. Colonies were fixed after 8 days, using 50% trichloroacetic acid and incubating cells at 4°C for 1 hour. Then colonies were washed 5 times with water, dried, stained with SRB 0.4% diluted in 1% acetic acid for 30 minutes at room temperature and washed 4 times with 1% acetic acid. Images were acquired using Gel Logic 1500 Imaging System (Kodak, Ronchester, NY, USA) and the number of colonies quantified using ImageJ software. The dye bound to the cells was solubilized with 10mM Tris (pH 10.5) and the relative absorbance of SRB was detected using plate reader VICTOR^3 (1420 Multilabel Counter-PerkinElmer, Turku, Finland) at the wavelength of 560 nm).

Cells doubling-time calculation

Doubling-time of cell lines was obtained by seeding $15x10^3$ cells in a 24-wells plate, performing replicates in order to have the starting time of the experiment (T0), 24 hours of growth and thus 48 and 72 hours. The count of cells per well was performed for each time point. The data obtained were normalized on T0 and reported in GraphPad. To determine the cells duplication time, the function exponential growth equation was used.

Measurement of mitochondrial complexes activity

Crude mitochondria were used to measure redox activities with a UV-Vis spectrophotometer (V550 Jasco) and were resuspended in a buffer containing 50 mM KPi pH 7,8, 1 mM EDTA, 2.5 mg/mL BSA, for all the respiratory complexes measurement except for complex I that requires a BSA concentration of 3.5 mg/mL. All enzymatic activities were performed under continuous stirring and at 37°C of temperature except were indicated. For CI and CII activity, spectrophotometric assay was performed at 600nm wavelength, by following the 2,6-Dichlorophenolindophenol (DCIP) reduction. Kinetic was calculated using the DCIP molar extinction coefficient (19.1 $mM^{-1}cm^{-1}$). The CIII and CIV activities were measured following cytochrome *c* reduction/oxidation at 550 nm (molar extinction coefficient 19.1 $mM^{-1}cm^{-1}$). The specific activity for each reaction was measured after subtraction of non-specific activity conducted in a separate assay, adding the specific complex inhibitor at the beginning of the reaction (CI: 1 µM Rotenone; CII: 5mM Malonate; CIII: 1µM Antimycin A; CIV: 300µM KCN) (Ghelli A *et al.*, 2013). For details about reaction components see Table 1. The activity of citrate synthase was evaluated in mitochondria suspended in a buffer containing 125mM TrisHCl, 0.1% Triton X-100, 100µM DTNB (ε= 13.6 mM^{-1}), 300µM Acetyl-CoA, pH 8. The

reaction was started by the addition of 500µM Oxaloacetate (Trounce *et al*., 1996) and measured at 412 nm at 30°C. Normalization was performed on both sample protein content (previously quantified in according to Bradford MM, 1976) and citrate synthase activity. Data are thus expressed as normalized redox activity.

Reaction components for mitochondrial complexes activity measurements	
COMPLEX I	- Buffer 50 mM KPi pH 7,8, 1 mM EDTA, 3.5mg/mL BSA - 5µL crude mitochondria - 60µM DCIP - 200µM NADH - 1µM Antimycin A **Starter**: 70µM DUB
COMPLEX II	- Buffer 50 mM KPi pH 7,8, 1 mM EDTA, 2.5mg/mL BSA - 5µL crude mitochondria - 200mM ATP - 50µM DUB - 80µM DCIP - 1µM Antimycin A - 1µM Rotenone - 300µM KCN **Starter**: 10µM Succinate
COMPLEX III	- Buffer 50 mM KPi pH 7,8, 1 mM EDTA, 2.5mg/mL BSA - 5µL crude mitochondria - 20µM oxidized cytocrome c - 1µM Rotenone - 300µM KCN **Starter**: 50µM DBH_2
COMPLEX IV	- Buffer 50 mM KPi pH 7,8, 1 mM EDTA, 2.5mg/mL BSA - 20µM reduced cytocrome c - 1µM Rotenone **Starter**: 5µL crude mitochondria

Table 1. Reaction components for mitochondrial ETC complexes activity measurements.

Oxygen consumption rate measurement

Oxygen Consumption Rate (OCR) was measured using the Seahorse XFe^{24} Extracellular Flux Analyzer (Seahorse Bioscience) as previously described (Iommarini *et al*., 2013). Cells were seeded ($40x10^3$ cells/well) into XFe^{24} cell culture plate and allowed to attach for 24 hours. For the measurement of β-oxidation rate, cells were seeded 48 hours before the assay, and underwent a 24 hours starvation-step in a medium containing 1% FBS, 0.5 mM Glucose, 1 mM Glutamine and 0.5 mM L-Carnitine. The day of the assay, the cell culture media was replaced with XF media (Seahorse Bioscience) with 10 mM Glucose, 1 mM sodium pyruvate

and 2 mM of Glutamine. For the β-oxidation rate, the assay medium requires 2 mM Glutamine, 0.5 mM L-Carnitine and XF-Palmitate (conjugated with BSA) was added directly in the wells. OCR was measured over a 3 minutes period, followed by 3 minutes mixing and re-oxygenation of the media. For Mito Stress Test, complete growth medium was replaced with 670µl of unbuffered XF media supplemented with 10mM glucose pH 7.4 pre-warmed at 37°C. Cells were incubated at 37°C for 30 minutes to allow temperature and pH equilibration. After an OCR baseline measurement, oligomycin, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), and rotenone plus antimycin A were sequentially added to each well to reach final concentrations of 1µM. For the β-oxidation assay, the FCCP was used at a concentration of 2.25 µM, as obtained from a specific titration assay in BSA, as the BSA conjugated to the Palmitate is able to partially complex the uncoupling agent and reduce its functionality. Three measurements of OCR were obtained following injection of each drug and drug concentrations optimized on cell lines prior to experiments. At the end of each experiment, the medium was removed and SRB assay was performed to determine the amount of total cell proteins as described above. OCR was normalized to total protein levels in each well. Each cell line was represented in 8 wells per experiment (n=3 replicate experiments). Data are expressed as pmoles of O_2 per minute (OCR) normalized on protein content.

Migration assay

The scratch assay was used as simple *in vitro* wound healing to test wound closure performed by cell migration. Briefly, cell lines were seeded in a 24-wells plate with 500 µL of media, in the following quantities: $5x10^5$ cells/well for $A2780^{mock}$ and $A2780^{MCJ}$ cell lines, $6x10^5$ cells/well for $A2780cis^{mock}$ cell line and $7x10^5$ cells/well for $A2780cis^{MCJ}$ cell line. Cells were incubated overnight at 37°C and 5% CO_2, reaching an approximate confluency of 95%. The day after, scratch was performed manually with a 200µL pipette tip and wells were washed twice with 1x PBS and medium was replaced. Images were taken with optical microscope (4x) connected with an optical camera system, at the starting time (T0) and after 24, 48 and 72 hours from the scratch. Wound areas were obtained from the analysis of the images with ImageJ software. Data were presented as percentage of wound area, normalized on T0 shown as 100% of wound area. For each well, three or four images were taken. Assay was performed in three independent biological replicates, each in three technical replicates.

Incucyte - ROS production analysis

ROS production measurement (in terms of H_2O_2) was performed with the incubation of cells with Cell-ROX probe (Thermo Scientific) and by using IncuCyte S3 Live-Cell Analysis System. Cells were seeded $40x10^3$ in a 96-wells plate and 24 hours after were incubated with Cell-ROX probe that is a Deep Red reagent for measuring cellular oxidative stress in live cell imaging, with absorption/emission maxima at ~644/665 nm. In the case of treatment with antioxidant N-acetylcysteine (NAC), treatment with 5mM NAC was performed before adding the probe. After the incubations in fresh medium, the plate was placed in the dedicated Incucyte incubator. The set acquisition protocol provides for the acquisition of 3 images/well every 10 minutes for a duration of 10 or 24 hours (as reported in the results). At the end of the experiment, a specific mask was applied to all the wells of the 96-wells plate, capable of recognizing the deep red signals and therefore an oxidative stress signal. The raw data were exported from the instrument and represented in GraphPad, by expressing them in Red integrated mean intensity as RCU x $\mu m^2/mm^2$.

DNAJC15 gene sequencing

Whole genomic DNA was extracted from A2780 and A2780cis cells with the Mammalian Genomic DNA Miniprep Kit (Sigma) according to the manufacturer's protocols. Sanger sequencing was performed using the following primers:

Fw1_ ATTCAAGAACGTCCCAGTGC
Rv1_ CTACAAGGGAGCCAGAGAGG
Fw2_ CTGATACAGTGAGCAGCAGA
Rv2_ AGGGTCTACTCATGAATCACTGT
Fw3_ AACATGAAATTTTGCCCTCCAA
Rv3_ TGAGTCTTCTAACTAAAGCCACG
Fw4_ GTCATGCCACCTCATACGTG
Rv4_ GGTACTTCACAACTTTGCCTGT
Fw5_ AGTGGAATTAAGTGGAAAGCTGT
Rv5_ CGCACAAACTTCCATCTCAACT
Fw6_ TGGGTAGTTGTAGGACTTCACT
Rv6_ TGGTGGAAGATAAGACTGTGGT

Genomic DNA (20ng) was used for the amplification with a set of 6 primer pairs. PCR amplification was performed using KAPA2G Fast PCR Kits (Sigma) in a 9700 thermal cycler. The purified PCR product was used for direct sequencing with BigDye kit version 1.1 (Applied Biosystem). Sequences were run in an ABI 3730 Genetic Analyzer automated sequencing machine. Electropherograms were analyzed with Sequencer4.9 software (Applied Biosystems).

RNA extraction

The pellets obtained by trypsinizing about $7x10^5$ cells in biological triplicate, were resuspended in 1mL of TRI-reagent (Sigma). 200µL of Chloroform were then added, vortexing for 15 seconds and leaving at room temperature for 15 minutes. The samples were then centrifuged at 12000g for 15 minutes at 4°C (in swing/out rotor). Thus, the supernatant transparent phase containing the RNA was transferred to a new eppendorf and 500µL of 2-propanol was added, mixing the whole. Then left at room temperature for 10 minutes and centrifuged at 12000g for 10 minutes at 4°C (Fix rotor). The supernatant was then removed and the RNA pellet was washed twice with 75% EtOH (adding ethanol, vortexing and centrifuging at 7500g for 5 minutes at 4°C). The pellets were dried under a chemical hood at room temperature and then resuspended in 50µL of RNAse free water and the samples heated under stirring at 55°C for 15 minutes. Subsequently, the integrity of the RNA was assessed by electrophoretic run on a 1% agarose gel, and the concentration and purity of the samples verified using Nanodrop2000 (Invitrogen) systems for nucleic acids quantification.

RNA reverse-transcription

The extracted RNA was converted into cDNA by using the High-Capacity cDNA Reverse Transcription kit (Applied Byosistems) following the manufacturer's protocols, which is based on the use of the reverse transcriptase enzyme. For each sample 300ng of RNA was reverse-transcribed in a final volume of 20µl.

Quantitative Real-Time PCR

The obtained cDNA samples were analyzed by quantitative PCR in real-time (qRT-PCR) by using SYBR Green (Promega) probe as intercalating fluorophore in the DNA double strands. This method allows the simultaneous amplification and quantification of the cDNA. In fact, since it is intercalated in the double-stranded cDNA, the fluorescence increases as the amplification product increases. At the end of each amplification cycle, the fluorescence value

is measured, which represents the quantity of double-stranded product synthesized-up to that point. Therefore, the emitted fluorescence is directly proportional to the amount of cDNA. The gene expression analysis was conducted with specific primers designed on the following target genes:

TARGET GENE	PRIMERS
DNAJC15	Fw 5'-TTTCGGATCTGGAAACCTCTAG-3'
	Rv 5'-TCTCGCCTACTCATTTTCTGTT-3'
ABCG2	Fw 5'-TAGCAGCAGGTCAGAGTGTG-3'
	Rv 5'-TCATTATGCTGCAAAGCCGT-3'
ABCC1	Fw 5'-GGACTCAGGAGCACACGAAA-3'
	Rv 5'-ACGGCGATCCCTTGTGAAAT-3'
ABCC2	Fw 5'-TACCAATCCAAGCCTCTACC-3'
	Rv 5'-AGAATAGGGACAGGAACCAG-3'
ABCC4	Fw 5'-CCGTGTACCAGGAGGTGAAG-3'
	Rv 5'-ACAAGGGATTGAGCCACCAG-3'
ABCC6	Fw 5'-GATGAGCTTCGCAGTGTTCC-3'
	Rv 5'-CACCACCAGAGACAGGCATA-3'
ABCA1	Fw 5'-GTGTTGTCAAGGAGGGGAGA-3'
	Rv 5'-GGCACCTGAACCTTCCATTG-3'

TARGET GENE	PRIMERS
ABCA3	Fw 5'-GCAGGGCACTTGTGATCAAC-3'
	Rv 5'-CTGTGGTTGAAGGGGTGCT-3'
PGC-1A	Fw 5'-GCCAAACCAACAACTTTATCTCTTC-3'
	Rv 5'-CACACTTAAGGTGCGTTCAATAGTC-3'
PGC-1B	Fw 5'-GAGTCAAAGTCGCTGGCATC-3'
	Rv 5'-AACTATCTCGCTGACACGCA-3'
PPRC1	Fw 5'-GACCTGAAACTTCAAGAGTACCAAA-3'
	Rv 5'-TGAGGCTTATTGTAGAGCTGAGTC-3'
TFAM	Fw 5'-AATCTGTCTGACTCTGAAAAGGA-3'
	Rv 5'-TGTGCGACGTAGAAGATCCT-3'
N-CADHERIN	Fw 5'-GGTGGAGGAGAAGAAGACCAG-3'
	Rv 5'-GGCATCCTCCACAGT-3'

The *U2AF2* gene was used as control. The Applied Biosystems 7900HT Fast Real-Time PCR System associated with the Sequence Detection Systems (SDS) Software was used for the amplification and quantification reaction of the transcripts. At the end of the quantification, the CT values for each sample were obtained and used in data processing. The values obtained were normalized on CT values of *U2AF2*, and used in the calculation of the ΔΔCt following the subsequent formula: ΔΔCt = ΔCt (control) - ΔCt (experiment). The control relates to the cDNA extracted from the mock cell lines and the experiment instead is represented by the cDNA extracted from MCJ-over-expressing clones. This value was raised at the power of 2 ($2^{-\Delta\Delta Ct}$) to define the fold change. Data reported in the results were represented as $2^{-\Delta Ct}$ when performed a general comparison of mRNA levels. Instead, were

presented as Fold Change when perfomed the comparison between mock- and MCJ-over-expressing cell lines.

