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INNOVATIVE MITOCHONDRIAL AND METABOLIC TARGETS TO ENHANCE CISPLATIN RESPONSE: STUDIES ON CANCER CELLS WITH ACQUIRED AND INTRINSIC RESISTANCE

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ABSTRACT

Cisplatin is one of the most potent anticancer agents used in the treatment of various solid tumors. Unfortunately the onset of resistance is the main limit of this therapy and severely compromises the treatment effectiveness. Although several studies regarding cisplatin resistance have been performed, the molecular mechanisms are not completely understood. Classically, cisplatin is studied as a DNA-damaging chemotherapy agent, but more recent investigations showed that only 5-10% of intracellular platinum is bound to nuclear DNA, while the great majority of the intracellular drug can interact with a variety of cellular component including proteins, RNA and mitochondrial DNA. MtDNA, unlike nDNA, does not possess efficient repair systems; therefore it is more susceptible to the onset of mutations often associated to cancer development, loss of tumor suppressor, activation of oncogenes and mitochondrial dysfunctions related with an increase of glycolytic activity. The Warburg effect indicates the alteration of energetic metabolism used by tumor cells as a strategy to adapt and grow independently from the substrate availability.

This evidence suggested us to verify the hypothesis that a similar metabolic strategy might be of relevance in cisplatin resistance.

Therefore, our aim was to investigate the energetic metabolism and the mitochondrial dynamic of cisplatin-resistant and sensitive cancer cells with different experimental approaches, in order to reveal targets useful to overcome the resistance.

In our laboratory we have already revealed that cisplatin resistant ovarian cancer cell line C13, as compared to sensitive line 2008, exhibits metabolic changes. Indeed, resistant clone showed a different mitochondrial and metabolic profile characterized by an increase of glucose and glutamine uptake, a decrease of the mitochondrial membrane potential and mitochondrial mass.

In this scenario, we proceeded to phenotype other cancer cells that present acquired or intrinsic resistance in order to identify new targets to sensitize to cisplatin treatment.

Our results pointed out alterations in mitochondrial fusion and fission in chemoresistant cancer cells. Moreover data obtained by real time q-PCR showed that resistant clones, with an imbalance toward fission process, present a faster mitochondrial turn-over using mitophagy as a mitochondrial quality control mechanism. Furthermore the data showed a mitochondrial network differently organized in resistant variants underlining a probable implication of dynamic process in resistance mechanisms.

Having regard to the data about metabolic reprogramming, breast cancer cells that have an innate resistance to cisplatin were evaluated.

The expression of c-Myc nuclear transcription factors, involved in the metabolic reprogramming of tumor cells, has been evaluated highlighting a different expression of some of its target genes involved in the glycolylisis and glutaminolysis, besides an increased dependency of glucose in cisplatin resistant cells.

RIASSUNTO

Il cisplatino è uno dei più potenti agenti antitumorali utilizzati nel trattamento di vari tumori solidi. Purtroppo l'insorgenza della resistenza è il limite principale di questa terapia e compromette gravemente l'efficacia del trattamento. Anche se sono stati eseguiti numerosi studi per quanto riguarda la resistenza al cisplatino, i meccanismi molecolari non sono del tutto chiari. Classicamente, il cisplatino è studiato come agente chemioterapico che crea danno a livello del dna ma studi più recenti hanno dimostrato che solo il 5-10% del platino è legato al DNA nucleare, mentre la maggior parte del farmaco intracellulare può interagire con diverse componenti cellulari tra cui proteine, RNA e DNA mitocondriale. Il DNA mitocondriale, a differenza del DNA nucleare, non possiede sistemi di riparazione efficienti ed è quindi più suscettibile alla comparsa di mutazioni spesso associate allo sviluppo del cancro, alla perdita di oncosoppressori, attivazione di oncogeni e ad alterazioni della funzionalità mitocondriale correlata ad aumento dell'attività glicolitica. L'aumento della glicolisi anaerobica, anche in presenza di alte concentrazioni di ossigeno (effetto Warburg), è l'alterazione del metabolismo energetico utilizzata dalle cellule tumorali come strategia per adattarsi e crescere in modo indipendente dalla disponibilità del substrato. Queste evidenze scientifiche ci hanno suggerito di verificare l'ipotesi che una simile strategia possa essere rilevante nell'insorgenza della resistenza al cisplatino.

Pertanto, lo scopo di questo studio è stato quello di indagare il metabolismo energetico e la dinamica mitocondriale delle cellule tumorali sensibili e resistenti al cisplatino con diversi approcci sperimentali, al fine di rivelare utili targets per superare questa importante forma di resistenza.

Nel nostro laboratorio abbiamo già dimostrato che la linea di carcinoma ovarico resistente al cisplatino C13, rispetto alla linea sensibile del 2008, presenta cambiamenti metabolici. Infatti, il clone resistente ha mostrato un profilo mitocondriale e metabolico differente, caratterizzato da un aumento della dipendenza da glucosio e glutammina, una diminuzione del potenziale di membrana e della massa mitocondriale.

In questo scenario, lo studio ha proseguito con la valutazione del meccanismo di resistenza cisplatino fenotipizzando altre cellule tumorali che presentano resistenza acquisita o intrinseca.

I nostri risultati indicano un'alterazione dei meccanismi di fusione e fissione mitocondriale nelle cellule tumorali chemioresistenti. I dati ottenuti dalla q-PCR Real Time hanno dimostrato che i cloni resistenti, che presentano uno squilibrio verso processo di fissione, attivano un turn-over

mitocondriale più veloce, utilizzando la mitofagia come meccanismo di controllo della qualità mitocondriale.

Inoltre i dati ottenuti hanno mostrato un network mitocondriale diversamente organizzato nelle resistenti sottolineando una probabile implicazione della dinamica mitocondriale nei meccanismi di resistenza.

Per quanto riguarda i dati relativi alla riprogrammazione metabolica, sono state prese in esame cellule del cancro al seno che hanno una resistenza innata al cisplatino.

È stata valutata l'espressione del fattore di trascrizione c-Myc che è coinvolto nella riprogrammazione metabolica delle cellule tumorali, per di più si è evidenziata una diversa espressione di alcuni geni bersaglio di c-Myc coinvolti nella glicolisi e glutamminolisi, oltre che una maggior dipendenza dal glucosio nelle linee resistenti di carcinoma al seno.

ABBREVIATION

Abs	Absorbance
ADP	Adenosine Diphosphate
AIF	Apoptosis Inducing Factor
Akt	Protein kinase B
AMPK	Adenosine Mono Phosphate Kinase
ANT	Adenine Nucleotide Translocator
ATP	Adenosine Triphosphate
ATP7A e ATP7B	Copper-Transporting P-type ATPase
BER	Base Excision Repair
CAD	Caspase-Activated DNase
CDDP	Cis-diamminedichloroplatinum II
CoA	Coenzyme A
CTR1	Copper Transport protein 1
DMEM	Dulbecco's modified Eagle's medium
DRP1	Dynami-Related Protein 1
ERCC1	Excision Repair Cross-Complementation Group 1
FAD	Flavin Adenine Dinucleotide
FBS	Fetal Bovine Serum
FIS1	Fission Protein Homolog 1
GAPDH:	glyceraldehyde 3-phosphate dehydrogenase
GSH	Glutathione
GTP	Guanosine 5'-Triphosphate
HIF	Hypoxia Inducible Factor

HR	Homologous Recombination Repair
ICAD	Inhibitor of Caspase-Activated DNase
KEAP 1	Kelch-like ECH-associated protein 1
LC3	Microtubule-Associated Protein 1A/1B-Light Chain 3
MEF	Mouse Embryonic Fibroblasts
MFI	Mean Fluorescence Intensity
Mff	Mitochondrial Fission Factor
MFN1	Mitofusin-1
MFN2	Mitofusin-2
MME	Membrana Mitocondriale Esterna
MMI	Membrana Mitocondriale Interna
MMP	Mitochondrial Membrane Permiabilization
MMR	Mismatch Repair
MOMP	Mitochondrial Outer Membrane Permiabilization
mtDNA:	mitochondrial DNA
mRNA	Messenger RNA
mTOR	Mammalian Target Of Rapamycin
NAD	Nicotinamide Adenine Dinucleotide
NER	Nucleotide Excision Repair
NF-Kb:	Fattore Nucleare-Kb
NRF2	Nuclear factor (erythroid-derived 2)-like 2
OPA1	Optic atrophy 1
PBS	Phosphate Buffered Saline
PIK3	Phosphatidylinositol 3-Kinase

PKA	Protein-Kinase A
PTP	Permeability Transition Pore
ROS	Reacting Oxygen Species
RPMI	Roswell Park Memorial Institute
RT PCR:	Real-Time Polymerase Chain Reaction
SQSTM1	Sequestosome 1
TNF	Tumor Necrosis Factor
TOM20:	Translocase of Outer Mitochondrial Membranes
TRAP1	Tumor necrosis factor Receptor Associated Protein 1
VDAC	Voltage-Dependent Anion Channels

INTRODUCTION

1. CURRENT CANCER THERAPY

Cancer is the main cause of death in economically developed countries (The Global Burden of Disease: 2004 Update. Geneva: World Health Organization; 2008.). There were 14.1 million new cases and 8.2 million deaths in 2012. The most commonly diagnosed cancers were lung (1.82 million), breast (1.67 million), and colorectal (1.36 million); the most common causes of cancer death were lung cancer (1.6 million deaths), liver cancer (745,000 deaths), and stomach cancer (723,000 deaths), (http://globocan.iarc.fr).

The incidence of cancer is increasing in countries economically developed due to aging and the growth of the population, as well as, the adoption of cancer-associated lifestyle choices, including smoking, physical inactivity, and western diets. The prevention is able to help to reduce an important percentage of the worldwide burden of cancer by implementing programs for tobacco control, vaccination, and early detection and treatment, as well as public health campaigns promoting physical activity and healthier dietary patterns. Nevertheless, there is still much to learn about the causes of several types of cancer. (Jemal A., et al., 2011).

Cancer cells differ from normal cells in many ways that allow them to escape the proliferation control mechanisms and to become invasive. In addition, cancer cells tend to evolve spontaneously towards a growing autonomy and increase the ability to colonize and expand into different tissues because they are able to ignore signals that normally lead to apoptosis.

Cancer was listed as a genetic complex multi-step disease, due to the acquisition of mutations of oncogenes and tumor suppressor genes (Ortega AD. et al., 2009). Oncogenes derive from proto-oncogenes that, once undergone the mutation, alter their protein synthesis, through the production of wrong proteins (Liu, E.T. et al., 2004; Balmer A.M. et al., 2005). The tumor suppressors, instead, usually control the over-expression of oncogenes, but, in different types of cancer, they are destroyed or altered leading to a decrease of their functionality.

Cancer cells may be able to influence the normal cells, molecules and blood vessels that surround and feed the tumor area known as the microenvironment. For instance, cancer cells can induce nearby normal cells to form blood vessels that supply tumors with oxygen and nutrients that allow the tumor to grow. Although the immune system normally removes damaged or abnormal cells from the body, cancer cells can mutate enough and become able to escape the surveillance mechanisms of the immune system: many cancers produce chemical messengers that

inhibit the actions of immune cells or other cancers have defects in the way that antigens are presented on their cell surface.