In silico bioinformatic analysis

All analyses were performed into R programming environment. The Cancer Genome Atlas (TCGA) ovarian carcinoma dataset was used to perform gene-centered virtual knockdown (https://doi.org/10.1038/nature10166). TCGA cohort was divided into high-expressing and low-expressing *DNAJC15* patients by dividing TCGA into quantiles of expression (all patients below 25% were assigned to low-expression, while patients above 75% were assigned to the high-expression cohort). DESeq2 (v 1.30.0) R package was used to perform differential expression analysis, while enrichR package was used to perform pathway enrichment analysis. Survival analysis was performed using the survival R package (v 3.2-7). Master Regulator Analyses was performed by comparing high-expressing and low-expressing *DNAJC15* patient samples in TCGA datasets with the viper algorithm [PMID: 27322546] using default parameters and the co-expression network derived from TCGA OV samples. In brief, a gene-by-gene signature of differential expression is generated, and a combined value for each co-expression network is generated by weighting every gene's likelihood in the network, providing a final Normalized Enrichment Score for each TF member, which is positive if the network is upregulated in high-expression patients and negative if it is downregulated, as in Mercatelli D *et al.*, 2020.

Statistical analysis

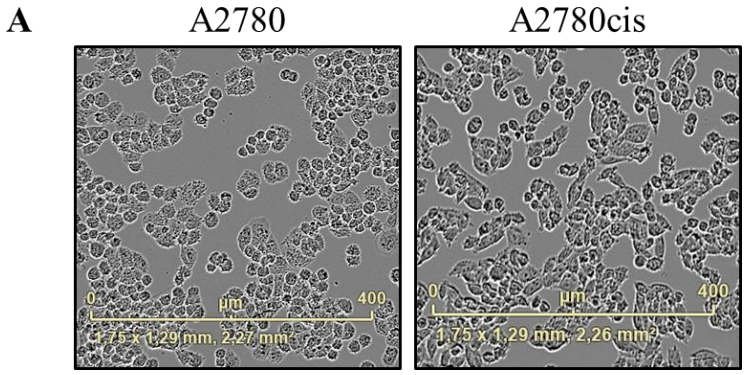
GraphPad Prism 8 was used to calculate the significance of data. All analyzes were performed a minimum of three times. All data are presented as mean ± standard deviation and Student's t-test was used to determine the significance of results. A p-value ≤ 0.05 was considered as significant (*), p-value ≤ 0.01 was considered as very significant (**), p-value ≤ 0.001 and ≤ 0.0001 were considered as extremely significant (***/****).

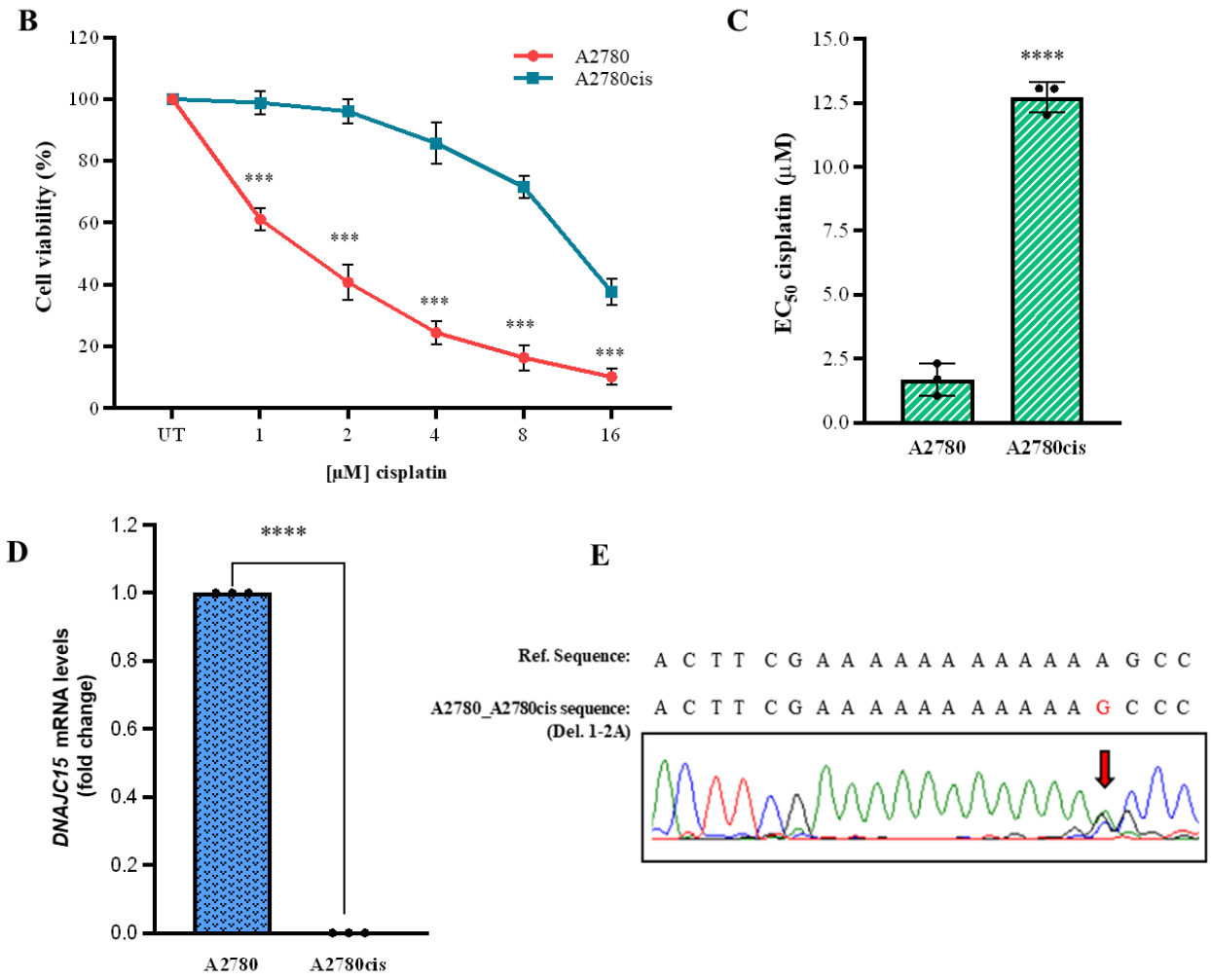
RESULTS

1. Understanding the impact of MCJ expression on the OC *in vitro* chemoresistance occurrence

1.1 The over-expression of mitochondrial chaperone MCJ increases the sensitivity to cisplatin of OC chemoresistant cell line.

As already mentioned in the introduction, the lack of MCJ expression is a common feature of OC when becomes aggressive and resistant to pharmacological treatments. To investigate whether MCJ has a role in the molecular mechanisms underlying chemoresistance in OC, we used chemosensitive and its cisplatin-chemoresistant counterpart OC cell lines, namely A2780 and A2780cis. It is interesting to note that A2780 shows a population of round epithelial-like cells, instead A2780cis is composed by a mix of round and elongated mesenchymal-like cells (Figure 5A). The evalution of cell viability after treatment with different concentrations of cisplatin revealed a significantly higher sensitivity of the A2780 (EC_{50}=1.8 µM) compared to A2780cis (EC_{50}=12.8 µM) cell line (Figure 5B-C). Further, according with the data reported in literature, cisplatin chemoresistance was associated with the lack of *DNAJC15* expression gene (Figure 5D). To exclude that the non-expression was due to the loss or mutations of the gene, we sequenced *DNAJC15* in both cell lines reporting that the gene was not lost, conserved its integrity and harboured only a small 1-2 bp deletion out of the coding sequence (Figure 5E). Hence, we decided to generate MCJ-over-expressing and control counterpart cell lines by transduction with the *DNAJC15*-lentiviral-DDK-tagged or empty-lentiviral-DDK-tagged vectors of both chemosensitive and chemoresistant cells Transduced A2780 and A2780cis cells were treated with puromycin and clonal selection was performed in order to avoid the contamination from non-transduced cells.

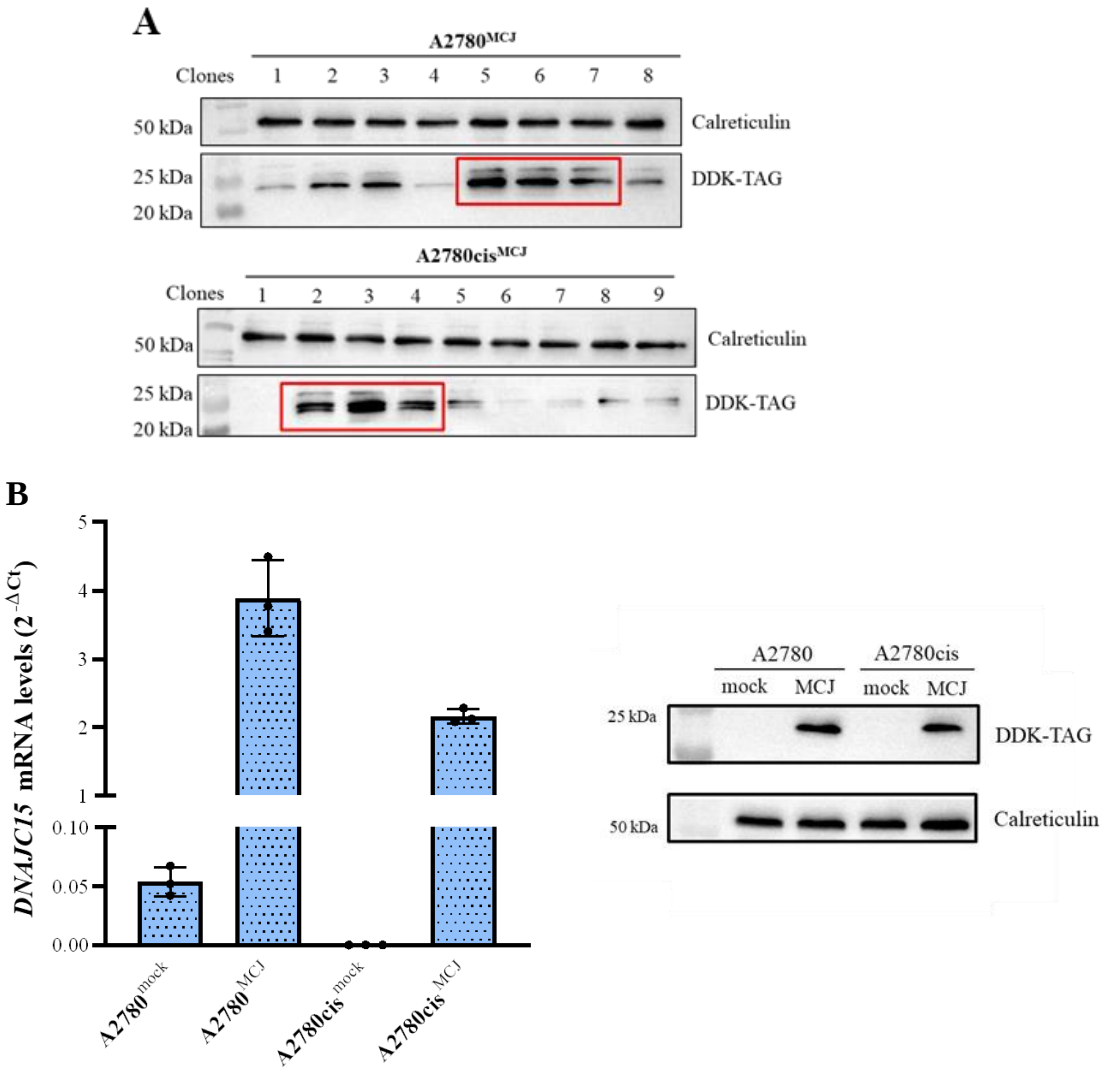


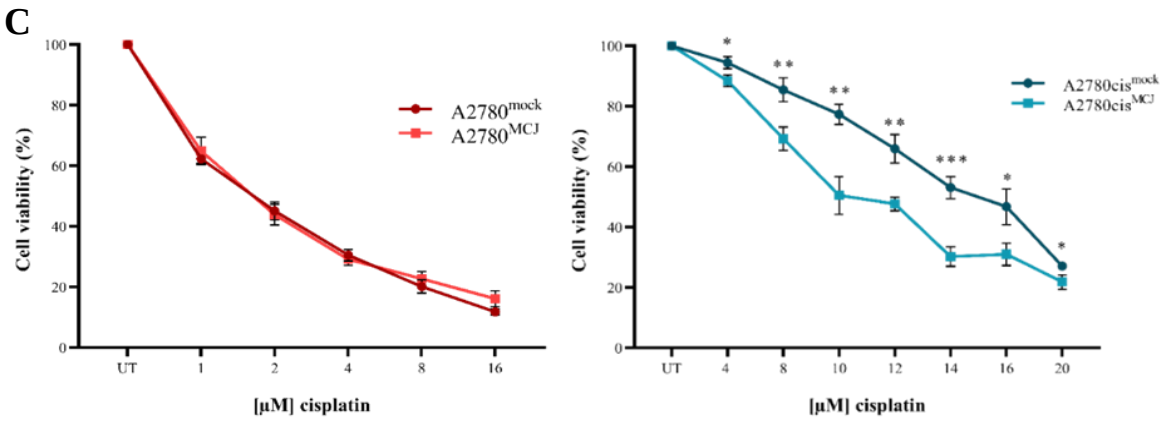


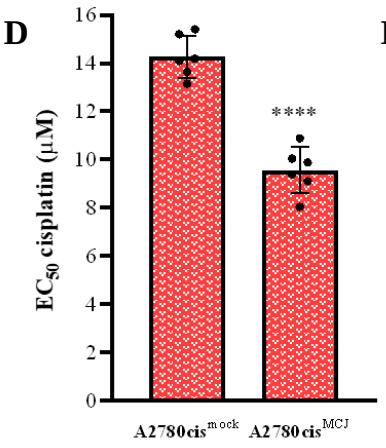
Figures 5. [**A**] Morphology images of A2780 and A2780cis cell lines visualized with optical microscopy (20X). [**B**] Cell viability of OC chemoresistant and chemosensitive cell lines treated with different cisplatin concentrations for 72h. Data (mean ± SD; n=3) are expressed as % of viable cells considering the untreated (UT) as 100%. [**C**] Cisplatin EC_{50} in A2780 and A2780cis cell lines was calculated after 72h of treatment, as the concentration that results in a 50% decrease in the number of cells compared to that of the untreated control. Data are mean ± SD (n=3). [**D**] Analysis of *DNAJC15* expression in A2780 and A2780cis cell lines measured by qRT-PCR. Data are mean ± SD (n=3). [**E**] Electropherograms of *DNAJC15* gene sequence. The red arrow indicates the position of the 1-2 A deletion in both cell lines taking as reference the wildtype sequence. All p-values were obtained with Student's t-test (***$p \leq 0.001$; ****$p \leq 0.0001$).

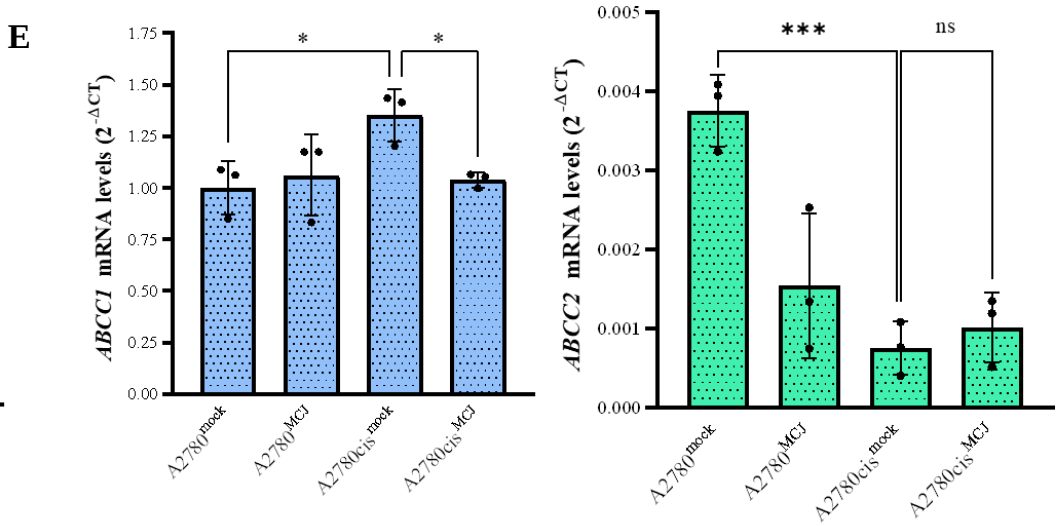
The evaluation of MCJ expression allowed us to identify three positive clones for each cell line (Figure 6A). These clones were then pooled together and the MCJ expression was determined as mRNA and protein levels (Figure 6B). The obtained pools hereinafter referred to as $A2780^{MCJ}$ and $A2780cis^{MCJ}$ were used for the further experiments. Interestingly, the MCJ over-expression was able to restores the chemosensitivity of the A2780cis cell line (Figure 6C) and, in turn, markedly decreases its cisplatin EC_{50} (Figures 6D). It is well known that the expression of the multi-drug resistance transporters proteins namely MDRs or ATP-

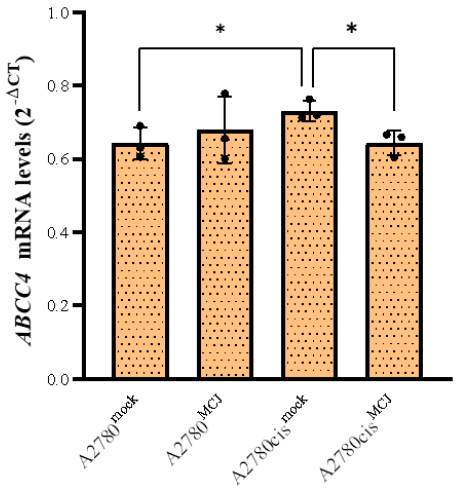
binding cassette (*ABC*) transporters contribute to the chemoresistance onset regulating drugs efflux and the activation of signaling cascades involved in cell proliferation, migration and tumorigenesis (Ween MP *et al.*, 2015). To assess whether the rescue of the chemosensitivity in the MCJ-over-expressing chemoresistant cell line was associated with the multi-drug resistance occurrence, the expression levels of common cisplatin-related and unrelated ABC transporters (*ABCC1*, *ABCC2*, *ABCC4*, *ABCA1*, *ABCA3*, *ABCC6* and *ABCG2*) were evaluated by using qRT-PCR. We found that the mRNA expression levels of *ABCC1*, *ABCC4*, *ABCA3* and *ABCG2* genes were significantly higher in the chemoresistant cell line comparing with the chemosensitive one (Figure 6E). Moreover, the expression of such genes became lower in the MCJ-over-expressing chemoresistant cell line, suggesting a putative involvement of MCJ in the molecular mechanisms underlying chemoresistance. Nonetheless, the expression of *ABCC6* transporter showed no significant difference between cell lines and the expression of cisplatin-specific *ABCC2* and *ABCA1* was higher in the chemosensitive cell line than chemoresistant cells.

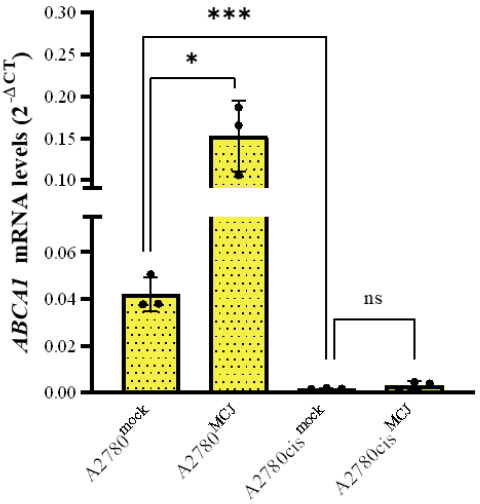


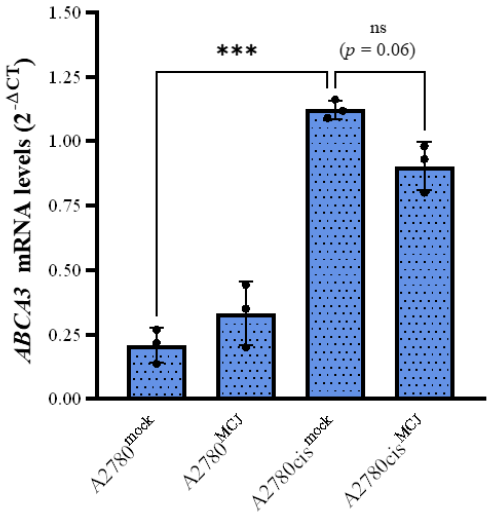

C
Cell viability (%)
A2780mock
A2780MCJ
[µM] cisplatin
A2780cismock
A2780cisMCJ
[µM] cisplatin

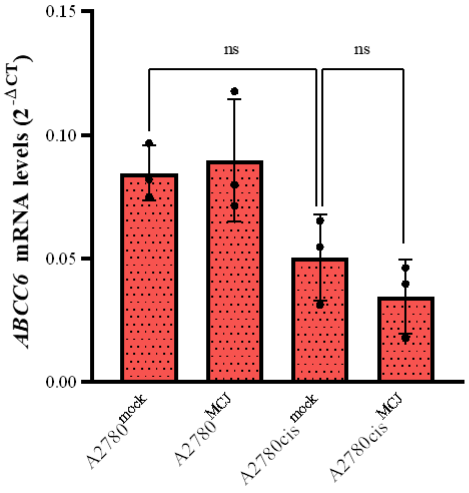

D
EC50 cisplatin (µM)
****
A2780cismock
A2780cisMCJ

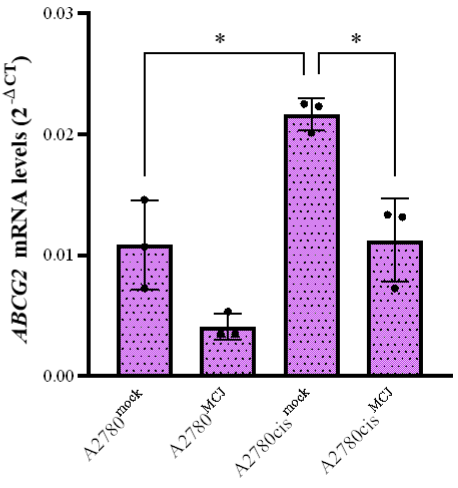

E
ABCC1 mRNA levels (2-ΔCT)
ABCC2 mRNA levels (2-ΔCT)
***
ns
A2780mock
A2780MCJ
A2780cismock
A2780cisMCJ


ABCC4 mRNA levels (2-ΔCT)


ABCA1 mRNA levels (2-ΔCT)
***
ns


ABCA3 mRNA levels (2-ΔCT)
***
ns
(p = 0.06)

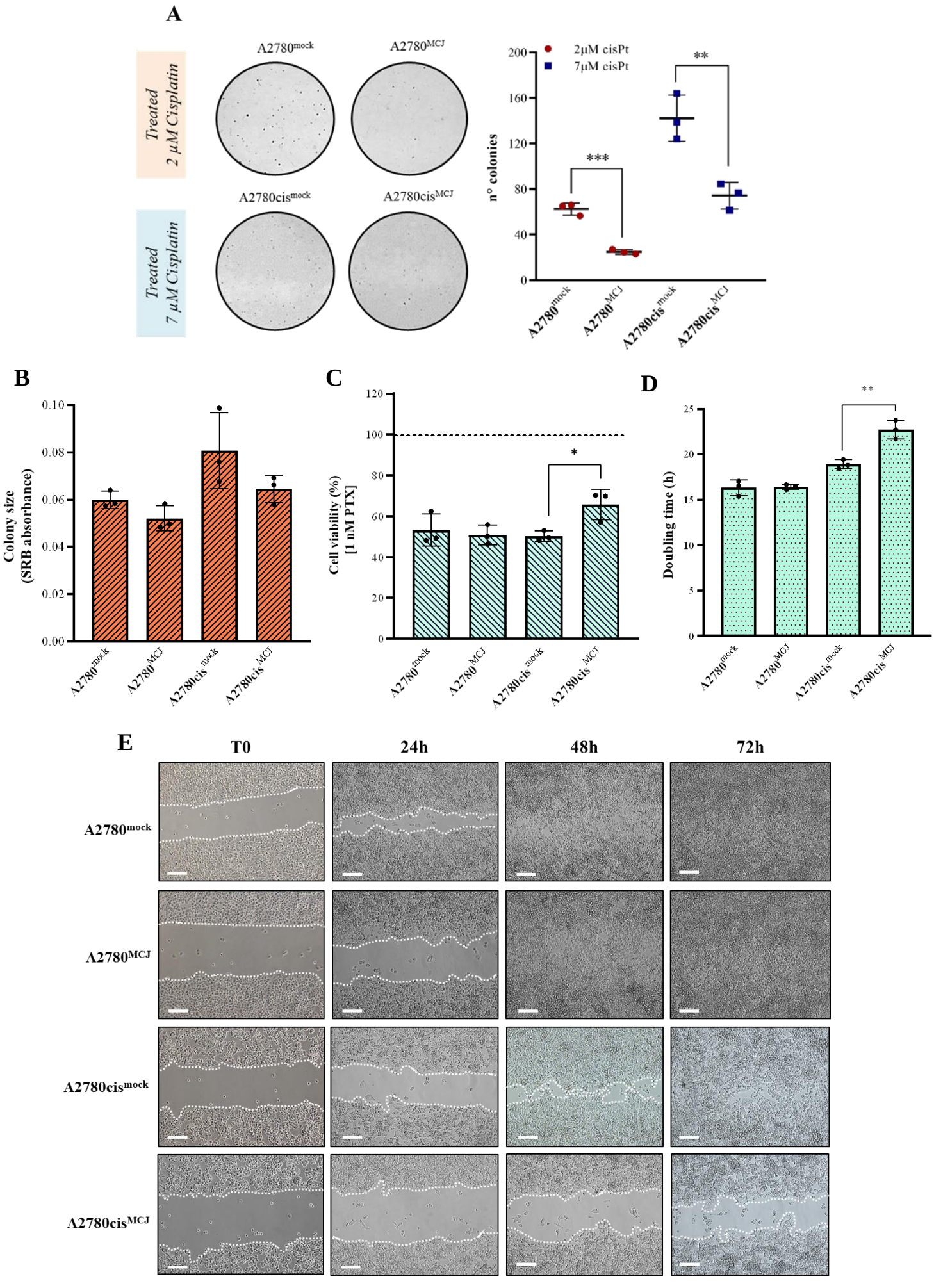

ABCC6 mRNA levels (2-ΔCT)
ns
ns


ABCG2 mRNA levels (2-ΔCT)

Figures 6. [**A**] Western blotting analysis of MCJ protein levels in puromycin selected clones after the transduction with *DNAJC15*-lentiviral-mediated DDK-tagged vector in 8 selected clones of the chemosensitive cell line and 9 selected clones of the chemoresistant one. The MCJ expression was determined by using a specific antibody against the DDK tag of over-expression. Red squares indicate the clones that were pooled together for each cell line and used for further experiments. [**B**] MCJ protein and *DNAJC15* mRNA expression levels in the pool of clones $A2780^{mock}$/$A2780^{MCJ}$ and $A2780cis^{mock}$/$A2780cis^{MCJ}$, determined by Western blot and qRT-PCR analysis respectively. Data are mean ± SD; n=3) [**C**] Cell viability of OC chemoresistant and chemosensitive cell lines treated with different cisplatin concentrations for 72h. Data (mean ± SD; n=6) are expressed as % of viable cells considering the untreated (UT) as 100%. [**D**] Cisplatin EC_{50} in A2780 and A2780cis cell lines was calculated after 72h of treatment, as the concentration that results in a 50% decrease in the number of cells compared to that of the untreated control. Data are mean ± SD (n=6). [**E**] *ABCC1*, *ABCC2*, *ABCC4, ABCA1*, *ABCA3, ABCC6* and *ABCG2* mRNA levels determined by qRT-PCR. Data (mean ± SD; n=3) are expressed as relative gene expression ($2^{-\Delta Ct}$). All p-values were obtained with Student's t-test (*p ≤0.05; **p ≤ 0.01; ***p ≤ 0.001; ****p ≤ 0.0001).