Cancer therapy uses different strategies whose effectiveness is variable depending on the type of cancer, the speed with which the treatment is undertaken, and many other factors. Surgical therapy keeps a critical role in cancer treatment, even if it has become less invasive, often performed endoscopically, with the consequent advantage of being more acceptable to the patient, who has a post-operative path favored in general also by the progress of anesthetic techniques. It is often accompanied with radiotherapy and chemotherapy to increase the percentages of survival (Gatenby R.A. 2009).

The complex of pharmacological therapies and techniques employed in their administration, used in the tumors treatment, has the purpose to stop the proliferation of cancer cells throughout the body, especially in the secondary localization level of metastases. Since different treatments are applied to the patient, drug therapy aims to be more effective and less aggressive. (Deberardinis R. J., et al., 2008). Therefore chemotherapy uses biological therapies, which attack the molecular mechanisms of cancer with drugs, specifically targeted on each type of tumor; when this is not possible, chemotherapy uses high doses of drugs. The anticancer drugs may have several targets in the cell: in general different classes of drugs are distinguished (alkylating agents, antimetabolites, intercalating agents and mitotic inhibitors), but in the final analysis the pharmacological target is constituted by the DNA.

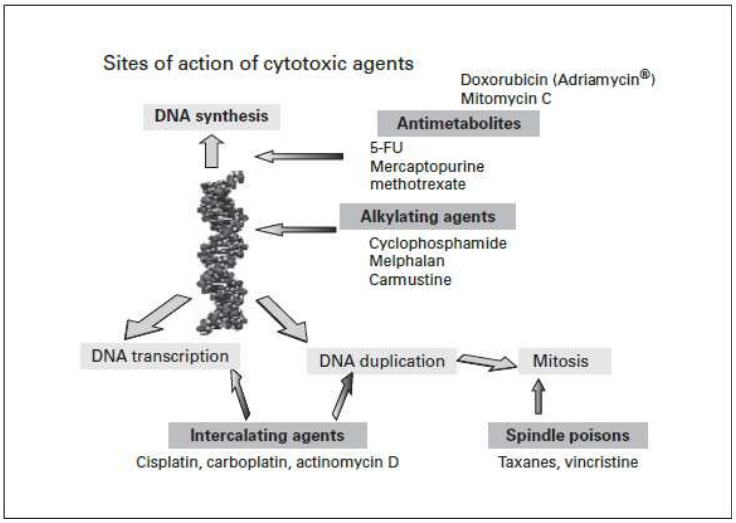


Fig.1: Sites of action of cytotoxic agents (Luqmani Y.A., 2005).

Chemotherapy has several side effects, in order to reduce them, as far as possible, these drawbacks have been devised in particular strategies for administering therapy. In the first place the chemotherapy is carried out in an intermittent manner, according to schemes that provide curative cycles lasting several days, alternated with rest intervals up to 3-4 weeks: in this way it is possible to the normal cell populations to recover between one cycle and the others, taking advantage to the tumor cells, generally slower to recover the damage. In order to further reduce the side effects it is necessary to use the association of more antitumor drugs (polychemotherapy), increasing the overall effect due to different mechanisms of action, and decreasing at the same time the toxicity. In some cases, chemotherapy is used as a precautionary measure (adjuvant chemotherapy) after surgery for tumor removal, to eradicate any microscopic metastases which have already spread at the time of the transaction. Often chemotherapy is used even before the surgery; in this case the chemotherapy, neoadjuvant, has the aim to reduce the tumor mass in order to make it more easily removable with surgical interventions.

Overall, the viability and success of chemotherapy are related to factors from both the patient (age, general health, performance status) and the tumor (biological characteristics of the degree of response to chemotherapy, total tumor mass extension): especially in the latter case the phenomenon of resistance to anticancer drugs assumes considerable importance, event which unfortunately almost occurs during the course of chemotherapy (Luqmani Y.A., 2005).

2. DRUG RESISTANCE AND CHEMOTHERAPY

Resistance to chemotherapy and molecularly targeted therapies is the major problem of the current cancer research. The investigation of the chemoresistance of malignant cells is needed also for oncology practice because drug resistance is often considered to be a cause of therapy failures and the impact on survival could be highly significant.

Cancer cells are constantly using a variety of tools, involving genes, proteins and altered pathways, to ensure their survival against antineoplastic drugs. The mechanisms of resistance to 'classical' cytotoxic chemotherapeutics are complex and multifactorial (Koberle et al., 2010).

A variety of factors have been demonstrated to be involved in chemoresistance, including the reduced intracellular concentrations of drugs, alterations in drug targets, activation of prosurvival pathways, ineffective induction of cell death and interactions between cancer cells and the tumor microenvironment (Shi W. J. and Gao J.B., 2016).

Resistance to antineoplastic chemotherapy is a process that involves a specific drug, a specific tumor and a specific patient. Chemoresistance can be divided into innate chemoresistance, intrinsically present before the administration of chemotherapy, and acquired chemoresistance when it can develop during antineoplastic drug administration (Meads M. B. et al., 2009). An example of intrinsic resistance is provided by cells of non-neoplastic normal peritumoral tissues. In fact, in the woman's breast invasive carcinomas treated in neoadjuvant manner with highly aggressive protocols as a result of healthy breast peritumoral tissue biopsies, it is noted the total absence of damage induced by chemotherapeutic protocol. If on the one hand this example demonstrates a physiological and natural resistance to chemotherapy by normal cells, on the other, it allows to hypothesize that the chemosensitivity is an acquired characteristic. (DeVita V.T., et al., 2008).

The acquired drug resistance may be due to several factors such as the cell cycle kinetics of the biochemical and pharmacological causes (Gottesman M. M., 2002). Today chemosensitivity and acquired chemoresistance represent a vast field of research because the two properties characterize the profile of each tumor. To date the input that generates the chemoresistance, especially those acquired, is little known but it is reasonable to think that the genetic instability, underling cancer, can induce mutations that produce phenotypic drug resistance. Spontaneous mutations can occur within the tumor cells population and develop resistant clones (cells derived from the division of a single cell, which all have the same genetic heritage) capable of withstanding to chemotherapeutic drugs due to different biochemical mechanisms (Weinstein I. B., 2002). The selective advantage gained promotes the proliferation of resistant clone, which allows the entire population to be insensitive to treatment: the tumor can growth unstoppably. In some cases the cancer cells are even capable of synthesizing a cell membrane protein which extrudes cytotoxic molecules, keeping intracellular drug concentration below a cell-killing threshold and cancer cells become resistant to multiple drugs. (Ullah M. F., 2008). With the increasing array of anticancer agents, improving preclinical models and the use of high-throughput screening techniques are now opportunities to understand and overcome drug resistance through the clinical assessment of rational therapeutic drug.

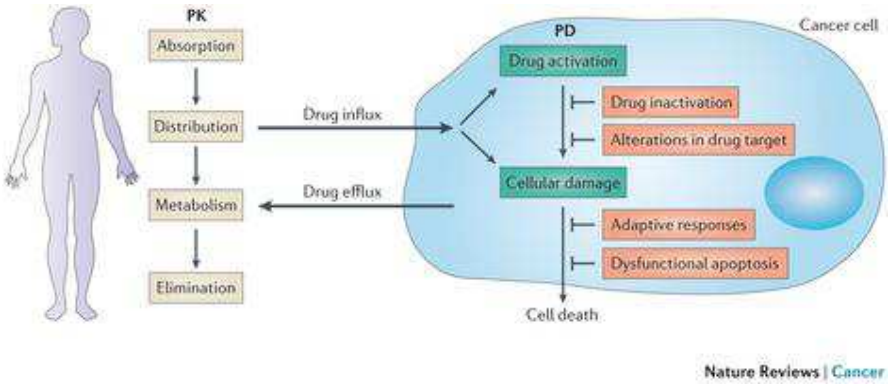


Fig. 2: General principles of drug resistance.

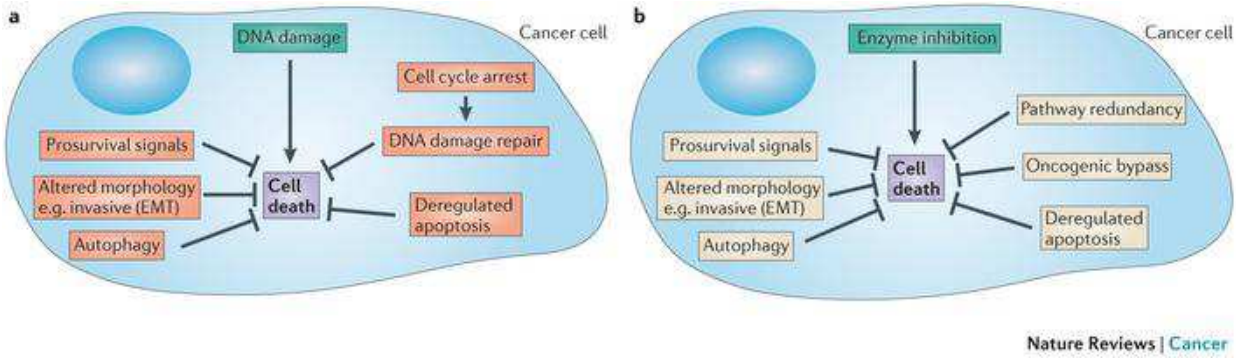


Fig. 3: Summary of downstream factors that influence drug resistance.

3. CISPLATIN

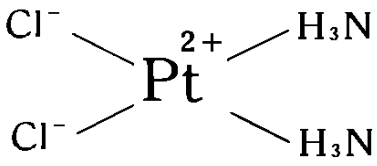


Fig. 4: cis-diamminedichloroplatinum II

Cisplatin is one of the best known alkylating chemotherapeutic agents, and it's mainly used for the treatment of solid tumors, especially testicular cancer, ovarian cancer and the head and neck cancers (Rosencweig M. et al., 1977; Vokes E.E. et al., 1992). It is effective against various types of cancers, including carcinomas, germ cell tumors, lymphomas, and sarcomas. (Dasari S. and Tchounwou P.B., 2014). It is an antineoplastic agent discovered by Rosenberg, that through *in vitro* studies discovered cisplatin as a neutral complex, formed by the dissolution of platinum electrodes in places chloride ammonium (Bloemink JM and J. Reedijk, 1996). Also certain electrolysis products of platinum mesh electrodes were capable of inhibiting cell division in *Escherichia coli* creating much interest in the possible use of these products in cancer chemotherapy. In fact they didn't lead to division, inducing, instead, a damage in DNA replication (B. Rosenberg et al. 1965; Rosenberg B. et al., 1967; Brown S.J. et al., 1994) .

3.1 MECHANISM OF CYTOTOXICITY

Cisplatin has a planar structure (Fig. 4) formed by a central atom of platinum in the oxidation state of +2 to which are linked two chlorine atoms and two NH3 molecules thus obtaining a framework-planar geometry. The molecule exists in two isomers *cis* and *trans* but it is only the first has a strong anti-tumor activity. The mechanism of action of alkylating agents in general is to transfer their alkyl groups to various cell constituents, in particular the most significant target is the DNA (Fig. 5) (Lemke T.K. D.A. and Williams, 2008). These modify the bases of DNA, interfering with replication and transcription and leading to mutations. Cross-linking atoms in the DNA, preventing strand separation for synthesis or transcription, also cause damage. Base mispairing between strands is also induced by alkylation (Luqmani, 2005).

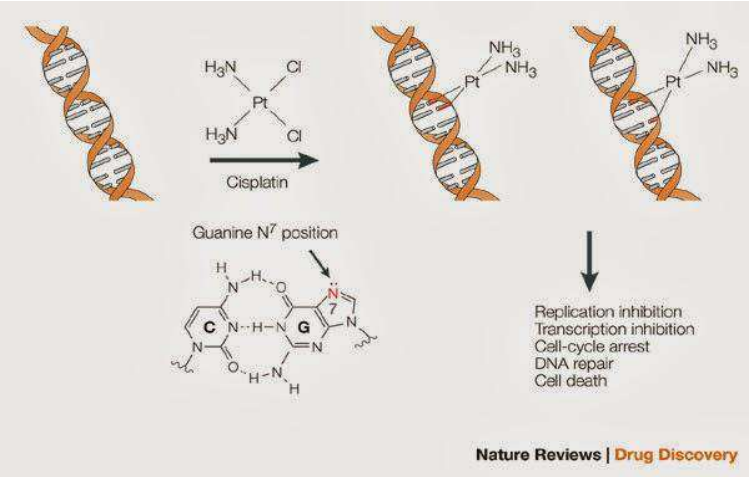


Fig. 5: Main adducts formed after binding of cis-DDP to DNA: 1,2-intrastrand cross-link, interstrand cross-link, monofunctional adduct, and protein-DNA cross-link. The main site of attack of cis-DDP to DNA is N7 of guanine.

The activity of cisplatin is conditioned by the amount of chlorine ions present in the environment: in the blood, in which the concentration of the ions is high, it is mainly present in the neutral form with the two chlorines bonded to the central platinum; in the cell, the decrease of the concentration of the ions causes the outgoing of chlorine atoms, which are replaced by water molecules: the molecule is positively charged and thus can react with nucleophilic groups (DNA, RNA and proteins) (Kartalou M. and Essigmann J.M., 2001).

In DNA, the sites for the binding of cisplatin are mainly the N'-7 atom of guanine, by binding preferably the nitrogen in position 7. Cisplatin forms monofunctional adducts, which may evolve into bifunctional, blocking the replication and/or preventing the transcription (Fig. 5) (Fichtinger-Schepman A.M.J. et al., 1985). The crosslinks created, which may be intrastrand or interstrand,

cause alterations of the double helix structure of DNA and consequently an altered interaction with proteins involved in replication and transcription processes (G. Villani et al., 1994). In particular, preventing or making difficult to the polymerase to bind with DNA, a DNA duplication failure occurs, and this block primarily affects tumor cells, whose feature is a fast replication. In addition to preventing these fundamental processes, cisplatin seems to damage cancer cells by inducing apoptosis (Florea A.M. and Büsselberg D., 2011); also the production of ROS induced by cisplatin can lead to irreversible damage to the cells (Brozovic A. et al., 2010).

The drug is primarily used intravenously for the treatment of ovarian cancer, testicular and advanced bladder cancer. It is primarily eliminated by kidney, and only 10% takes the bile duct. The main side effect is nephrotoxicity, due to the activation of inflammatory mechanisms inside cells, apoptosis, oxidative stress induction and early vasoconstriction that causes ischemic injury. This effect is enhanced in patients already suffering a disease on renal tract level in therapy with other drugs that have the same toxicity. Other side effects are ototoxicity, neurotoxicity and myelosuppression, and alterations in liver function; cisplatin is also a strong emetogenic. There are many precautions in this regard and medications that can help reduce these problems (Lemke T.K. And Williams D.A., 2008). There is, however, another problem associated with the use of cisplatin, common to other chemotherapeutic drugs: the induction of resistance, either intrinsic or acquired, that seriously compromises the efficacy.

Therefore, the occurrence of resistance to platinum is the major problem that undermines efforts to effectively treatment. One approach to overcome this limitation is to elucidate the mechanisms responsible for drug resistance and then develop ways to treat resistance effectively or prevent its occurrence.

3.2 MECHANISM OF RESISTANCE

Despite the development of new targeted anticancer therapies, mechanisms of protection against cytotoxic compounds will continue to act as the main limit to successful treatment of cancer. More knowledge about these resistance mechanisms may help to design strategies to circumvent resistance. In vitro studies suggest that cisplatin resistance can result from mutations and epigenetic events at the molecular and cellular levels causing cancer relapse and failure of treatments. Cisplatin resistance appears to be a mutifactorial phenomenon (Fig. 6), including reduced accumulation of platinum compounds by either active efflux/sequestration/secretion or impaired influx, detoxification by GSH conjugates, metallothioneins and other antioxidants,

increased levels of DNA damage repair (nucleotide excision repair and mismatch repair), changes in DNA methylation status, alterations of membrane protein trafficking as a result of defective organization and distribution of the cytoskeleton, overexpression of chaperones, up- or down-regulated expression of microRNA (miRNA1), transcription factors and small GTPases, inactivation (Shen D.W. et al., 2012).

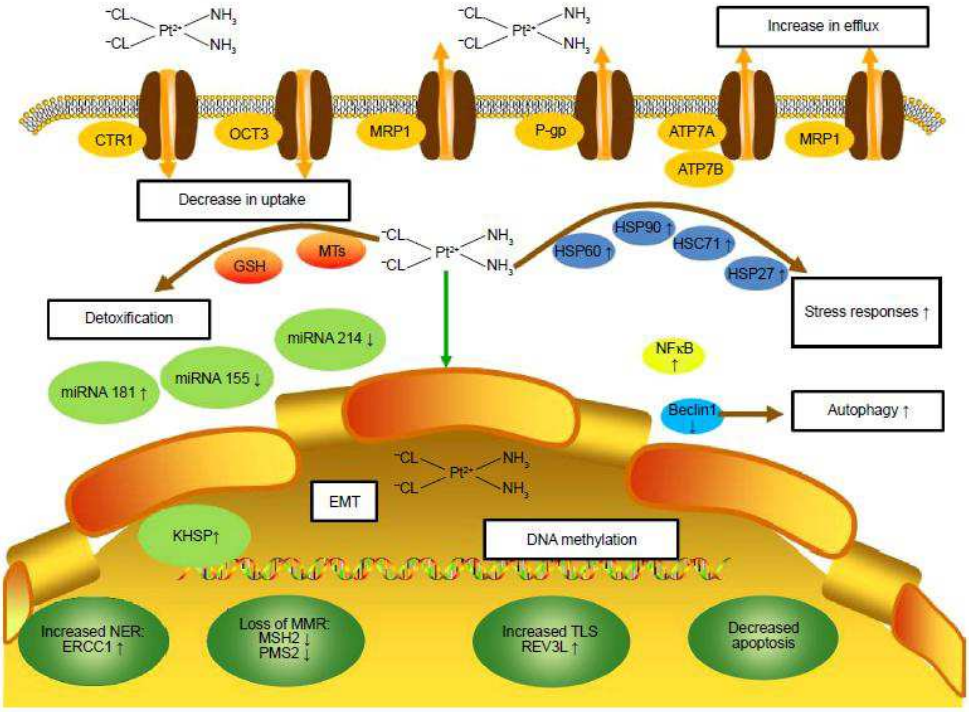


Fig. 6. Cisplatin resistance mechanisms. Many mechanisms may contribute to cisplatin resistance including reduced uptake, increased efflux, increased detoxification, inhibition of apoptosis and increased DNA repair (increased NER, loss of MMR, and increased TLS); alteration in DNA methylation and expression of stress-response chaperones. (Haiyan Zhu et al., 2016).

Reduced drug accumulation

The predominant cause of cisplatin resistance is the reduced effective concentration of intracellular drug (Haiyan Zhu et al., 2016). This can be due to the decrease in uptake, the increase in efflux, and the inactivation by thiol-containing proteins, which results in reduction in cisplatin–DNA adduct formation and ultimately leads to resistance to cisplatin.

The compounds of platinum is taken up within the cell by passive diffusion (not saturable) or through facilitated transport: some copper transporter (CTR1), seem to be involved in the influence of cisplatin (Song I.S. et al., 2004; Holzer A.K. et al., 2006) and could therefore have a role in resistance. The cells with a decreasing expression of these receptors appear to be resistant to

the drug due to their rapid cytoplasmic internalization, consequently to the exposure to the chemotherapeutic drug, limiting further uptake (Safaei R. et al., 2004-2005). Mouse embryonic fibroblasts (MEF) null for CTR1 provide 3.2-fold increased resistance as compared with transfected cells. The other shuttle coppers between the Golgi and the plasma membrane, ATP7A and ATP7B, are instead involved in the efflux of the drug (Safaei R. et al,. 2004; Samimi G. et al., 2003): the copper, in fact, competes with cisplatin for the cellular uptake, but is also able to reduce the outflow of the chemotherapeutic drug, increasing its accumulation and toxicity (Stewart D.J. et al., 1995).

The overexpression of the receptor ATP7B, decreasing the competition between the two elements is associated with a worse prognosis in patients with cancer esophageal level (Higashimoto M. et al., 2003) and squamous cancer cells of the head and neck (Miyashita H. et al,. 2003), suggesting an implication in the resistance. Patients, whose carcinomas expressed high levels of ATP7B, had a significantly poorer prognosis than patients with tumors that expressed low levels of ATP7B (Nakayama K. et al., 2001).

The main protein involved in the effective elimination of the drug from the cell, acting as an efflux pump is the P-glycoprotein, an adenosine 5'-triphosphate (ATP)-binding cassette (ABC) transporters, located on the cell membrane and able to recognize the various types of anticancer drugs, including compounds cisplatin-based (Gottesman M.M. and Ling V., 2006). Its over-expression, observed in resistant cells in particular of ovarian tumors, sarcomas and breast tumors, can determine the failure of chemotherapy and worsen the prognosis (Rodrigues F.F.O. et al., 2008; Hoffmann B.C. et al., 2010).

In addition, other ABC transporters , including multidrug resistance proteins (MRPs), MRP1, MRP2, MRP3, and MRP5, (ATP-dependent pump), might mediate cisplatin resistance by increasing cisplatin export. (Galluzzi L. et al. 2012).

Drug Inactivation by thiol containing species

In the cytoplasm, platinum-based agents become acquo species and can avidly react to intracellular nucleophilic species, such as glutathione (GSH), methionine, metallothioneins (MTs), and thiol-containing proteins. An overexpression of these compounds may lead to resistance through the binding and inactivation of the drug, which subsequently can be expelled from the cell through a series of membrane protein, like outflow pumps GS-X (Siddik Z.H. et al., 2003).

GSH, which is a thiol-containing tripeptide (Glu-Cys-Gly), can bind to cisplatin, through adduct formation, to prevent cisplatin from binding to DNA and other targets, quench proapoptotic reactive oxygen species. Moreover it helps to maintain the redox environment while maintaining reduced sulfhydryl groups (Haiyan Z., 2016).