1.2 MCJ over-expression impairs clonogenic and migration capability of chemoresistant OC cell line.

To assess whether the MCJ expression could affect the *in vitro* tumorigenic potential of OC cell lines in terms of ability to form colonies and migration, clonogenic and scratch wound healing assays were performed. The MCJ over-expression induced a significant reduction of number colonies but not their size after 8 days of cisplatin treatment of both OC chemosensitive and chemoresistant cell lines (Figure 7 A-B). In the clinical scenario, the standard chemotherapy approach for OC care provides the combined treatment with cisplatin and paclitaxel (PTX), a drug that disrupts microtubule dynamics of cells with high proliferation rate (Zheng HC *et al*., 2017). Hence, we evaluated the effect of PTX treatment on the proliferative ability of MCJ-over-expressing resistant cell line uncovering its lower sensitivity to this drug compared with mock resistant counterpart (Figure 7C) and a higher time for cells duplication and division (Figure 7D). This higher doubling time may be responsible of the increased resistance to PTX compound that usually acts on highly proliferating cells. Furthermore, MCJ over-expression was able to significantly reduce the *in vitro* migration ability of the chemoresistant OC cell line only (Figure 7E).


A
Treated 2 μM Cisplatin
Treated 7 μM Cisplatin
A2780mock
A2780MCJ
A2780cismock
A2780cisMCJ
2μM cisPt
7μM cisPt
n° colonies
B
Colony size (SRB absorbance)
C
Cell viability (%) [1 nM PTX]
D
Doubling time (h)
E
T0
24h
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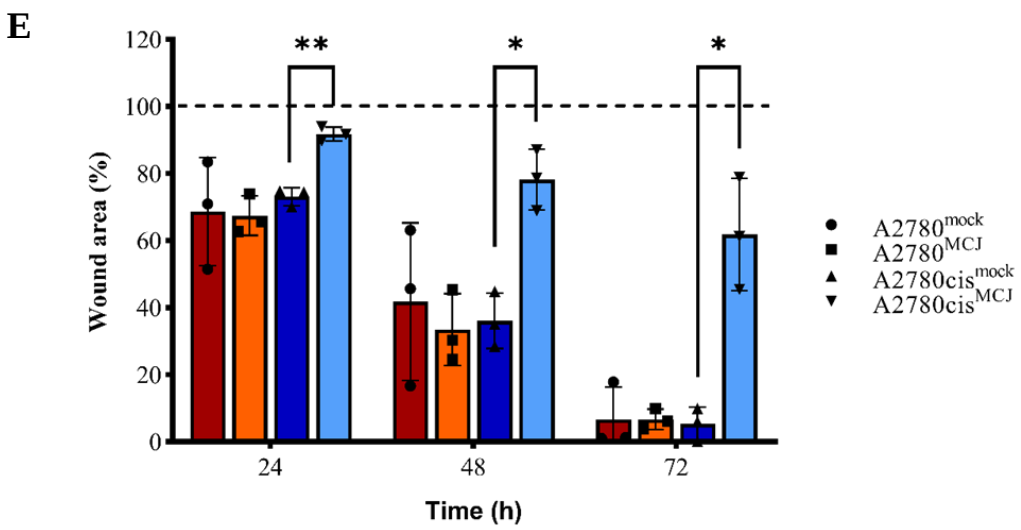
72h
A2780mock
A2780MCJ
A2780cismock
A2780cisMCJ



Figures 7. [**A**] Clonogenic assay after 8 days of treatment with cisplatin (2 µM for chemosensitive cells and 7 µM for chemoresistant cells). Data are mean ± SD (n=3). One representative experiment of three different is shown. [**B**] Colony size obtained with the measurement of the SRB absorbance (565 nm) from the previous clonogenic experiment. Data are mean ± SD (n=3). [**C**] Cell viability of OC chemoresistant and chemosensitive cell lines treated with 1nM of PTX for 72h. Data (mean ± SD; n=3) are expressed as % of viable cells considering the untreated (UT) as 100%. [**D**] Doubling-time of both chemosensitive and chemoresistant cell lines determined by counting cells after 72h from the seeding. The count at 72h was normalized on the number of cells at time zero. Data are mean ± SD (n=3). [**E**] Scratch wound healing assay of both chemosensitive and chemoresistant cell lines was performed and images were taken after 24h, 48h and 72h from the scratch (T0) using an optical microscope (10X). One representative experiment of three different is shown. White bars indicate the length of 100 µm. The wound areas data were obtained with ImageJ analysis and were normalized to the time zero of the experiment (reported as 100%). Data are mean ± SD (n=3). All p-values were obtained with Student's t-test (*p ≤0.05; **p ≤ 0.01; ***p ≤ 0.001).

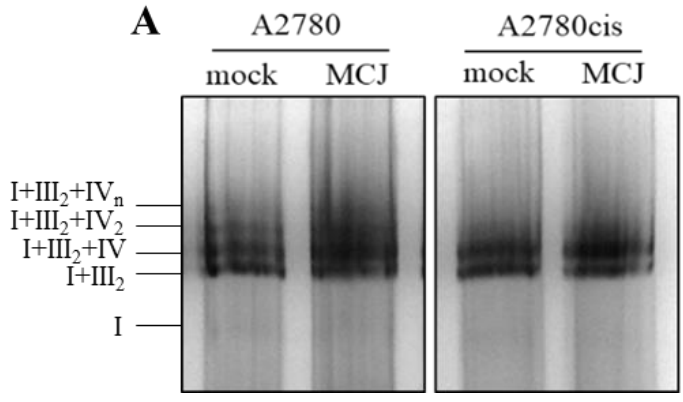
Overall, data reported above suggest that the MCJ expression is able to make the OC chemoresistant cell line more sensitive to cisplatin treatment and less able to migrate and proliferate *in vitro*.

2. Unravelling the role of MCJ as molecular player in mitochondrial biogenesis, energetics and metabolism in OC

2.1 MCJ expression influences the mitochondrial bioenergetics

Data in literature report MCJ as negative endogenous interactor of ETC complexes and regulator of their organization in supercomplexes in non-cancer background (Hatle KM *et al.*, 2013). Taking advantage by our cisplatin-chemosensitive and chemoresistant OC models, we aimed to investigate the mitochondrial respiratory chain activity and efficiency and whether

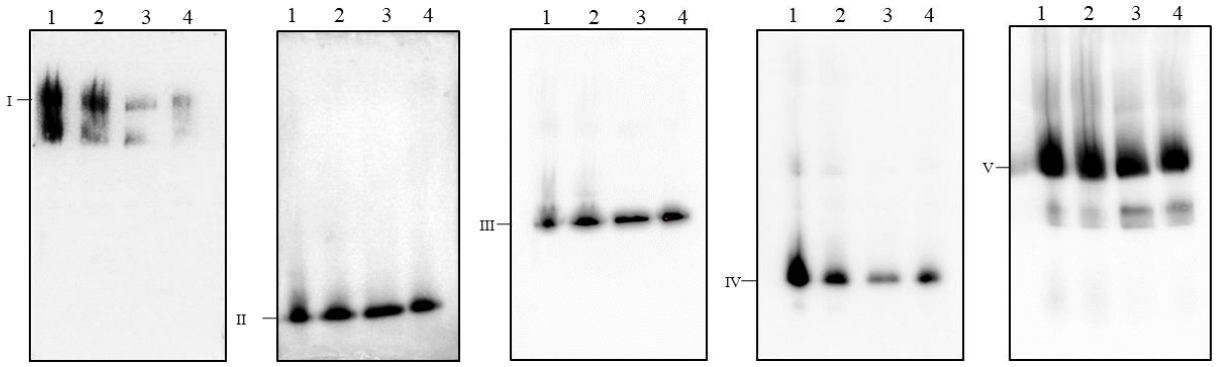
the MCJ over-expression may alters the energetic profile in a cancer background. We first analyzed the supramolecular organization of mitochondrial complexes in native supercomplexes. Chemosensitive cells showed more supercomplexes species with different compositions and stoichiometries of respiratory complexes than chemoresistant cells (Figure 8A). In addition, the MCJ over-expression seems to slightly increased supercomplexes levels without modifying the number and type of species in both chemoresistant and chemosensitive cell lines. However, the preliminary analysis of expression levels of isolated respiratory chain complexes under native conditions, displayed a decrease of complex I (CI) and complex IV (CIV) in chemoresistant cells compared to chemosensitives (Figure 8B). Furthermore, MCJ over-expression seems to trigger an enrichment of isolated complex V (CV) in the chemoresistant cells. Furthermore, the spectrophotometric analysis of respiratory complexes activities, showed a tendency to decrease of complex III and IV in the chemoresistant cell line compared to chemosensitive one (Figure 8C). Noteworthy, when MCJ was over-expressed, chemoresistant cells showed a significant increase of CI, CII and CIII activities associated with a slight decrease of CIV function, mirroring the amount of this enzyme (Figures 8C and 8B). The analysis of the oxygen consumption rate profile (OCR) of chemosensitive and chemoresistant cell lines showed a higher basal and ATP-linked OCR in sensitive rather than in chemoresistant cells, allowing us to define them high-OXPHOS and low-OXPHOS, respectively (Figure 8D). Interestingly, both chemoresistant and chemosensitive MCJ-over-expressing cell models showed a significant increased of basal and ATP-linked OCR compared to the mock counterparts. However, no significance in both maximal and spare respiratory capacity was observed by comparing all cell lines. These data suggest that MCJ, triggering the ETC activity, may regulate and enhance the basal OXPHOS ability of chemoresistant cell lines, whithout increasing the maximal energetic efficiency of mitochondria probably due to an increased ATP demand of cells under basal conditions.



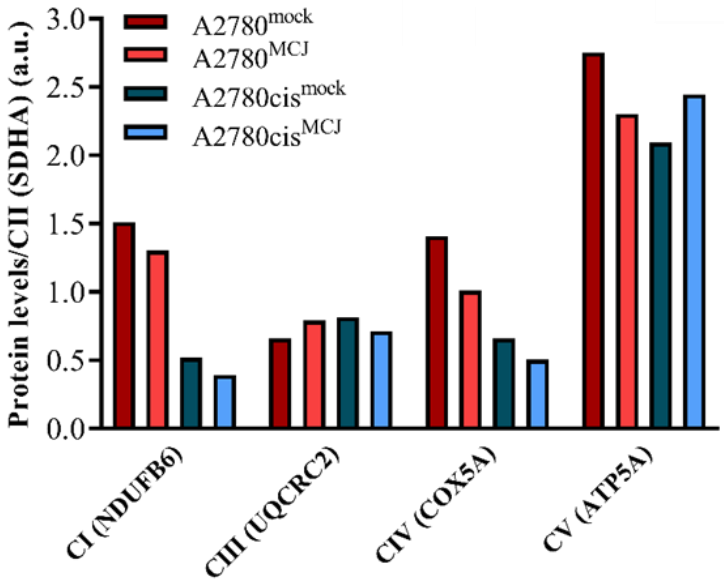
B

Samples:

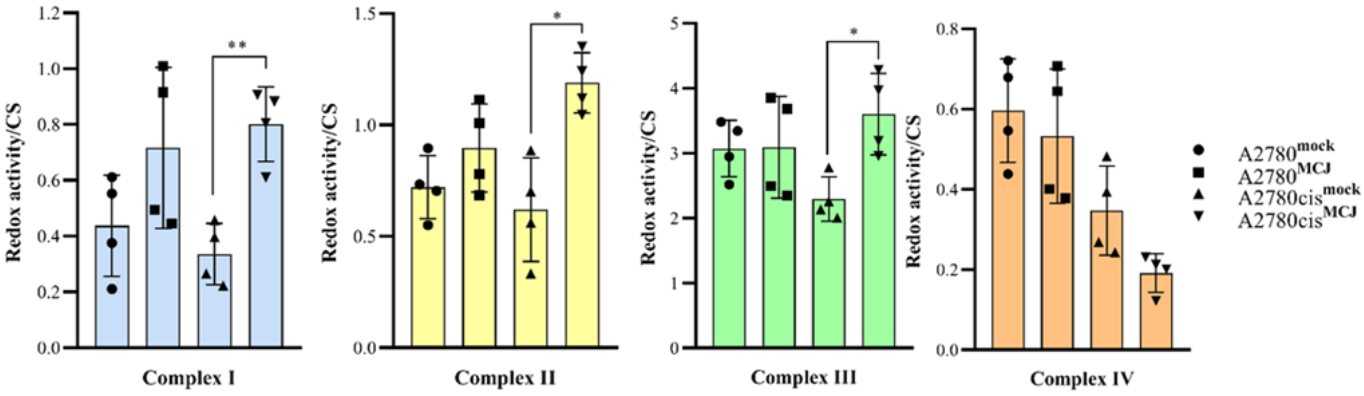
1. A2780^{mock}
2. A2780^{MCJ}
3. A2780cis^{mock}
4. A2780cis^{MCJ}