Methionines and metallotionine are low-molecular-weight thiol-containing proteins comprised of several cysteine and aromatic amino acid residues (Siegsmund M.J. et al., 1999). MTs contain sulfide groups and are involved in the zinc and copper homeoastasis and detoxification (Siddik Z.H. et al., 2003). They can confiscate the largest amount of cisplatin; the presence of these compounds is linked to the resistance in ovarian carcinomas, cervical and lung (Kavanagh J.J. et al., 2005; Li M. et al., 2009). Therefore the association of MT levels with cisplatin resistance may be tissue specific and may play a minor role depending on the cellular environment.

Increased repair of platinum-DNA adducts

The well known mechanism of cisplatin is the alteration in the structure of the DNA molecule, then, the extent of the damage and the amount of DNA damage determine survival or cell death. (Cobo M. et al., 2007). There are several mechanisms of DNA repair, among which nucleotide excision repair (NER) and mismatch repair (MMR) are the predominant DNA repair mechanisms. NER is an ATP-dependent multiprotein complex that recognizes the binding induced on DNA by 1,2-intrastrand cross-links, and subsequently excises the DNA that includes as 27- to 29-base-pair oligonucleotides. The gap that remains is then filled by DNA polymerase (Chaney S.G. and Sancar A. J., 1996). In particular, many studies have shown that an high gene expression of the excision repair cross-complementation group 1 (ERCC1), is a single-strand DNA endonuclease and forms a tight heterodimer with ERCC4 to incise DNA on the 5' side of bulky lesions such as DNA–cisplatin adducts and is related to the increase in the DNA repair capacity (Galluzzi L. et al., 2012), in particular in the tumor ovary resistant cells (Selvakumaran M. et al., 2003). In addition, cell lines that develop resistance in vitro after exposure to cisplatin chemotherapy were found to have increased expression of ERCC1 (Ferry K.V. et al., 2000). Other repair mechanisms can be the HR (homologous recombination repair), capable of repairing the damage created at the level of the double helix, due to the intersection of the platinum through inter-chain cross bonds to DNA and the BER (base excision repair). Studies have also shown a greater tolerability to DNA damage, (Kelland L.R., 2000), caused by the MMR (mismatch repair), a control mechanism of mismatches,

ATP dependent, generated during DNA replication, which usually acts in case of the DNA polymerase errors (Martin LP. et al., 2008).

The mismatch repair system consists of three steps and involves at least five: MLH1, MSH2, MSH3, MSH6, and PMS2; the decrease of this system can contribute partly to the development of tolerance in case of DNA damage; and it has been observed that tumor lines, with a deficiency of MMR, are 2-3 fold more resistant to cisplatin treatment (Stojic L. et al., 2004). Epigenetic silencing of MMR seems to occur more often through Mut L homologue 1 (hMLH1) promoter hypermethylation, as it has been shown in ovarian, endometrial, gastric, and colorectal carcinoma, among others (Peltomaki P., 2003; Geisler J.P. et al., 2003; Bignami M. et al., 2003).

Genetic alteration and apoptosis inhibition

Cisplatin-induced apoptosis is essential for the anticancer effect of cisplatin. Cisplatin stimulates apoptosis by triggering the extrinsic death receptor pathway or the intrinsic mitochondrial pathway. The resistant cells show a decrease of apoptotic mechanism: this seems to be caused by an increase of the genes, proteins and pathways of anti-apoptotic signal and/or a reduction of those pro-apoptotic (Scatchard K. et al,. 2012). Multiple proteins such as the Bcl-2 family proteins and p53 and several signaling pathways including mitogen-activated protein kinase (MAPK) pathway and nuclear factor-κB (NF-κB) pathway are involved in the extrinsic and intrinsic apoptosis pathways. Dysfunction of these proteins and signaling pathways may lead to the development of cisplatin resistance (Fig.7).

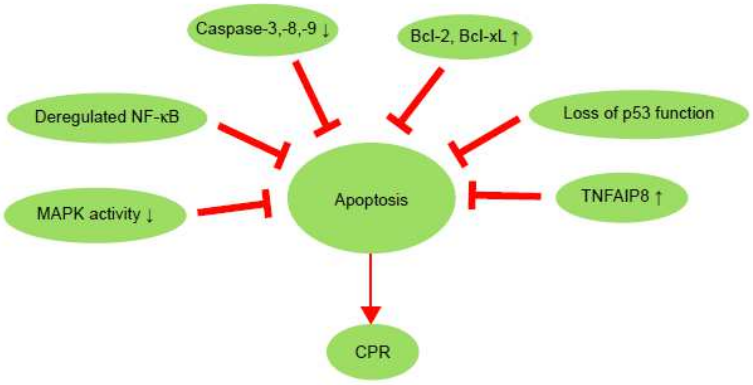


Fig. 7: Multiple molecules and signaling pathways that inhibit apoptosis are involved in cisplatin resistance. (CPR, cisplatin resistance; MAPK, mitogen-activated protein kinases; NF-κB, nuclear factor-κB; TNFAIP8, tumor-necrosis-factor-α-induced protein 8) (Haiyan Z., 2016).

In fact, one of the main genes involved in the regulation of the cell cycle, apoptosis and proliferation, is the tumor suppressor p53. It has been observed that its alteration is associated with cisplatin resistance (O'Connor P.M. et al., 1997), moreover other studies reported that the frequency of protein mutations is greater in resistant ovarian cancer cell lines than the sensitive counterpart (Kigawa J. et al., 2001).

Another important pathway is the PIK3/Akt/mTOR, key regulator in cell survival and involved in all aspects of tumor biology as the transformation of cells, growth, proliferation, migration, apoptosis and DNA repair. It has been observed that an increase of activity contributes to the drug resistance through various mechanisms such as, for example, inhibition of apoptosis (Saal L.H. et al., 2007; Liu C. et al., 2009).

Other proteins involved in resistance are those of the Bcl-2 family, which participate in the apoptosis, acting on the complex that regulates the permeability of the external mitochondrial membrane (MOMP). The family contains proteins pro and anti-apoptotic: there are evidences that show as an alteration of their balance with a decrease of the first and an increase of the second may lead to a reduction of the effectiveness of the drug in some types of cancer such as ovarian cancer and lung cancer (Han J.Y. et al., 2003; Williams J. et al., 2005).

MAPKs play critical roles in the complex intracellular signaling network, which regulates gene expression in response to various extracellular stimuli. (Dean M. et al., 2005). The correlation between MAPK activation and CPR has recently been recognized. CPR cancer cells often have reduced MAPK activity; inhibition of JNK, p38 kinase, or ERK attenuates cisplatin-induced apoptosis and cell death (Basu A. and Tu H., 2005).

Finally numerous in vitro and in vivo studies, have shown that constitutive activation of NF-κB, gene involved in immunoregulation, inflammation, growth regulation, apoptosis, and carcinogenesis, inhibits chemotherapy-induced apoptosis in different types of cancer, including cervical cancers (Venkatraman M. et al., 2005).

3.3 CISPLATIN AND MITOCHONDRIA

The cisplatin due to its alkylation capacity is able to form adducts with the nucleophilic species of DNA, RNA and proteins (Fichtinger-Schepman A.M.J., 1985), in particular it seems that many consequences of its cytotoxicity are due to binding to the nuclear DNA (nDNA). The only damage to nDNA, however, does not explain the high cytotoxic effect of the chemotherapy drug nor its tissue specificity (Marullo R., 2013). In this regard, recent studies have shown that the cisplatin

intracellular concentration, binding to nuclear DNA, is about 5-10%, thus, it is significant the drug concentration that can interact with other cellular components, such as proteins, RNA, endoplasmic reticulum, lysosomes (Mandic A. et al ,. 2003), including the mitochondrial DNA (mtDNA) (Arnesano F. et al., 2008). Mitochondria contain their own DNA (mtDNA) that is transcribed and translated to synthesize 13 proteins of the mitochondrial electron transport chain (Fernandez-Silva P. et al., 2003). Mitochondrial DNA has no efficient repair systems like nuclear DNA and therefore is more sensitive to oncogenic mutations and insults of toxic substances (Preston J.J. et al., 2001). Also the adducts that are formed between cisplatin and the mitochondrial DNA have a low spin speed (Olivero O.A. et al., 1997). Recent literature has shown that cells depleted of mitochondrial DNA show significant resistance to cell death mediated by a range of chemotherapeutic agents (Park et al., 2004; Montopoli et al. 2009). A mechanism that could explain the effect on the mitochondria in the treatment with cisplatin, involves the formation of ROS: it has been observed that exposure to chemotherapy increases the expression of reactive oxygen species, consequently, to a decrease in mitochondrial respiratory chain (Santandreu F.M. et al., 2010), linked to respiratory partial decoupling (Stöckl D. et al., 2007).

Also antioxidants counteract the cytotoxicity induced by cisplatin in tumor cells, showing that oxidative stress is important for its antiproliferative activity (Santandreu F.M. et al., 2010). The radical species produce damage either directly or by inducing the activation of pro-apoptotic intracellular pathways (Madesh and Hajnóczky M.G., 2001). In fact, the increase in the intra-mitochondrial calcium, dysregulated during apoptosis induced by the drug, interacting with the ROS products, allows the opening of the PTP (permeability transition pore), which causes the rupture of the outer mitochondrial membrane and therefore apoptosis (Desagher S. and Martinou J.C., 2000; Harris M.H. and Thompson C.B., 2000; Korsmeyer S.J. et al., 2000). Moreover the opening of the PTP results in the release of cytochrome c and consequently in the activation of caspases (Duchen M.R., 2000; Fiskum G., 2000; Hajnóczky G. et al., 2000). Even the only alteration of the channels anionic-voltage dependent VDAC, mitochondrial porins ion channels, localized in the outer membrane, has been indicated as a mechanism able to control the pro-apoptotic factors, without the complete opening of PTP (formed by VDAC and ANT), in particular by mediating the release of cytochrome c. These channels can be a cisplatin target, they have two cysteines in their domain and two methionines and the chemoterapeutic drug can form adducts with cellular proteins (K.J. Cullen et al., 2007). The closure of these porins may cause a defect in the exchange between ATP and ADP: the result is the inhibition of the F0F1-ATPase, which causes

hyperpolarization and release of cytochrome c after the loss of integrity of the outer membrane (Vander Heiden M.G. et al., 1999). Another mechanism that appears to be implicated in the activation of apoptosis is mediated by a family of proteins, the Bcl-2 (Bax and Bak) which controls the permeability of the outer mitochondrial membrane. The Bcl-2 family includes not only pro-apoptotic factors but also inhibitors of cell death (Bcl-2, Bcl-Xl) (Oltvai Z.N. and Korsmeyer S.J., 1994; Reed J.C. et al., 1996). The mechanism by which apoptosis on the part of these proteins is induced is still little known but it seems that, consequently to the stimulus by apoptotic agents such as cisplatin, pro-apoptotic factors can interact with one another and form homo- and hetero-dimers: in particular the formation of homodimers of Bax promotes apoptosis and this is opposed to hetero-dimerizations of Bcl-2 / Bax (Oltvai Z.N. and Korsmeyer S.Y., 1994).