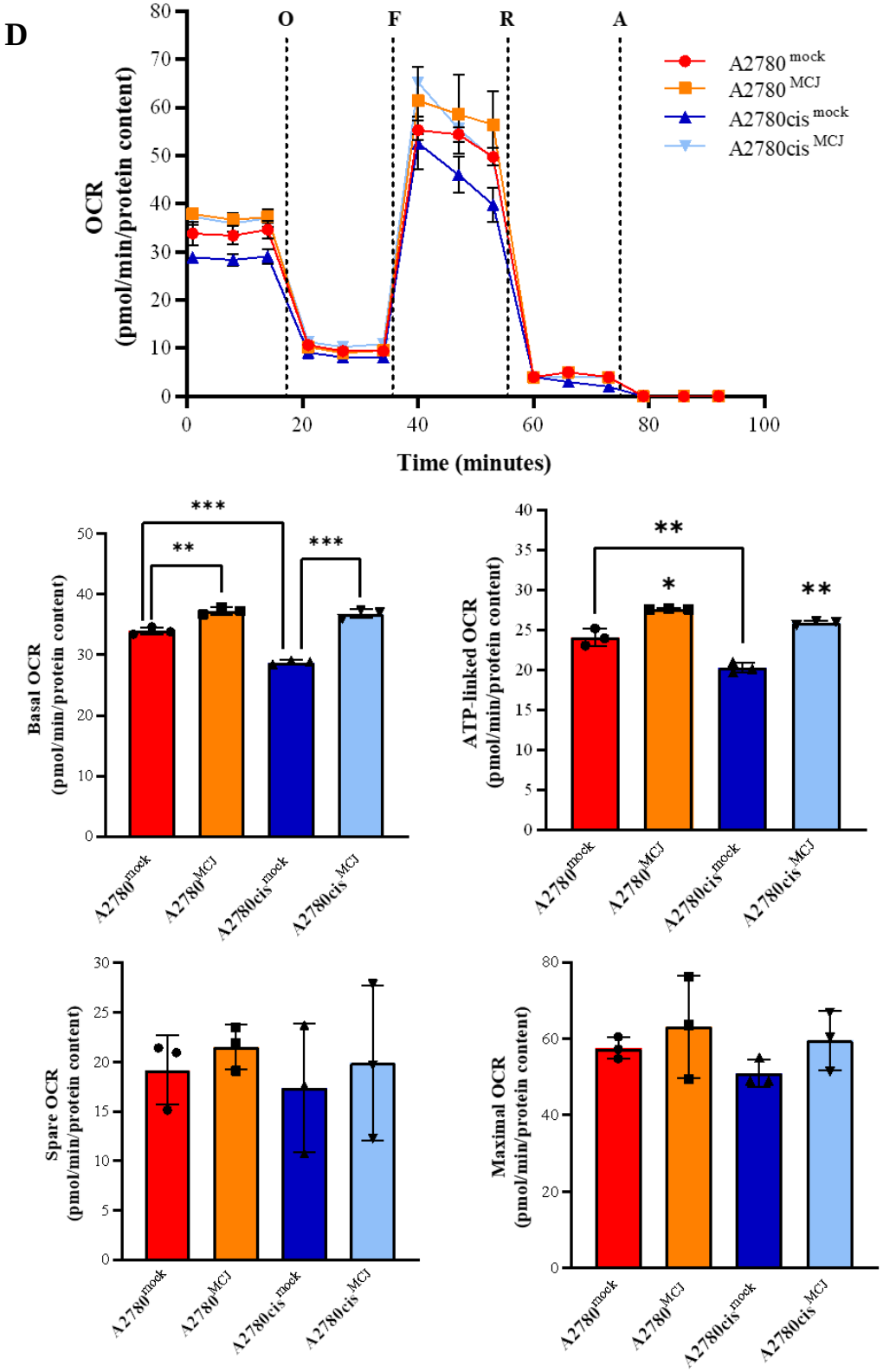


CI – NDUFB6 | CII – SDHA | CIII – UQCRC2 | CIV – COX5A | CV – ATP5α



C

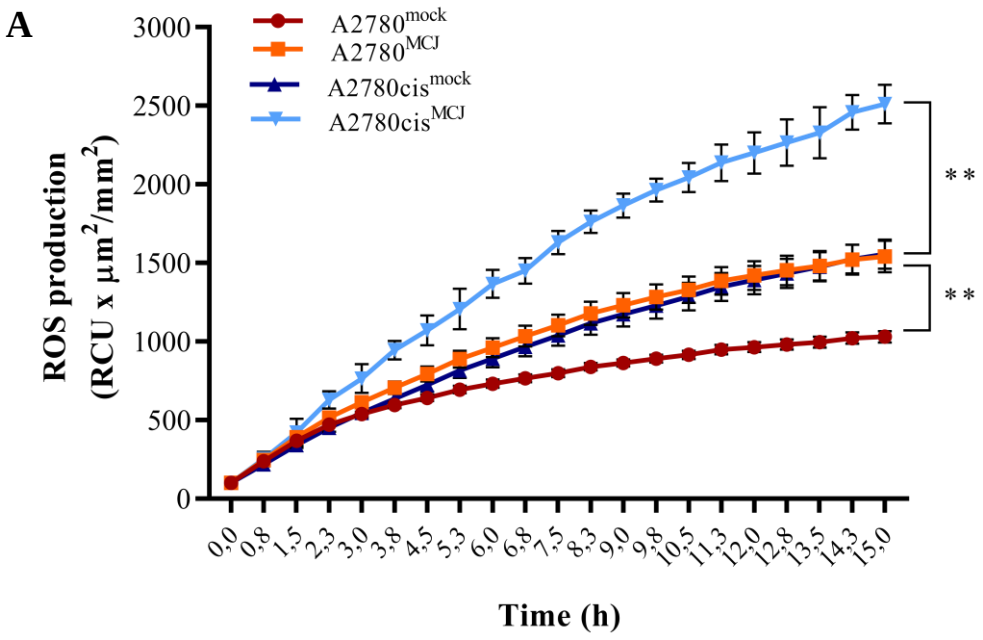


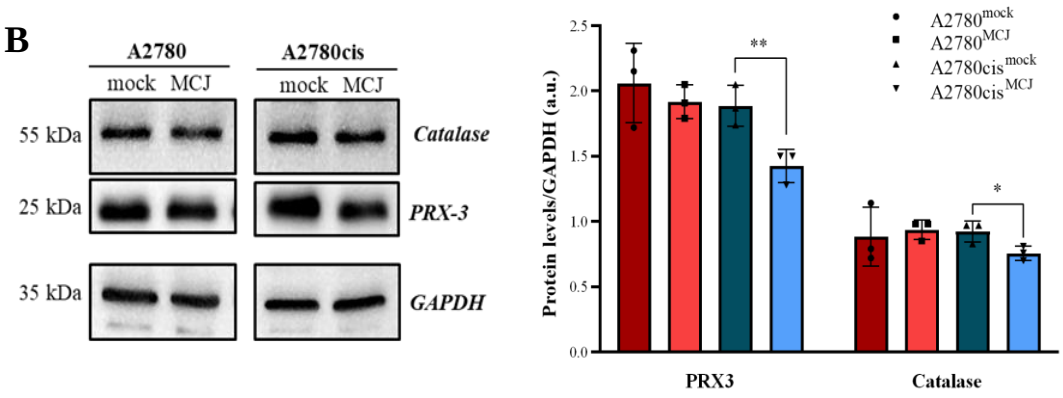


Figures 8. [**A**] Complex I in-gel activity of mitochondrial supercomplexes in Blue Native Page performed on crude mitochondria extracted from OC chemoresistant and chemosensitive cell lines. Representative experiment of two different is reported. [**B**] Western blot and densitometric analysis of isolated mitochondrial complexes on crude mitochondria extracted from OC cell lines, by using antibodies against NDUFB6 (CI), SDHA (CII), UQCRC2 (CIII), COX5A (CIV) and ATP5A (CV). One preliminary experiment is shown (n=1). Samples 1, 2, 3

and 4 are respectively: A2780^{mock}, A2780^{MCJ}, A2780cis^{mock} and A2780cisMCJ. In the densitometric analysis, CII (SDHA) was used as loading control. [**C**] Spectrophotometric redox activity of ETC complexes performed by using crude mitochondria from OC cells. Data are mean ± SD (n=3). Data were normalized on total protein content and citrate synthase activity (as control of total mitochondrial activity). [**D**] Oxygen consumption rate (OCR) representative traces and Basal, ATP-linked, Maximal and Spare OCR bar graphs of OC cell lines are shown. Data were obtained by using XF-96 Seahorse Analyzer. Inhibitors Oligomycin (O), FCCP (F), Rotenone (R) and Antimycin A (A) were all used at the concentration of 1µM. Data are mean ± SD (n=3). All p-values were obtained with Student's t-test (*p ≤0.05; **p ≤ 0.01; ***p ≤ 0.001).

It is well known that mitochondria are an important source of ROS (Murphy MB 2009). As a double-edged sword, ROS can play a key role in many signalling pathways, but their over-production can lead to cellular damage (Zhao RZ *et al.*, 2019). Therefore, we also analyzed the oxidative stress profile in OC cell models, comparing the chemosensitive and chemoresistant cell lines. Measuring ROS production as H_2O_2 levels, we noticed that the chemoresistant cell line showed a ROS production significantly higher than the chemosensitive one (Figure 9A). In addition, MCJ over-expression leads to a significant increase of ROS production in both cellular contexts (Figure 9A). Moreover, we evaluated the expression levels of two common antioxidant enzymes, catalase and peroxiredoxin-3, and we found their expression significantly lower in the MCJ-overespressing chemoresistant cell line (Figure 9B). All toghether, these results suggest that MCJ over-expression is able to induce an increase in oxygen consumption in the OC cell lines and a redox imbalance probably due to an increase of respiratory chain activity.



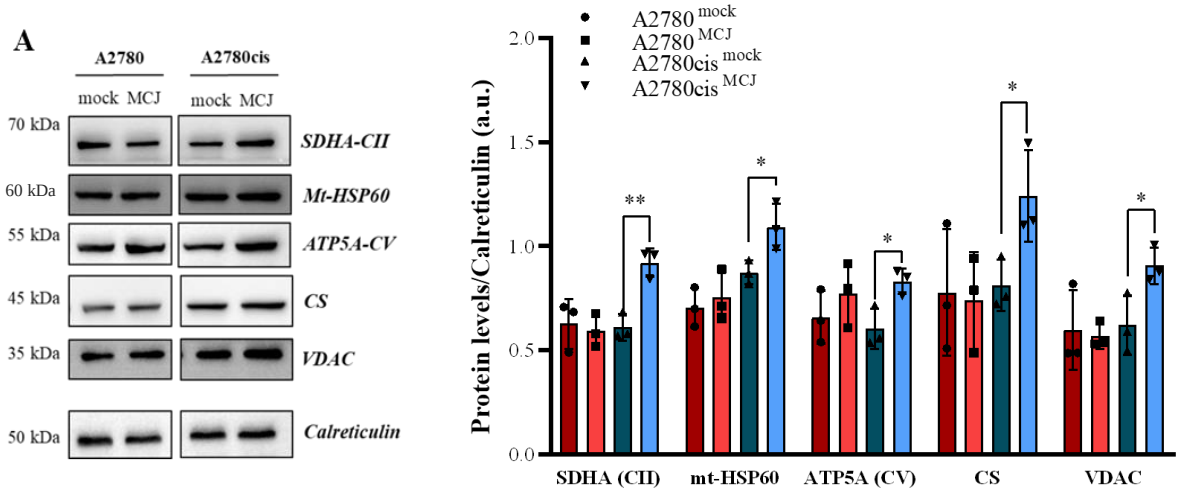


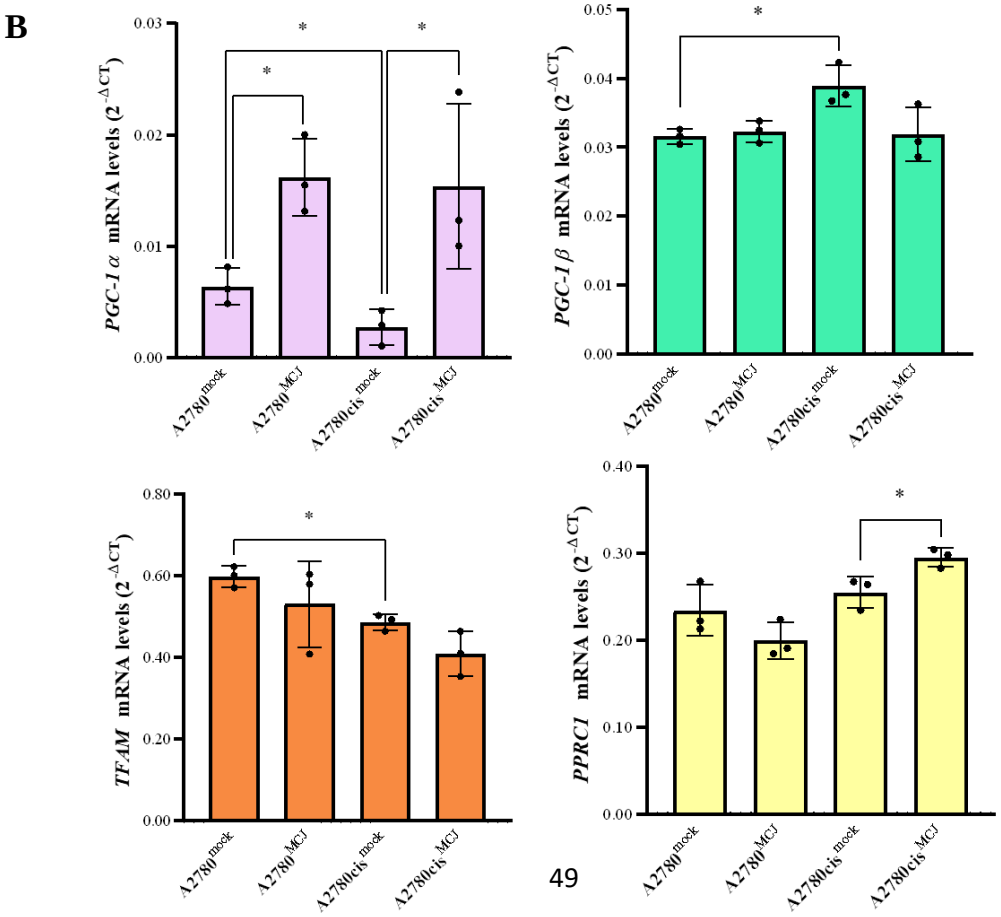
Figures 9. [**A**] ROS production levels in OC cell lines were determined using Cell-ROX probe for 10h with Incucyte Live Imaging. Data (mean ± SD; n=3) were normalized on the T0 (showed as 100%) and represented as Red Integrated Intensity per mm^2. [**B**] Western blot and densitometric analysis of antioxidant enzymes in OC cell lines by using antibodies against Catalase and Peroxiredoxin-3 (PRX-3). One representative experiment of three different is reported. GAPDH was used as loading control. Data are mean ± SD (n=3). All *p*-values were obtained with Student's t-test (*$p \leq 0.05$; **$p \leq 0.01$).

2.2 MCJ triggers mitochondrial biogenesis and may modulates lipids metabolism in OC chemoresistant cells.

Since we observed that MCJ is capable to increase mitochondrial respiration, we aimed to investigate if this modification in mitochondrial functionality might be due to mitochondrial biogenesis in OC chemoresistant cells. Hence, we firstly evaluated the expression levels of several mitochondrial proteins, and we reported that the amount of all proteins was significantly higher in the MCJ-over-expressing chemoresistant cell line compared with its mock counterpart (Figure 10A). In order to elucidate how MCJ could be involved in the increasing of mitochondrial proteins, we analyzed the expression levels of the main mitochondrial biogenesis regulators. Hence, we further evaluated the mRNA expression levels of *PGC-1α*, *PGC-1β*, *PPARGC1* (*PPRC1*) and *TFAM* (Wu Z *et al*., 1999; Jornayvaz FR and Shulman GI, 2010). It is noteworthy that the lower expression levels of *PGC-1α* and *TFAM* were accompanied by an increase in *PGC-1β* mRNA amount in the chemoresistant cell line, probably due to the activation of a compensation mechanism (Figure 10B). Importantly, we found significantly higher levels of *PGC-1α* and *PPRC1* in the MCJ-over-expressing chemoresistant cell line comparing with the mock counterpart (Figure 10B), suggesting a possible MCJ-mitochondrial biogenesis axis. In addition, it has been demonstrated that mitochondrial biogenesis mediators as *PGC-1α* and *PPRC1* can promote the expression of genes that encode for subunits of ETC complexes and for key enzymes of TCA cycle, as well as the expression of genes that encode key mediators of lipids metabolism (Bhalla K *et al*., 2011; Piantadosi CA and Suliman HB, 2012). Moreover, it has been reported that OC

progression and metastasization is strongly dependent on lipids storage and FAs β-oxidation (Tucker SL *et al*., 2014). Noteworthy, the demonstration that a reduction in MCJ expression improves hepatocyte capacity to mediate FAs β-oxidation (Barbier-Torres L *et al*., 2020) led us to investigate the β-oxidation metabolism in OC models. In this regard, we found that the levels of β-oxidation were higher in the chemoresistant cell line compared to the chemosensitive counterpart (Figure 10C) and decreased in the MCJ-over-expressing chemoresistant cell line. All toghether, these data suggest that this chaperone may be able to trigger the mitochondrial protein content and may influence the lipids catabolism in the chemoresistant cell line, making it metabolically similar to che chemosensitive models.





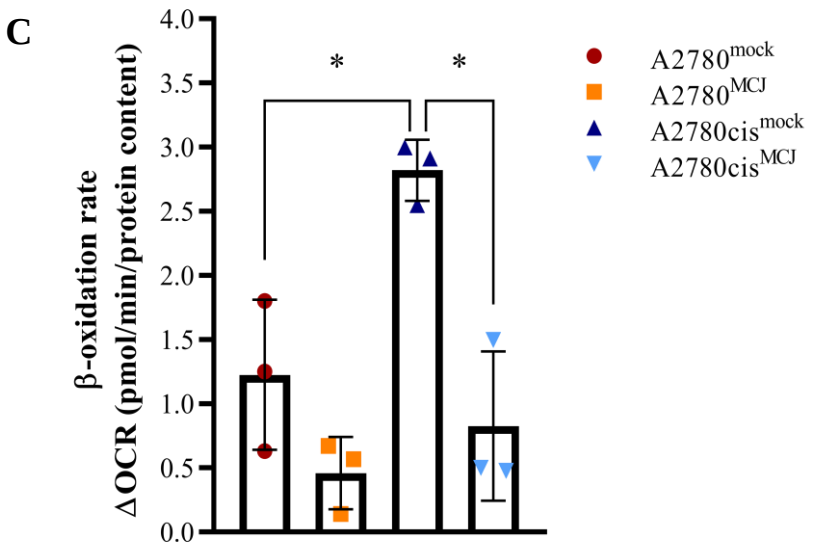
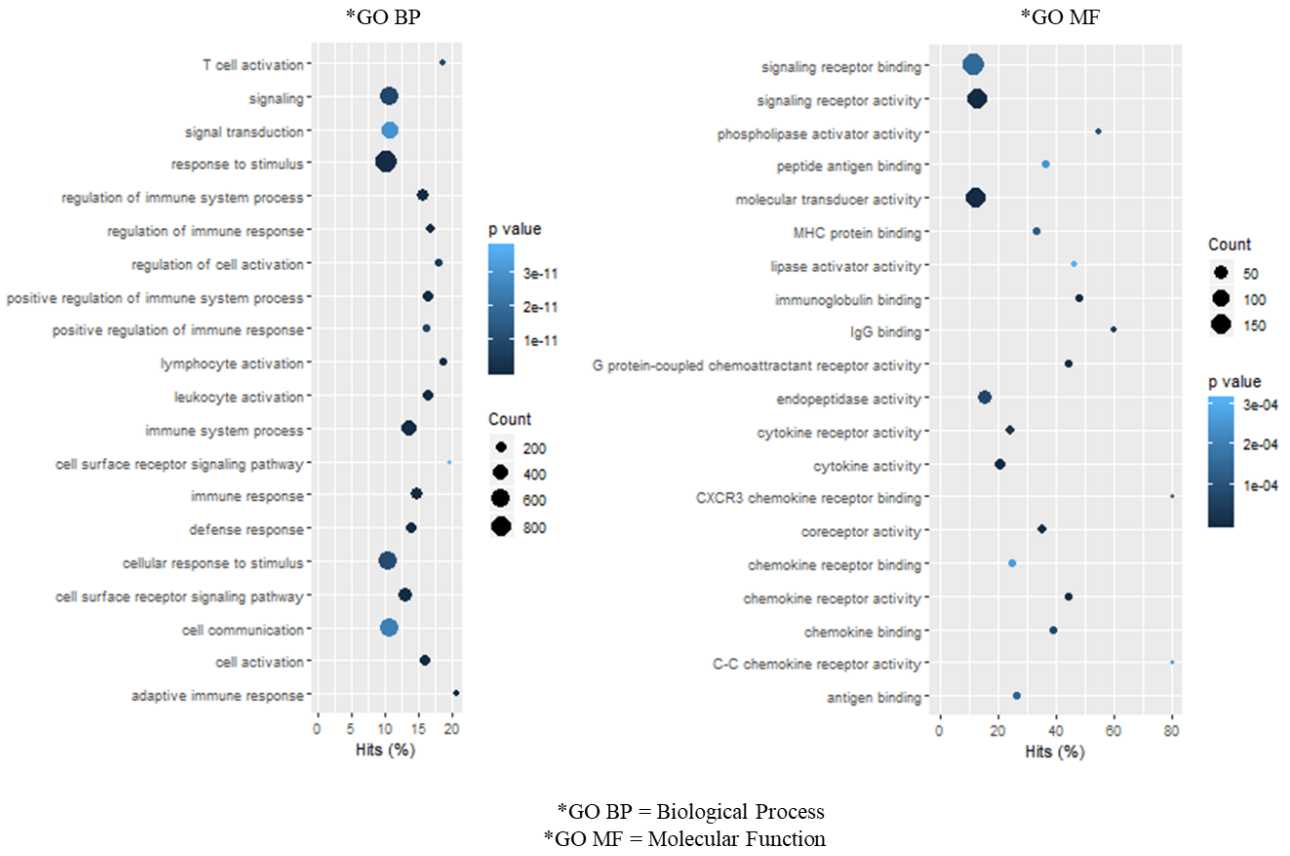


Figure 10. [**A**] Western blot and densitometric analysis of several mitochondrial proteins in OC chemosensitive and chemoresistant cell lines by using antibodies against SDHA (CII), mt-HSP60, ATP5A (CV), citrate synthase (CS) and VDAC. One representative experiment of three different is reported. Calreticulin was used as loading control. Data are mean ± SD (n=3). [**B**] Analysis of mRNA levels of mitochondrial biogenesis markers (*PGC1α*, *PGC1β*, *PPRC1* and *TFAM*) in OC cell lines performed with qRT-PCR and expressed as relative expression. Levels of *PGC1α* were shown as Fold Change *versus* mock counterparts. Data are mean ± SD (n=3). [**C**] Basal β-oxidation rate was obtained performing the basal ΔOCR calculation between the untreated and etomoxir-treated cells. Data were obtained by using XF-96 Seahorse Analyzer. Inhibitors Oligomycin, Rotenone and Antimycin A were all used at the concentration of 1μM. FCCP was used at the concentration of 2.25μM. Data are mean ± SD (n=3). All *p*-values were obtained with Student's t-test (*$p \leq 0.05$; **$p \leq 0.01$).

3. MCJ induces extra-mitochondrial oncogene pathways alteration.

3.1 MCJ leads to the alteration of canonical β-catenin onco-pathway.

To unravel the effect of the MCJ expression on the whole cellular network pathways, we performed an *in silico* analysis of the expression of *DNAJC15* in the TCGA (The Cancer Genome Atlas) dataset of OC samples by using EnrichR (Mercatelli D *et al.*, 2020). In particular, by performing a gene ontology correlation, this analysis revealed that the expression of *DNAJC15* correlated to an enrichment of molecular pathways involved in cells communication and signaling trasduction (Figure 11). The latter, enforce us to enrol which is/are the molecular pathways that are triggered by the alteration of mitochondrial bioenergetics and ROS production, and are able to regulate chemoresistance and cell proliferation in OC cell models.

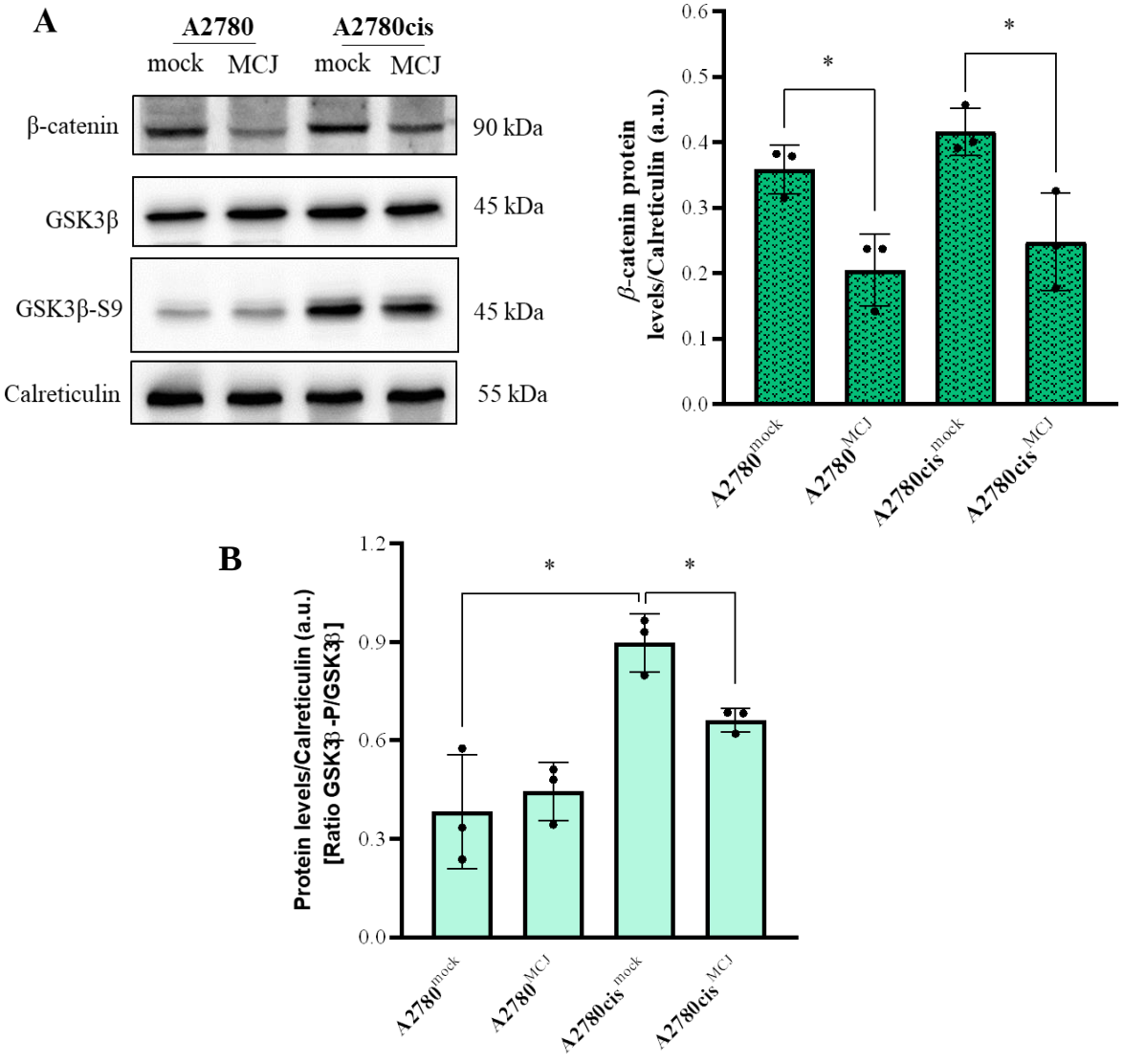


*GO BP = Biological Process
*GO MF = Molecular Function

Figures 11. *In silico* analysis on TCGA ovarian serous adenocarcinoma datasets (n=78). Gene ontology analysis was performed by comparing high-expressing and low-expressing *DNAJC15* patient samples in TCGA datasets, using default parameters. The analysis was performed using EnrichR. The Count represent the size of the corresponding gene set (or pathways), while the percentage of hits is the number of the significant genes belonging to that pathway.