Once dimers are created, these are transported in the MME, causing an alteration of the potential and the creation of large pores (MOMP) (Waterhouse N.J. et al., 2002), which facilitate the release of pro-apoptotic factors , normally present in the inter-membrane space, such as cytochrome c, in the cytoplasm level. To confirm the role of the alteration of these proteins in the cisplatin activity, was observed that high levels of Bcl-2 in ovarian cancer cells expression seem to confer resistance to chemotherapeutic treatment (Eliopoulos A.G. et al., 1995), as well as an increase of Bcl-Xl (Gauthier E.R. et al., 1996; Han Z. et al., 1996). However, the increased expression of the protein Bak is common before an apoptotic event.

Cytochrome c, implicated in the mechanism of programmed cell death, is a soluble protein and positively charged, located in the intermembrane space (Sancho-Martinez S. et al., 2012). Citocrome c is involved in the mitochondrial respiratory chain through interaction with the complex III and IV (Hatefi Y., 1985; Mathews F.S., 1985), once translocated in the cytosol during apoptosis, it interacts with other proteins and activates Apaf-1, leading to the formation apoptosome (Hill M.M. et al., 2004). This, through ATP hydrolysis, recruits and activates pro-caspase 9 (Zou H. et al., 1999), which cleaves and actives the pro-caspase. These effector caspases, like caspase-3, activate the endonuclease (CAD), usually complexed with its inhibitor (ICAD) (Sakahira H. et al., 1998); once activated, the CAD degrade chromosomal DNA, causing chromatin condensation, either through reorganization of the cytoskeleton and the disintegration of the cell into apoptotic bodies (Nunez R. et al., 2010). A study of intestinal epithelial cells, revealed a positive correlation between the mitochondrial density and the sensitivity to cisplatin: the reduction of mitochondrial concentration, raising the threshold for the onset of an event

apoptotic or necrotic, it gives the cell an advantage against death induced by chemotherapy (Qian W. et al., 2005).

Furthermore mitochondrial damage by cisplatin has been increasingly studied as a mediator of systemic toxicity such as gastrointestinal toxicity, ototoxicity (Devarajan P. et al., 2002) and nephrotoxicity (Park M.S. et al., 2003). Indeed, mitochondria are thought to be a major target for cisplatin in cancer cells (Tacka K.A. et al., 2004) and alterations in mitochondrial function (reduced mitochondrial respiration and ATP production) have been investigated in cancer cell resistance (Harper M.E. et al., 2002).

4. REGULATION OF MITOCHONDRIAL DYNAMICS

Mitochondria are intracellular organelles involved in several cellular processes, they produce the energy currency (ATP) through respiration and regulate cellular metabolism. They also constitute a crucial structure for the control of apoptosis and the regulation of intracellular calcium (Sukhorukov V.M. et al., 2012); moreover they are involved in many other activities such as gluconeogenesis metabolism of fatty acids, steroid hormone and porphyrin synthesis and interconversion of amino acids. The ability to perform various functions is given by their ability to change shape (may be present in fact in spherical form or elongated). Their distribution within the cell is functional: although they are present in many sites, mitochondria tend to concentrate where there is a high demand for energy or where there is the need to perform the metabolic activity (Anesti V. and Slide L., 2006).

Mitochondrial dynamic is request for different mitochondrial functions and in literature, it has been demonstrated to be involved in cytotoxicity of chemotherapeutic drugs (Kong B. et al., 2015).

4.1 MITOCHONDRIAL METABOLISM AND MORPHOLOGY

Mitochondria are elongated structures that are surrounded by a double membrane each formed by a phospholipid bilayer. The two membranes are quite distinct in appearance and in physico-chemical properties, thus determining the biochemical function of each membrane (Pernas and Scorrano, 2016).

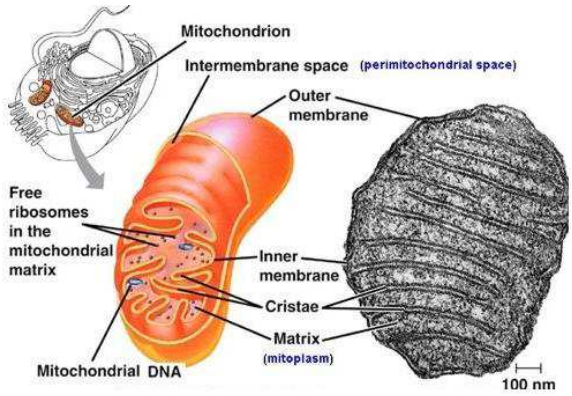


Fig.8: Mitochondrial morphology and structure. (http://www.federica.unina.it/smfn/citologia-ed-istologia/perossisomi-mitocondri/).

The outer membrane (OMM) completely encloses the mitochondrion, defines the boundary with the cytosol and is structurally similar to the plasmatic membrane. It is mainly composed of lipids (50%) and from proteins, the most abundant are the porins: these form broad channels which permit entry to proteins up to 5000 (ATP,NAD,CoA,ions,nutrients), and are able to open and close to reversibly on the basis of the conditions of the cell. On the contrary, the inner membrane (IMM) encloses and convolutes into the mitochondrial matrix, forming cristae, which develop inside the organelle and modify their structure and shape according to the energy demand. The inner membrane is freely permeable only to oxygen, carbon dioxide, and water. (NelsonD.L. and Cox M.M., 2002). The IMM is formed mainly from proteins and cardiolipin, which, being formed by four chains of fatty acids, reduces the permeability to protons, thus creating an electrochemical gradient between the space inter-membrane and the matrix, important for the transduction of energy. In space inter-membrane there are proteins that participate in the apoptotic process such as cytochrome c and b and that have an important role in oxidative phosphorylation (NelsonD.L and Cox M.M., 2002).

The matrix contains a lot of water-soluble proteins and different enzymes, as well as ribosomes and mtDNA (it encodes a number of proteins involved in the transport of the electrons, process localized in the mitochondrial cristae). The matrix is the site in which occurs mainly the oxidation of pyruvate and fatty acids to form CO2 and reduction of coenzymes NAD and FAD FADH and NADH, while in the internal mitochondrial membrane occurs the electron transport to oxygen and is present the complex F0F1 or ATPsintasi. The cells use two processes for the ATP production, a key molecule for their functionality: glycolysis and oxidative phosphorylation. The glycolysis occurs in the cytosol and is constituted by a series of reactions that lead to the cleavage of a glucose molecule into two phosphoglyceraldehyde molecules and these metabolites into two

pyruvate molecules with the formation of two ATP molecules and two NADH for each glucose molecule consumed; all this can occur in the absence of oxygen (glycolysis anaerobia) with the final transformation of pyruvic acid in lactate, or in the presence of oxygen (aerobia), with complete oxidation of pyruvic acid in water and carbon dioxide in the Krebs cycle. The third phase of respiration, i.e. the oxidative phosphorylation, takes place at the level of the internal mitochondrial membrane and leads to the production of ATP through a system consisting of five complexes (Fig. 9), four of which are oxidoreductases: the complex I (NADH dehydrogenase), the complex II (succinate dehydrogenase), the complex III (cytochrome C reductase) and complex IV (cytochrome c oxidase); then there is the ATP synthase or V complex where there is the real synthesis of ATP, starting from ADP molecules. Three of the four complexes of oxide reductase, function as proton pumps by coupling the electron transport of hydrogen of NADH, up to oxygen, final acceptor, with the transfer of protons from the mitochondrial matrix to inter-membrane space. Then, it generates a proton gradient that is used by the ATP synthase to catalyze the phosphorylation of ADP to ATP, bringing as final result to the obtainment of 36 molecules of ATP (Stock D. et al., 2000; Boekema E.J. and Braun H.P., 2007).

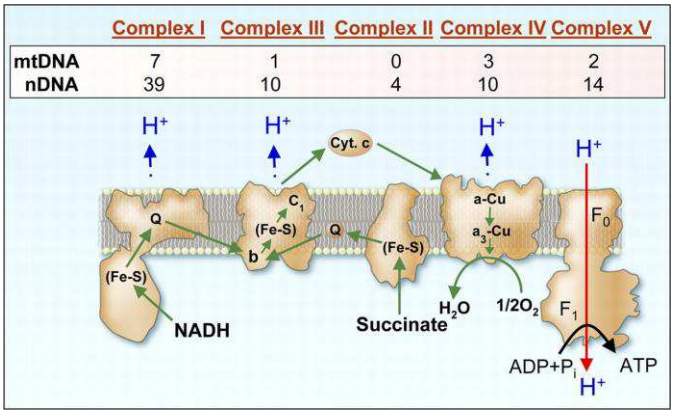


Fig.9: The schematic depiction of the five complexes (I-V) of the respiratory chain localized in the lipid bilayer of the internal mitochondrial membrane. (Scarpulla, 2008).

The mitochondria cannot be created ex novo, they originate from mitochondria already formed. Mitochondria are dynamic organelles interconneted to form a network. The network will be changed in response to numerous stimuli both endogenous and exogenous e.g. the level of nutrients, leading to a shift toward the fusion or fission mitochondrial (Hales K.J et al., 2010); the adjustment is controlled sophistically during the cell cycle and defects of this can be connected to human pathologies (Westermann B., 2010) as Parkinson (Laar V.S.V. and Berman S.B., 2009), diabetes (Liesa M. et al., 2009), cancer (Grandemange S. et al., 2009) and Alzheimer (Zhu X. et al.,

2013). Some evidence emphasizes a link between mitochondrial bioenergetic functions and its architecture. In particular, besides its critical implication in mitochondrial quality control, mitochondrial dynamics has been recently linked to energy demand and supply balance.

4.2 KEY REGULATOR IN MITOCHONDRIAL METABOLISM

Mitochondria play a central role in bioenergetic and metabolism of amino acids and lipids, since they host β-oxidation, Kreb's cycle, and oxidative phosphorylation (OXPHOS). In normal condition, a large amount of ATP is required for cells replication: glucose participates with two ATP molecule synthesis through glycolysis and up to 36 ATPs through its complete catabolism by the TCA cycle and OXPHOS (oxidative phosphorylation) (Wu R, Racker E., 1959).

The glycolysis is the central pathway for the glucose catabolism. In addition to obtaining a limited amount of energy, glycolysis is a good source of intermediates for tracks biosynthetic (amino acids). The final product, two molecules of pyruvate, maintains the greater part of the energy from the departure of the glucose that will be formed, under aerobic conditions, from the most effective redox reactions of the citric acid cycle and by the oxidative phosphorylation (Nelson D.L. and Cox M.M., 2002). An alternative catabolic pathway that uses the glucose (phosphorylated) is represented by the pentose phosphate pathway. This pathway in addition to constituting an alternative branch of the glycolysis first reactions, supplies important precursors, tracks anabolic activities of nucleic acids and produces reducing power in the form of NADPH (fundamental in the biosynthesis of fatty acids) (Ramos-Montoya A, et al. 2006).