In this regard, as already mentioned in the introduction, the molecular pathway of Wnt/β-catenin is frequently hyperactivated when OC becomes more aggressive and it is also known that this signaling axis is influenced by the mitochondrial ROS production (Shayesteh L *et al.*, 1999; Shin SY *et al.*, 2004). In several types of cancer, when β-catenin is active can translocate into the nucleus and act as co-activator of transcription factors responsible of the expression of genes encoding for proteins involved in tumor aggressiveness and invasiveness, in multi-drug resistance and in cell proliferation (MacDonald BT *et al.*, 2009; Shang S *et al.*, 2017). The levels of β-catenin in the cytosol are finely regulated by glycogen synthase kinase 3 beta (GSK-3β), that can phosphorylate β-catenin and triggers its destabilization and proteasomal degradation (Yuan S. *et al.*, 2020). We therefore investigated in the β-catenin oncogene pathways and, interestingly, a significant down-regulation of its expression level was observed in both chemoresistant and chemosensitive MCJ-over-expressing cell lines (Figure 12A). Furthermore, the analysis of both total and phosphorylated (Ser-9) amount of

GSK-3β protein levels revealed an increased phosphorylated/total ratio suggesting that this kinase is more inactive in the chemoresistant cell line since the phosphorylation on Ser-9 is an inactivating post-translational modification (Figure 12B). Noteworthy, in chemoresistant cells, the MCJ over-expression leads to a reduction of the phosphorylated amount of GSK-3β, which means its re-activation. This latter may suggest a putative GSK-triggering mechanism that would turns off the canonical Wnt/β-catenin oncogene pathway in MCJ-over-expressing cells.

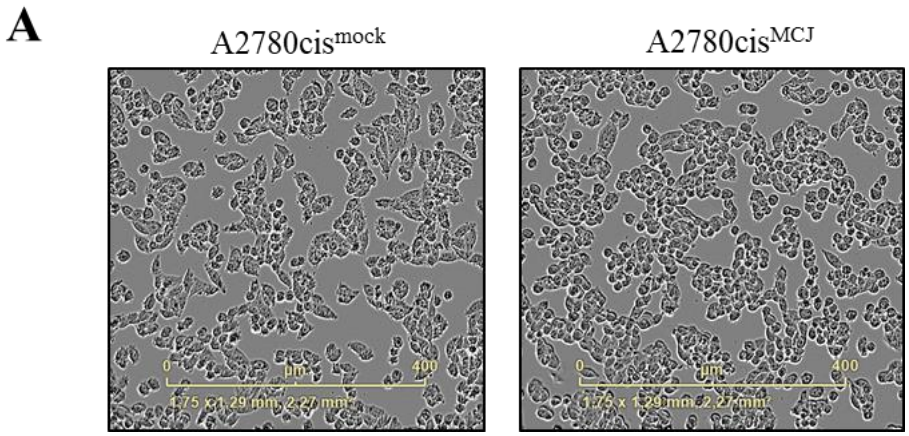


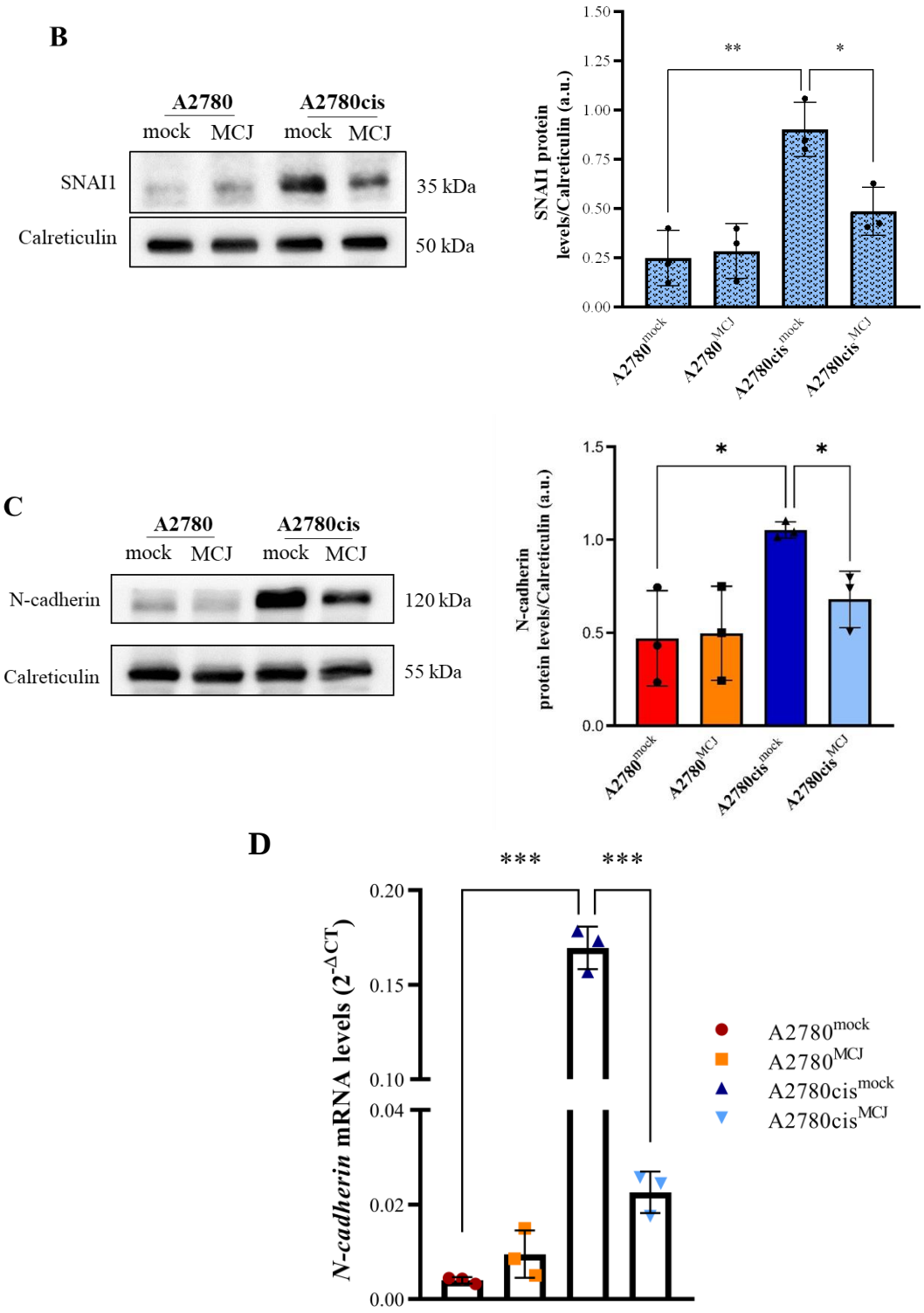
Figures 12. [**A**] Western blot and densitometric analysis of β-catenin pathway in OC chemosensitive and chemoresistant cell lines by using antibodies against β-catenin, glycogen synthase kinase 3 beta (GSK-3β) and glycogen synthase kinase 3 beta phosphorylated on Serine 9 (GSK-3β-P; inactivating phosphorylation). One representative experiment of three different is reported. Calreticulin was used as loading control. Data are mean

± SD (n=3). [**B**] Ratio of the phosphorylated form of GSK-3β normalized on the total GSK-3β, obtained by using data from the densitometric analysis of previous western blot. All *p*-values were obtained with Student's t-test (*$p \leq 0.05$).

3.2 MCJ reduces the mesenchymal phenotype of OC chemoresistant cell line.

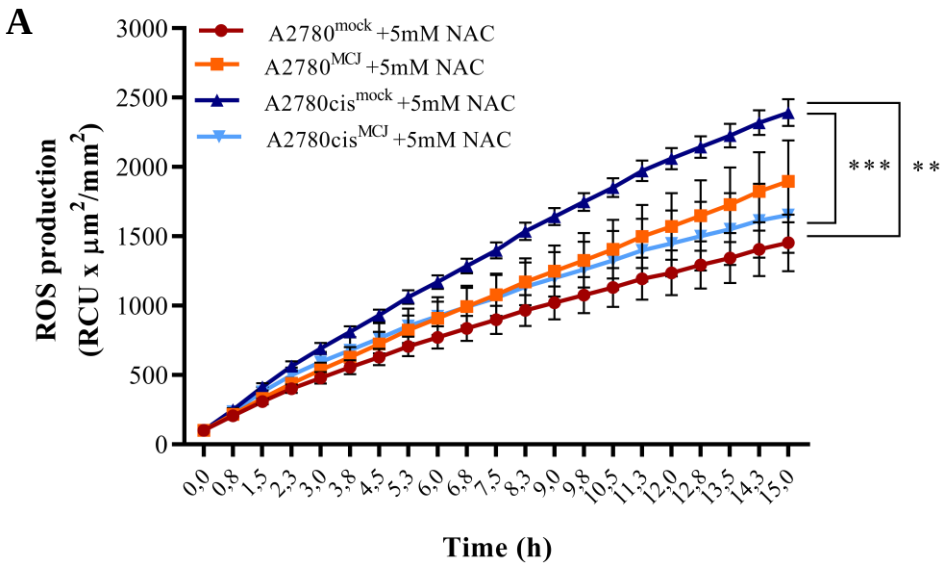
It is well known that β-catenin onco-pathway has a crucial role in regulating tumorigenesis, proliferation, migration as well as epithelial-mesenchymal transition (EMT). In this regard, we noticed that the chemoresistant cell line tends to change within the culture with the over-expression of MCJ chaperone, by increasing the abundance in round epithelial-like cells and reducing the elongated cells population (Figure 13A). We were therefore encouraged in investigating EMT in both chemosensitive and chemoresistant OC cell models. In particular, we evaluated β-catenin-downstream regulated genes, such as Snail Family Transcriptional Repressor 1 (SNAI1) and N-cadherin, both involved in the epithelial to mesenchymal transition (Zeisberg M and Neilson EG, 2009). SNAI1 protein expression resulted significantly higher in the chemoresistant cell line compared to the chemosensitive, and downregulated in the MCJ-over-expressing chemoresistant cell line (Figure 13B). Further, we found that both protein and mRNA level of N-cadherin were significantly higher in the chemoresistant cell line compared to the chemosensitive and downregulated in the MCJ-over-expressing chemoresistant cell line (Figure 13C-D), suggesting an MCJ-induced shutdown of pro-mesenchymal signaling in the chemoresistant cell line. Taken together, these data suggest that MCJ may be able to reduce the EMT by triggering the canonical oncogenic Wnt/β-catenin pathway.

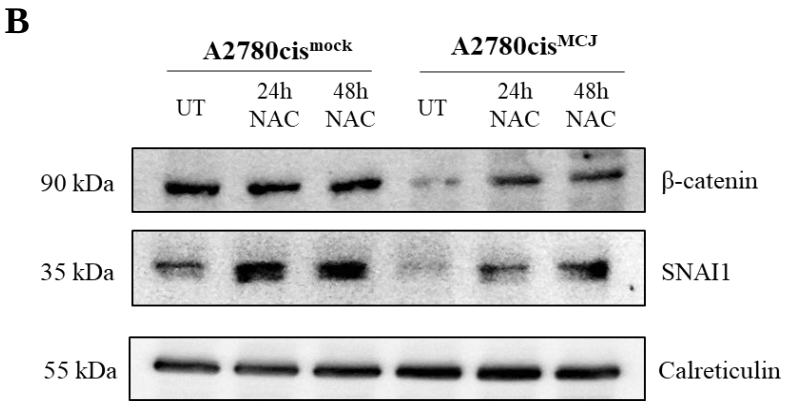


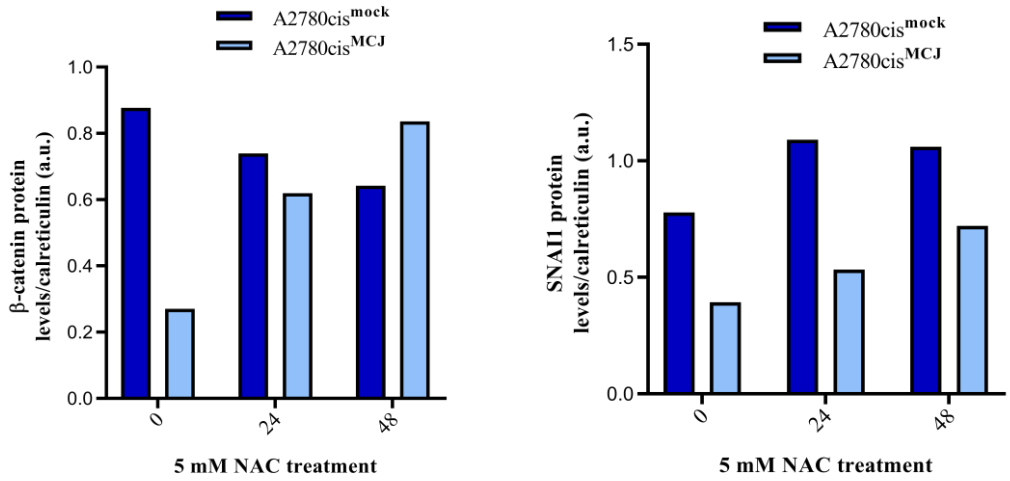


Figures 13. [**A**] Morphology of A2780cis^{mock} and A2780cis^{MCJ} cell lines was visualized with optical microscopy (20X). [**B**] Western blot and densitometric analysis of SNAI1 protein levels in OC chemosensitive and chemoresistant cell lines. One representative experiment of three different is reported. Calreticulin was used as loading control. Data are mean ± SD (n=3). [**C**] Western blot and densitometric analysis of N-cadherin protein levels in OC chemosensitive and chemoresistant cell lines. One representative experiment of three different is showed. Calreticulin was used as loading control. Data are mean ± SD (n=3). [**D**] mRNA expression levels of *N-cadherin* in OC chemoresistant cell lines. Data, reported as relative expression, are mean ± SD (n=3). [**D**] All *p*-values were obtained with Student's t-test (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$).

Since Wnt/β-catenin pathway can be regulated by ROS production that resulted increased in the MCJ-over-expressing chemoresistant cells, we evaluated the effect of the antioxidant N-acetylcysteine (NAC) on both β-catenin and its target SNAI1 expression levels. The treatment of chemoresistant cell lines with NAC, a GSH precursor that increases cell thiol levels and can thereby directly ameliorates cellular oxidative stress, significantly reduced ROS production in the MCJ-over-expressing chemoresistant cells, whereas no difference was observed between the two mock counterparts (Figure 14A). Moreover, we preliminarly found that treatment with NAC induces a recovery of both β-catenin and SNAI1 expression in the MCJ-over-expressing chemoresistant cell line in a time-treatment exposure dependent manner (Figure 14B), suggesting that MCJ may modulate Wnt/β-catenin and EMT signalling axis through its mitochondrial impact on ETC by enhancing ROS production.