Acetyl-CoA is the final product in which converge the main catabolic routes (β-oxidation of fatty acids, glycolysis, oxidation of amino acids). This compound is the main source that supplies the citric acid cycle. The main functions of the citric acid cycle are supplying the respiratory chain of fuel to synthesize ATP (in the form of reducing power, NADH and FADH2) and constitute a sort of compensation reservoir of metabolites whose flows are involved in all the main metabolic pathways (biosynthetic and degradative) (Nelson D.L. and Cox M.M., 2002).

Being at the heart of the entire intermediate metabolism, in normal conditions the dynamic equilibrium between the reactions that remove intermediates by cycle and those that instead supply the cycle, is carefully preserved. If, in particular situations, a considerable quantity of intermediates were subtracted to the citric acid cycle and used as precursors in other ways, the balance would be quickly restored by anaplerotic reactions. These reactions are regulated to maintain Kreb cycle, involved to sustain the non essential amino acids biosynthesis and fatty acids,

compatible with the needs of the cell. This occurs through the conversion of pyruvate to oxaloacetate by pyrivate carboxylase, and through glutamine metabolism (from glutamine to α-ketoglutarate). In addition to glucose, glutamine is catabolized in appreciable quantities for most mammalian cells growing in culture. Therefore glucose and glutamine both supply carbon, nitrogen, free energy, and reduce equivalents necessary to support cell growth and division (Nelson D.L. and Cox M.M., 2002).

In the final phase of the aerobic metabolism the electron transporters (NADH and FADH2) are oxidized releasing protons and electrons. The electronic transfer along the mitochondrial respiratory chain ends with the oxygen as the final acceptor, which is reduced to form water. The high energy released during the process is coupled to the transport of protons to pass through the inner mitochondrial membrane (impermeable to charged species).

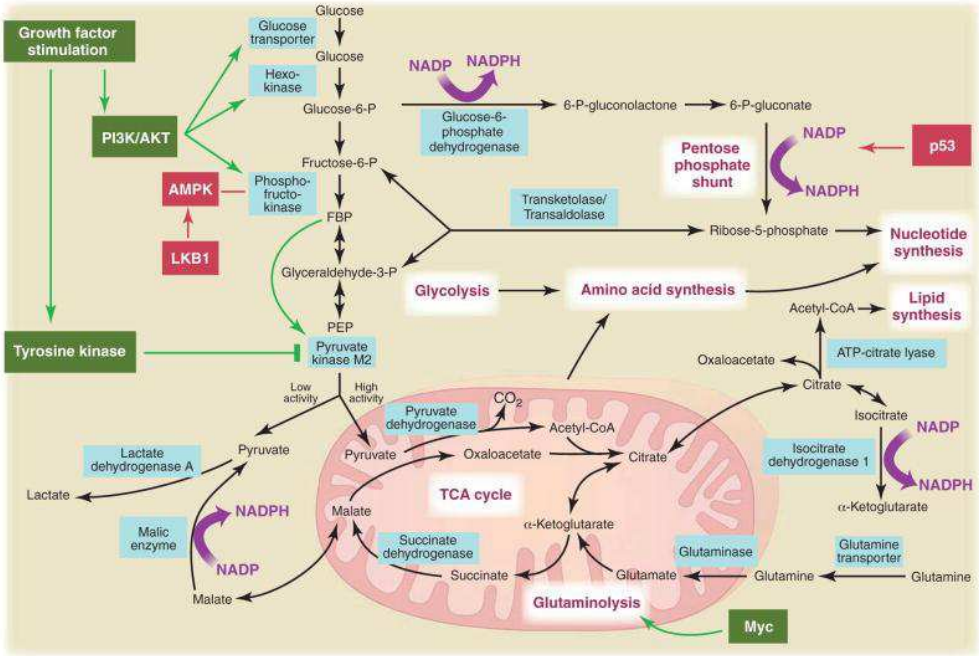


Fig.10: Metabolic pathways active in proliferating cells. (Vander Heiden M.G. et al., 2010).

4.3 KEY REGULATOR IN MITOCHONDRIAL FUSION AND FISSION

The mitochondrial morphology is determined by a balance between the fusion and fission organelles, in which the mitochondria form elongated tubules that continually divide and fuse to create a dynamic network interconnected (Chen H. et al,. 2003). Even if the importance of the dynamism of the mitochondrial network is recognized, many aspects of fusion and fission remain unclear. However processes seem to improve the ability to control efficiency and stability of the mitochondria and increase the oxidative capacity (Hoitzing H. et al., 2015). Between the steps

G1/S mitochondria create a single tubular network of large size and are in the hyperpolarized and highly coupled form: this leads to an increase in the production of energy for preparing the cell to the high expenditure necessary for the process of DNA synthesis (Mitra K. et al., 2009). Instead in the course of following steps S, G2 and M, the hyperfused mitochondrial network disassembles and becomes very fragmented; this massive breakdown is required for the process of mitosis and to facilitate the equitable division of mitochondrial content between daughter cells (Qian W., 2013). It has been widely demonstrated that the changes in the activity of the proteins of fusion and fission can cause alterations in the mitochondrial shape. In fact, when both fusion and fission are inhibited through modulation of these proteins, the mitochondrial morphology is similar to that observed in a basal state, thus demonstrating that the shape is the result of a balance between these two processes (Chen H. et al., 2003).

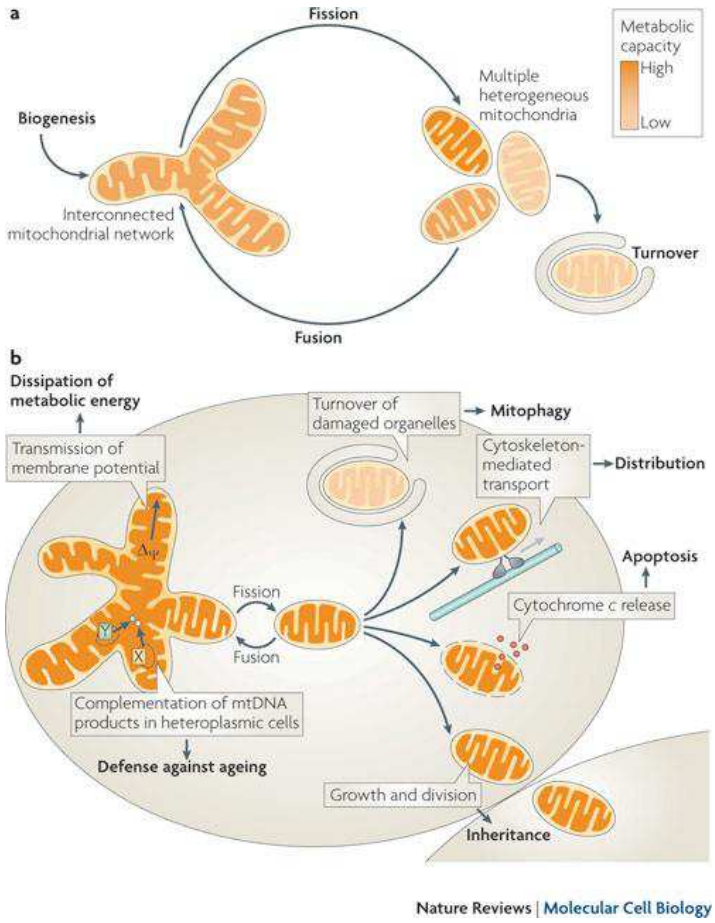


Fig. 11: Mitochondria life cycle. a) Mitochondrial morphology, mitochondrial fusion and fission alternate during the life of the mitochondria. The mitochondria which have a reduced metabolic capacity, are removed. b) The mitochondrial dynamics are involved in the modulation of important biological functions. (Westermann B., 2010).

However the mitochondrial morphology depends not only by the components of mitochondrial dynamic (the proteins involved in mitochondrial fusion or fission processes), but may also depend on other distinct proteins or mediators such as cytoskeleton proteins or other unknown cellular components. Mitochondria are able to respond to cellular signals by remodeling their morphology. In this regard, changes in the activity of mitochondrial fusion proteins can modulate the distribution and circulation of this organelle (Santel A. and Fuller MT., 2001). Thus, the mitochondrial elongation after starvation is necessary to maintain ATP production and to support mitochondria-dependent cellular growth (Gomes L.C. et al., 2011).

This leads to an increase in efficiency of bioenergy, required to optimize the production of ATP when in presence of limited nutrients available. On the other hand, the mitochondrial fragmentation, a division event that produces one or more daughters mitochondria, is associated with a depleted ATP production (Jheng et al., 2012), an increase in the mitochondrial uncoupling and nutrients storage, in order to avoid wastage of energy. Through the fusion and fission events, mitochondria share membranes, solutes, metabolites and proteins, as well as the electrochemical gradients.

FUSION:

At the molecular level, mitochondrial fusion is a two-step process, requiring the coordinated fusion of both outer mitochondrial membranes (OMMs) and inner mitochondrial membranes (IMMs) by separable sequential events (Malka et al., 2005; Song et al., 2009). In mammals, this process depends on distinct mitochondrial sublocalization of three fusogenic proteins: the mitofusins 1 and 2 (Mfn1 and Mfn2), located on the outer mitochondrial membrane and involved in outer membrane fusion (Eura et al., 2003) and the IMM-located optic atrophy 1 (Opa1) (Olichon et al., 2002) involved in inner membrane fusion. This fusion event allows a transfer of information via exchange of mtDNA, proteins, lipids, and metabolites (Jakobs S, et al.2003).

However it is not yet well elucidated how the outer and inner mitochondrial membranes coordinately fuse neither the factors that signal to a particular region of the mitochondrial membrane to start the tethering and fusion and then distribute in a correct manner the mitochondrial content. Thus, the study of the proteins controlling mitochondrial fusion and their activity is important.

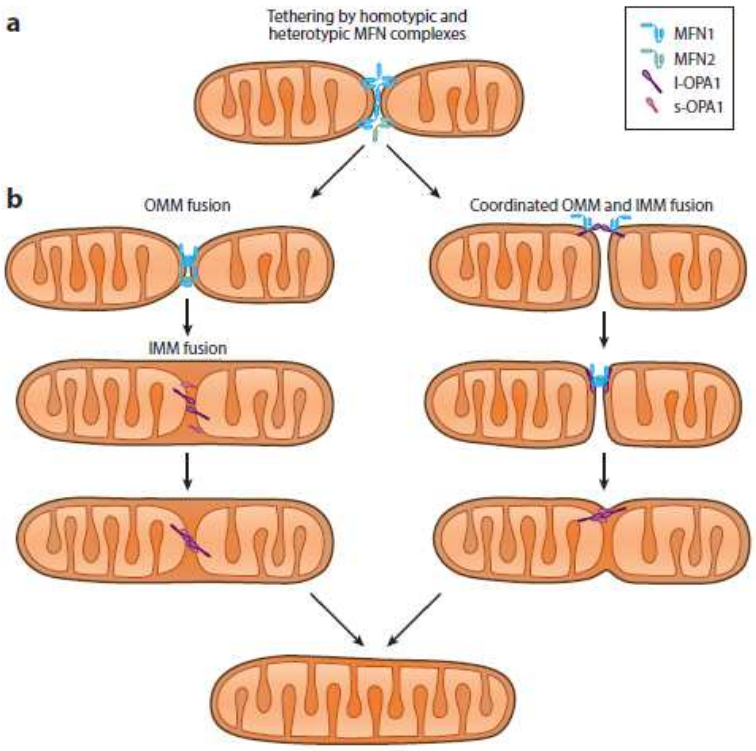


Fig.12: Schematic representation of OMM fusion that may occur in a step distinct from IMM fusion. (Pernas and Scorrano, 2016).