Figures 14. [**A**] ROS production levels of both chemosensitive and chemoresistant OC cell lines during treatment with 5mM NAC. The measurement was determined using Cell-ROX probe for 24h and performed with Incucyte Live Imaging. Data (mean ± SD; n=3) were normalized on the T0 (showed as 100%). [**B**] Western blot and densitometric analysis of β-catenin and SNAI1 protein levels in OC chemosensitive and chemoresistant cell lines in absence and in presence of 5mM NAC for 24h and 48h. Calreticulin was used as loading control (n=1) All *p*-values were obtained with Student's t-test (**$p \leq 0.01$; ***$p \leq 0.001$).

DISCUSSION

This experimental study is focused on ovarian cancer (OC) disease, which is a type of tumour renowned for its lethal and silent aggressiveness, that claims new victims each year always ranking fifth among the causes of death from cancer in women (Siegel RL *et al*., 2020). Nonetheless, numerous studies have been implemented to identify new therapeutic strategies to overcome the conditions of recurrence and resistance to chemotherapy that are both very frequent in this type of tumour (Riggs MJ *et al*., 2020; Marchetti C *et al*., 2019). It is interesting to note that OC, when becomes metastatic and develops resistance to therapies, tends to epigenetically downregulate the *DNAJC15* gene that encodes for MCJ, a mitochondrial co-chaperonine of the inner mitochondrial membrane (Shridhar V *et al*., 2001; Strathdee G *et al*., 2004; Fernández-Cabezudo MJ *et al*., 2016). Although in a non-cancer context MCJ is able to modify the cellular bioenergetic profile modulating the activity and assembly of mitochondrial respiratory chain complexes (ETCs) (Hatle KM *et al*., 2013), no data about its role in cancer biology are reported.

We aimed to assess whether the expression of MCJ can modulates the OC tumorigenic potential and the drug resistance onset. In particular, we unraveled whether and how MCJ affects mitochondrial bioenergetics to sustain cell proliferation of cisplatin-sensitive and resistant OC cell lines. The first evidence that we obtained was that the MCJ expression is lacking in cisplatin-resistance cells and its over-expression leads to a significant reduction of the cisplatin-resistance and of *in vitro* proliferative and migration capacities, suggesting that this chaperonin may modulate the tumorigenic potential of OC cell lines.

In this regard we also evaluated the expression levels of cisplatin-specific and not specific ABC transporters. The expression of some cisplatin-specific transporters, such as *ABCC2* (the most renowed cisplatin transporter) and *ABCA1*, was higher in the chemosensitive cell line than in the resistant one. However, it should be noted that MDR transporters are also involved in numerous physiologic mechanisms of the cells and could transport a *plethora* of substrates across the plasmamembrane, such that they cannot be related only to the condition of cisplatin-resistance (Higgins CF, 2001; Borst P and Elferink RO, 2002; Linton KJ, 2007). Nonetheless, it has been reported in the literature that the expression of *ABCC1* and *ABCC4* transporters, that could transport platinum compound, have an unfavorable impact on disease relapse and are good prognostic factors for ovarian cancer progression (Bagnoli M *et al*., 2013). In our data we showed both *ABCC1* and *ABCC4* transporters significantly higher, together with cisplatin-specific *ABCA3*, in the resistant cell line compared to chemosensitive

cells. All these latters became less expressed in the MCJ-over-expressing resistant cell line, thus suggesting a likely partial involvement of MCJ in the modulation of cisplatin chemoresistance. Moreover, no change in the MDRs gene expression was observed in the MCJ-over-expressing chemosensitive cell line, probably due to a lower expression of these genes at basal levels compared to the chemoresistant counterpart. However, the study of MDRs needs further investigations, including the analysis of their activity.

Since MCJ was first described as a negative endogenous inhibitor of mitochondrial ETC function, we investigated the bioenergetic state of cisplatin-sensitive and resistant OC cell lines. We interestingly found that the over-expression of MCJ causes a significant increase in the activity of the respiratory CI, CII, CIII and a slight reduction of CIV activity, supporting the finding that MCJ over-expression leads to a significant increase in the oxygen consumption in the chemoresistant OC cell line. These data clearly indicate that the expression of MCJ in cisplatin-resistant cells confer a respiratory profile similar to the cisplatin-sensitive counterpart. The increased basal oxygen consumption in the MCJ-over-expressing resistant cell line is probably due to the increased activity of CI, CII and CIII, despite the observed reduction in the activity of CIV. In this regard, the reduction of CIV activity of about 50% compared to sensitive cells, probably is not sufficient to affect the overall activity of the ETC, being over the 40% threshold estimated as the activity of CIV that reduces the oxygen consumption in mitochondria (D'Aurelio M *et al*., 2006; Rossignol R *et al*., 2003).

It is interesting to note, that our observations are apparently in contrast with the increase of CI activity and OXPHOS capacity reported in MCJ-knockout mouse and in doxorubicin resistant MCJ-lacking breast cancer cells (Hatle KM *et al*., 2013; Giddings EL *et al*. 2021). These discrepancies could be due to the different models used and drug-mediated resistance analyzed (doxorubicin *versus* cisplatin). Moreover, in our study we evaluated the impact of MCJ only in A2780/A2780cis cells that harbours a deletion in the *PTEN* gene and not canonical driver mutations in *TP53*, typical of most frequent high-grade serous OC (Bell D *et al*., 2011). Thus, to validate and generalize our findings a panel of different OC cell lines and patient's biopsies are needed.

The general finding that MCJ over-expression is able to trigger the ETC activity and oxygen consumption, prompted us to investigate if this bioenergetic phenomenon is due to the induction of a mitochondrial biogenesis mechanism. We found that *PGC-1α* and *PPRC1* levels and several mitochondrial proteins were significantly increased in the MCJ-over-

expressing chemoresistant cell line indicating that the ETC activity may involve the activation of mitochondrial biogenesis.

Recently, it has been demonstrated that the absence of MCJ in adipocytes leads to a FAs β-oxidation induction with a reduced lipid intracellular accumulation (Barbier-Torres L. *et al*., in 2020). As the expression of MCJ in OC cells increases the oxygen consumption dependent on the oxidation of glucose and pyruvate, we measured the ability of OC cells to perform a lipid-mediated respiration. The chemoresistant cell line showed a higher FAs β-oxidation rate compared to chemosensitive one that was reduced by the MCJ-over-expression, supporting our previous observation that MCJ is able to confer an energetic state similar to that of chemosensitive cells. It should be noted that, in agreement with data reported by Barbier-Torres L. *et al*., we showed that the β-oxidation was enhanced in the chemoresistant cell line that lacks of MCJ expression. Hence, a deep dissection of lipid metabolism in OC models is required since OC peritoneal dissemination and the response to chemotherapy seems to be strictly dependent on the modulation of biochemical pathways involved in the lipid metabolism (Zhao G. *et al*., 2019; Sawyer BT *et al*., 2020).

Interestingly, we have shown that the over-expression of MCJ triggers an activation of ETC accompanied by a significant boost in intracellular ROS amount as well as in a reduction of expression levels of antioxidant enzymes, suggesting an imbalance in the cellular redox mechanisms. Among the ROS-dependent mechanisms, the Wnt/β-catenin signalling is a pathway regulated by mitochondria in a ROS-related manner (Shayesteh L *et al*., 1999; Shin SY *et al*., 2004). Moreover, this signalling axis is able to induce the expression of downstream genes involved in multi-drug resistance (MDR) and stemness, such as *ABCG2*, epithelial-mesenchymal transition (EMT) and cell proliferation (MacDonald BT *et al*., 2009; Shang S *et al*., 2017). In this regard, the mechanism through which the ROS levels can lead to the repression of the β-catenin pathway is still debated in literature and not demonstrated yet. Interestingly, our data shown that β-catenin expression levels were significantly lower when MCJ was over-expressed, in both chemosensitive and chemoresistant cell lines. In addition, the reported downregulation of β-catenin was coherent with the re-activation of GSK-3β enzyme in the MCJ-over-expressing chemoresistant cell line. In the latter cells, the showed decreased levels of the mesenchymal markers, SNAI1 and N-cadherin, and of *ABCG2* suggested a switch towards an epithelial phenotype similar to chemosensitive cells. In this regard, it is interesting to note that the cisplatin-resistant cell line showed a mixed population of round-epithelial and elongated mesenchymal-like cells that, however, tends to lose the elongated component when MCJ was over-expressed, probably due to the specific loss of the

mesenchymal-like cells within the overall population. Furthermore, the lack of both β-catenin and EMT markers was rescued by NAC antioxidant treatment**,** suggesting that a ROS-β-catenin signaling axis may occur, upon the combination of MCJ expression and cisplatin-resistance.

CONCLUSIONS

Overall, the data reported in this study lead us to hypothesize a novel link between MCJ and β-catenin pathway during OC cisplatin-resistance occurrence (Figure 15). MCJ expression in the cisplatin-resistant cell line may attenuate FAs β-oxidation and trigger the ETC activity as cause or consequence of the activation of PGC-1α/PPRC1-mediated mitochondrial biogenesis. This mitochondrial perturbation is accompanied with ROS overproduction that may repress the Wnt/β-catenin onco-pathway. The lack in β-catenin pathway would cause the loss of expression of its target genes involved in the regulation of MDR, EMT and cell proliferation, triggering a low mesenchymal, low proliferative and low migratory phenotype making the OC chemoresistant cells more sensitive to cisplatin-based therapy.

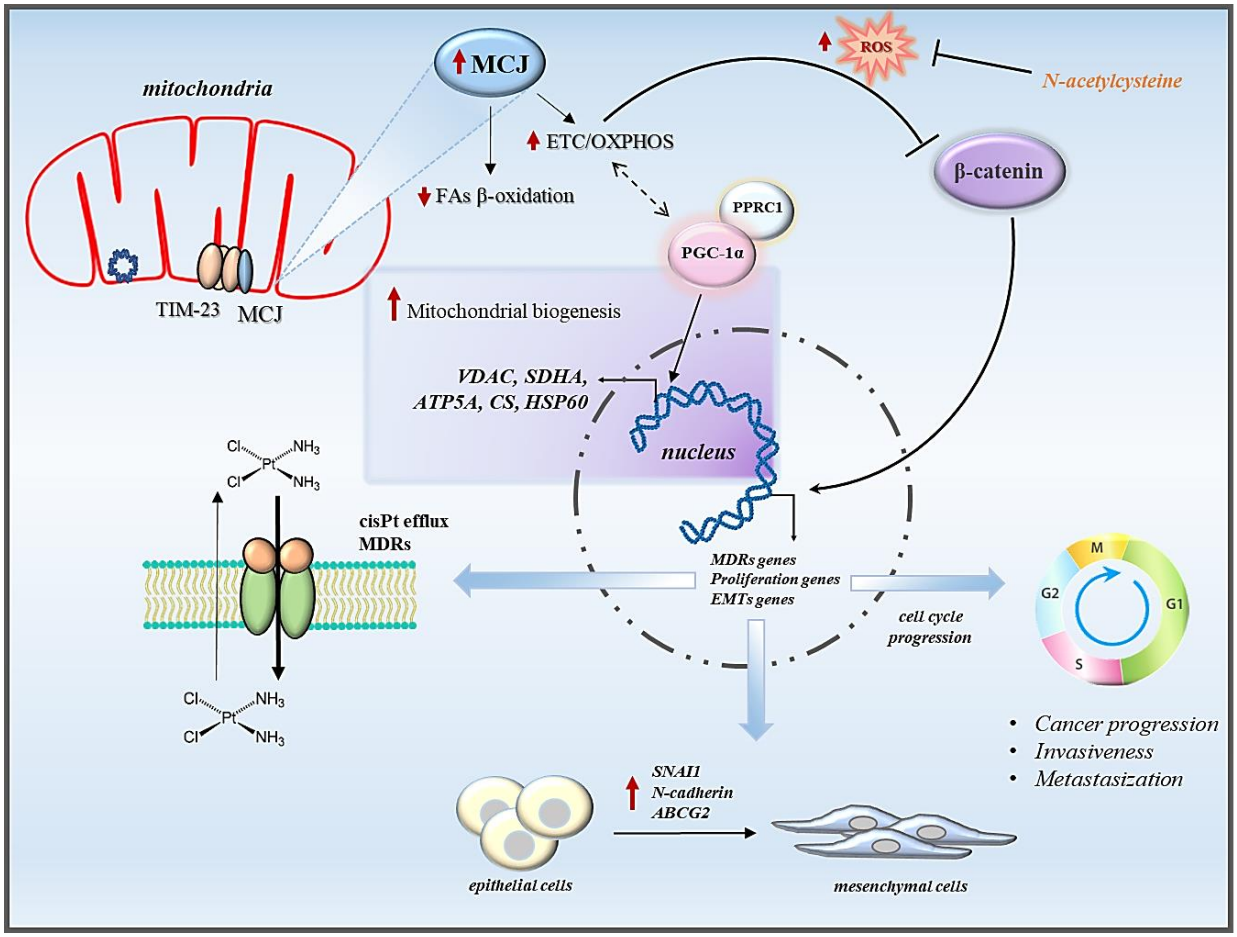


Figure 15. Scheme of the proposed MCJ-β-catenin axis in OC cisplatin-resistance occurrence.

FUTURE PERSPECTIVES

This work aimed to provide new insights and unravel novel mechanisms that make MCJ a newsworthy factor in cancer biology, in particular in OC disease. In this frame, future perspectives dwell in enforce the role of MCJ in the chemoresistance processes of OC and its impact on the mitochondrial bioenergetics state. For instance, in order to generalize what we observed in the A2780/A2780cis cell lines, others cisplatin-chemosensitive and chemoresistant cell lines will be used. In addition, pre-clinical mice models will be exploited to evaluate the contribution of MCJ in the *in vivo* OC proliferation and sensitivity to chemotherapy. Furthermore, the impact of the MCJ expression will be also studied in biopsies from cisplatin-sensitive and resistant OC patients' cohort. As well, it will be interesting to dissect the mechanisms with which MCJ may regulate the ETC activity that leads to both ROS accumulation and imbalance in β-catenin signaling. In addition, it will also be interesting to unravel the metabolism of chemoresistant cell lines by investigating bioenergetics-sustaining pathways such as glycolysis, glutaminolysis and lipids metabolism in depth.

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