Two mitochondria must come into close contact for the initiation of fusion (Meeusen S. et al., 2004) (Figure 12a). Then, the dynamin-related OMM proteins Mitofusin 1 andMitofusin 2, that hydrolyse GTP, form homotypic or heterotypic complexes (Song Z, et al., 2009) between two different mitochondria (Figure 3) and dimerize via their coiled domain, allowing mitochondrial tethering and fusion.

MITOFUSIN:

As demonstrated by gain- and loss-of-function studies, both Mfn proteins regulate mitochondrial fusion, although at different stages (Bach et al., 2003; Chen et al., 2003, 2005; Santel et al., 2003). Indeed, Mfn1 mediates mitochondrial docking and fusion more efficiently than Mfn2, probably due to its higher GTPase activity (Ishihara et al., 2004). Moreover, Mfn1 but not Mfn2 is required to mediate Opa1-driven mitochondrial fusion (Cipolat et al., 2004). When overexpressed, each protein is able to rescue the loss of the other and promote fusion (Chen et al., 2003). The MFN2 has multiple roles, whether or not related to fusion, and partly due to its tissue specificity. For example, it has been identified at the level of the endoplasmic reticulum, where it seems to control the morphology and the binding to the mitochondria (de Brito and Scorrano, 2008). It is also involved in the oxidative metabolism, in cell cycle, and in cell death; this

could explain the greater involvement of MFN2 in pathological processes (for example its mutation may lead to neurodegenerative disease of Charcot-Marie-Tooth type 2°) (Zuchner S. et al., 2004).

OPTIC ATROPHY 1 (OPA1):

OPA1 is required for fusion of the inner membrane, in a dependent-membrane potential manner, mtDNA maintenance and cristae morphology (Frezza C, et al., 2006). The role of this protein in cristae organization could be mediated by the ATP synthase whose oligomerization appears to have an impact on inner membrane curvature and cristae biogenesis.

OPA1 is located in the space intermembrane in its soluble form, or is linked to the internal mitochondrial membrane (Akepati V. R. et al., 2008). There are at least eight isoforms of OPA1, produced by alternative splicing and proteolytic processing by mitochondrial proteases which create the molecular structures of OPA1 distinguished in long and short form (Ishihara N. et al., 2006). Isoform 1 (contains only exon 4) and isoform 7 (contains exon 4 and 5b) are the dominantly expressed forms in human and mouse cells. These distinct isoforms have the ability to interact between them and, even if the function of each isoform is not clear, it seems necessary to the fusion of mitochondria the combination between a short form and a long one (Song Z. et al., 2007). In fact OPA1 is also regulated by proteolytic cleavage, which decreases the amount of long OPA1 isoforms and increases the amount of the short ones. In a Western blot analysis, human or mouse OPA1 is detected as five distinct bands between 100 and 80 kDa. The two bands showing a higher molecular mass (known as long isoforms) are thought to be a mixture of the translation of alternative splicing mRNA isoforms 1, 2, 4, and 7. The three bands showing a lower molecular mass are considered the products of proteolysis of the long isoforms and also the product of translation of splice isoforms 3, 5, 6, and 8. Despite being essential to this process, some studies have identified that an overexpression of OPA1 leads to a fragmentation of the mitochondrial network (Arnoult D. et al., 2005; Griparic L. et al., 2007). This may be due to an alteration of the proportion between the endogenous isoforms and oligomers, which determines, consequently, failure to control the mitochondrial morphology and therefore the fragmentation. Studies also report that a decrease of OPA1 results in the activation of apoptosis and leads in addition to mitochondrial cristae altered formations (Arnoult D. et al., 2005). The ability of the protein to retain the morphology of the cristae is due to the soluble isoform which creates a complex ,through self-oligomeric interactions with the long forms of OPA1 anchored at the level of the MMI, this

determines the magnitude of the joints at the level of the cristae and the amount of cytochrome c released during apoptosis (Frezza C. et al., 2006; Yamaguchi R. et al., 2008).

FISSION:

Fission is the other process used by mitochondria to respond to cytosolic needs and to change its functionality, a division event that produces one or more daughters mitochondria. Fission requires the coordination of cytoplasmic, cytoskeletal, and organellar elements and consists of three key steps: (a) marking of a fission site (Figure 13), (b) assembly of cytosolic dynamin related protein 1 (DRP1) dimers and oligomers into a spiral structure around mitochondria (Figure 13b), and (c) GTP hydrolysis and DRP1 helix constriction that split the mitochondrion (Figure 13c) (van der Bliek A.M., et al., 2013).

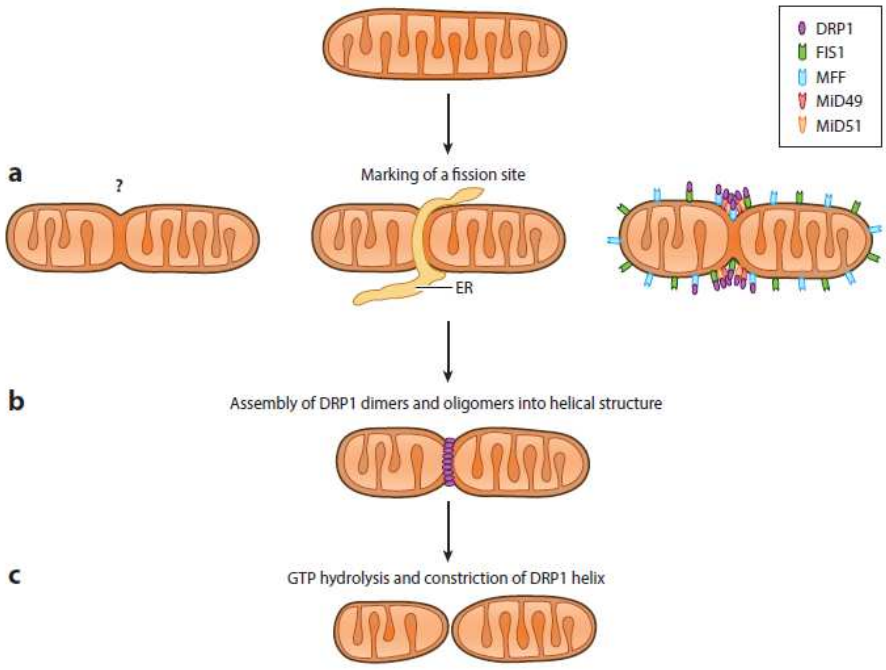


Fig.13: Mitochondrial fission is coordinated by DRP1, mitochondrial adaptors, and cytoplasmic elements. (Pernas and Scorrano, 2016).

Multiple receptors can recruit DRP1 to mitochondria; four mitochondrially localized adaptor proteins have been identified: mitochondrial fission factor (MFF);mitochondrial dynamics proteins of 49 kDa and 51 kDa (MiD49 and MiD51; the latter is also known as Mief1); and fission 1 (FIS1). However, the main proteins involved in the process of mitochondrial fission are the dynami-related protein 1 or DRP1 and fission protein homolog 1 or FIS1 which, during the fission process, localize at the level of the OMM.

DINAMIN RELATED PROTEIN 1 (DRP1):

DRP1 is a protein GTPase, GTP hydrolysis is required to obtain the mechanical force to ensure that the oligomers of the protein, once formed a spiral around the mitochondrial tubule in the cleaving point, have the capacity to tighten it and then return to finish the cleavage (Hoppins S. et al., 2007; Ingerman E. et al., 2005). Mutations at the level of the site of GTP binding lead to the formation of mitochondria extremely long and interconnected due to lack of fulfillment of the process (Yoon et al., 2001). Unlike the proteins described above, Drp1 presents no transmembrane domains, and usually is located in the cytoplasmic level; to carry out its activities at the level of the mitochondria or peroxisomes requires interaction with a protein anchored on the membrane of these: FIS1 (James D.I. et al., 2003; Yoon Y et al. 2003). Drp1 undergoes numerous modifications post-translational, and its effects on the mitochondrial dynamics are mainly controlled by its phosphorylation. In particular it can be phosphorylated at the level of the serine 616 through the complex Cdk1/CyclinB that induces the fission stimulating translocation into the mitochondria and the link with other proteins involved in the process. Instead the phosphorylation of serine 637, carried out by protein kinase A (PKA), leads to a decrease in the GTPase activity with consequent inhibition of fission (Chang C.R. and Blackstone C., 2010).

HUMAN-FIS1:

FIS1, is located mainly at the level of the outer membrane of the mitochondria: its structure C-terminal is essential for the mitochondrial localization, while the N-terminal part is needed to make future fission. FIS1 plays a role in recruiting DRP1 in certain cell types and under specific physiological conditions (Loson O.C et al., 2013), possibly in an isoform-specific manner. Its over-expression is the cause of the mitochondrial fragmentation, while its repression leads to the elongation, confirming its role as a limiting factor in the dynamics of the fission (James D.I. et al., 2003; Stojanovski D. et al., 2004); contrary to DRP1, to which also an over-expression does not alter the network. An increase of the expression is also involved in apoptosis, causing the release of cytochrome c; while its reduction can lead to resistant phenotypes to this process (Y.J. Lee et al., 2004). The fusion and fission usually possess a mutual balance, for example the inhibition of DRP1 expression leads to a down-regulation of fusion proteins such as OPA1 and the mitofusins (Ishihara N. et al., 2009).

DRP1 RECEPTORS:

Studies have shown that other multiple mitochondrially localized receptors can recruit DRP1 to mitochondria: mitochondrial fission factor (MFF) and mitochondrial dynamics proteins of 49 kDa and 51 kDa (MiD49 and MiD51; the latter is also known as Mief1). Mff over-expression results in a fragmented mitochondrial network, whereas its genetic ablation induces elongation and significantly reduces DRP1 recruitment at mitochondria (Loson O.C et al., 2013). The adaptors MiD49/51 specifically recruit DRP1 to mitochondria (Zhao J. et al., 2011); the knockdown of MiD51 induces elongation and fragmentation. MiD51 over-expression also induces elongation , cluster formation, and recruitment of inactive DRP1 to mitochondria (Zhao J. et al., 2011).

Components of the fission and fusion machinery have been shown to be regulated at the post-translational level through phosphorylation, ubiquitination and sumoylation. All these post-translational modifications allow mitochondria to adopt rapid and reversible morphological changes and to adapt to continuously changing environmental conditions.

Several experiments point to a role of balanced mitochondrial fusion and fission events in the maintenance of mitochondrial integrity. Enforced mitochondrial fusion by down-regulation of proteins responsible for fission such as Drp1 was found to lead to a drop in mitochondrial ATP production, drop in cell proliferation and increased autophagy (Parone PA.. et al., 2008).

Moreover proteins of the fission machinery as Drp1 and Fis1 appear to play a role later by triggering mitochondrial elimination also called mitophagy (Mijaljica D. et al., 2007).

4.4 KEY REGULATOR IN MITOPHAGY

Mitophagy is the process by which the mitochondria are eliminated and fission of mitochondrial network into individual units seems to be necessary for efficient mitophagy (Twig G, et al., 2008). This could proceed via proteolytically processing of the fusion protein OPA1 occurring in energetically compromised mitochondria or by an increased activity of the fission proteins, such as Fis1 or Drp1 (Gomes L.C. et al., 2008).

Mitophagy could constitute a quality checkpoint for the maintenance of mitochondrial bioenergetics. Indeed, elimination of damaged mitochondria could stimulate mitochondrial biogenesis and thereby maintain cells with efficient organelles.

For example, inhibition of autophagy in yeast results in increased ROS production and a decrease of mitochondrial OXPHOS associated with a higher mtDNA mutation rate (Zhang Y et al., 2007). Mitochondria are the main producers of ATP through the oxidative phosphorylation, but the accumulation of ROS, that is obtained by this process, can lead to their dysfunctions.

The altered mitochondria must be removed to maintain the homeostasis and turnover and this selective elimination is an important mechanism of mitochondrial quality control (Lemasters J.J., 2005; Kim I. et al. 2007).

To emphasize the importance of this process, recent studies have demonstrated that defects in the elimination of the mitochondria are associated with a wide range of human diseases, for example in neurodegenerative disorders (Johri A. and Beal M.F., 2012), to myopathies (Cotán D. et al., 2011) to aging (Yen W.L. and D.J. Klionsky, 2008) to heart disease and to those autoimmune diseases (Zungu M. et al., 2011; Zhou R. et al., 2011), as well as to tumorigenesis (Ravikumar et al. 2010).

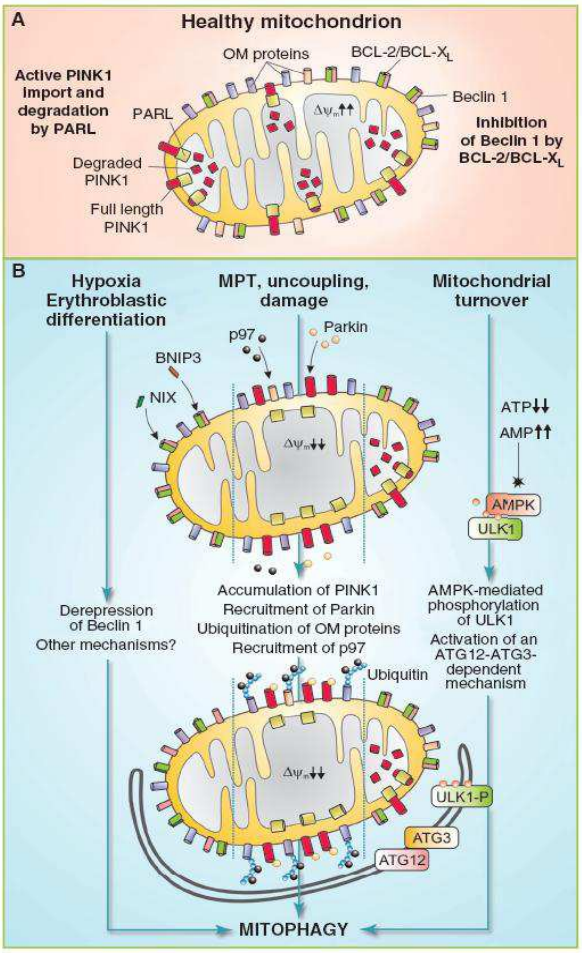


Fig. 14: The general process of selective autophagic degradation of mitochondria. When cells suffer different kinds of stress, damaged or excessive mitochondria are removed by mitophagy to maintain cellular homeostasis. (Green D.R., 2011).

The mitophagic process is regulated by five basic steps. The autophagic induction with assembly of phagopore, formed by an initial isolated membrane, which then will give origin to the autophagosome. The recognition of damaged mitochondria through the interaction with specific proteins receptors as BNIP3 or p62 which recognizes the ubiquitinated proteins of the external mitochondrial membrane including mitofusins and accumulates in the mitochondria damaged. They acts as a molecular adapters through the direct interaction with LC3 with whom form a complex able to recruit the mitochondrion damaged toward the autophagic machinery.

Then there is the autophagosome formation, followed by the fusion of autophagosome with the lysosome and the formation of autophagolysosome where the content is degraded by

lysosomal acidic proteases. Subsequently permeasis and lysosomal transporter export the degradation products in the cytoplasm where they can be reused to build macromolecules or can be metabolized.

Mitophagy serves to degrade damaged or dysfunctional mitochondria to match metabolic demand and orchestrate mitochondrial quality and quantity control in cellular homeostasis; many proteins play important roles in this process.

PINK1-PARKIN:

The best-studied mitophagy-related pathway in mammals is mediated by PINK1 (PTEN induced putative kinase 1) and Parkin. PINK1 and Parkin encode a mitochondrially targeted Ser/Thr kinase and a cytosolic RING-domain containing E3 Ub ligase, respectively. Parkin also regulates mitochondrial morphology and integrity in mammalian cells and is widely expressed in many tissues including brain, skeletal muscle, heart, and liver. (Narendra D. et al., 2008).

The translocation of Parkin to mitochondria requires the presence of PINK1, and that the accumulation of PINK1 on mitochondrial membrane surface causes the recruitment of parkin to mitochondria even in the absence of defects of Δψm (Matsuda et al., 2010; Narendra et al., 2010; Vives-Bauza et al., 2010). In addition, a novel role of PARK2 as a tumor suppressor has been verified in breast and ovarian cancer, highlighting that PARK2-dependent mitophagy might be associated with cancer biology (Poulogiannis G. et al., 2010).

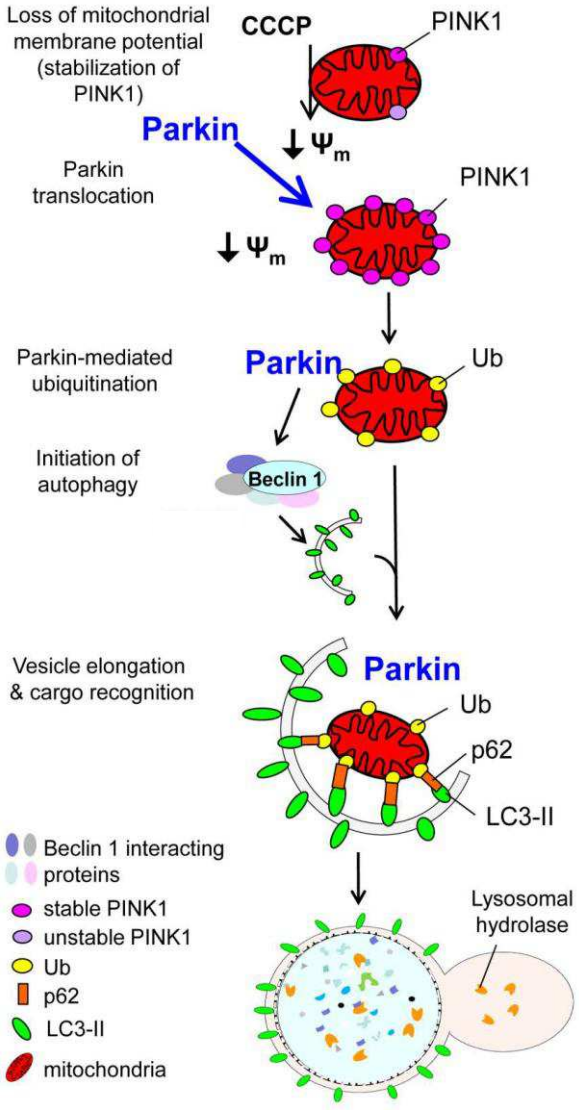


Fig. 15: Parkin-mediated mitophagy. The accumulated PINK1 directs Parkin to mitochondria with low Δψm. Parkin targets the depolarized mitochondrion with ubiquitin, which links to the phagophore through p62, following Parkin-mediated Beclin1 complex form. The ubiquitinated mitochondrion is engulfed within the autophagosome and degraded in lysosomes. (Sih-han Wang, 2012)

PINK1 locates either in the mitochondrial intermembrane space (IMS) or OMM and may be maintained at a low expression level in healthy mitochondria. Both the kinase activity and the mitochondrial localization of PINK1 seem to be essential for the recruitment of Parkin to the mitochondria.

Once the mitochondrial membrane potential is decreased, proteolysis or import of PINK1 is blocked so that it is stabilized and accumulated on the OMM where it recruits PARK2 to induce mitophagy (Narendra DP, et al., 2010).

Following translocation, PARK2 polyubiquitinates itself and its mitochondrial substrates such as VDAC1 (voltage-dependent anion channel) (Chan et al., 2011; Geisler et al., 2010), the MFN mitofusine1 and MFN2 (Gegg et al., 2010; Ziviani et al., 2010), which are essential for the maintenance of the correct melting process of mitochondria, Drp1 (Wang et al.,2011) that regulates the mitochondrial fission and Bcl-2 (Chen et al., 2010), producing the docking site for the Ub-binding adaptor SQSTM1/p62 to trigger autophagic degradation by binding to the LC3-interacting region (LIR) motif of LC3/GABARAP (GABA(A) receptor-associated protein) (Pankiv S. et al., 2007).

LC3:

LC3 is a specific marker of the autophagosome. It is a system similar to the ubiquitin and is expressed in many cell types when it is in a cytosolic, not active, form. Consequently to the autophagic stimulus it is activated and localizes in both the outer and the inner surface of the autophagosome having both a role in the membranes fusion, both in the degradation of selective content. Because of the synthesis and modification of LC3 is greater during the autophagic process, its expression can be useful as an indicator of the level of cellular autophagy (Barth S. et al. 2010).

Indeed the newly synthesized LC3 is immediately cleaved at its C-terminal end via autophagin (ATG4) into the cytoplasmic form of LC3-I (16 kDa). When autophagy is active, LC3-I is conjugated with phosphatidyl-ethanolamination (PE) into LC3-II (14 kDa). LC3-II is recruited via its lipid moiety to the inner and outer surface of autophagosome membrane. After fusion with lysosome, LC3-II is still presented in the single membrane autophagolysosome, which is the original outer membrane of autophagosome before fusion (Tanida I., et al., 2008).

During autophagosome formation, the diffused distribution of the cytosolic form, LC3-I translocates to a punctuate distribution of the membrane form LC3-II. p62/SQSTM1 (p62) provides

a link for the ubiquitinated targets to the autophagosome through its interaction with LC3. p62 is ultimately degraded by lysosomal enzymes in the autophagolysosome (Dieter A. Kubli and Åsa B. Gustafsson, 2012).

P62:

P62 is an autophagy adaptor, with the ability to interact with key signaling proteins through well-defined structural elements (Moscat J. and Diaz-Meco M.T., 2009). Thus, p62, due to the ubiquitin-associated (UBA) or LC3-interacting region (LIR) domains, can promote the autophagy process and the degradation of ubiquitinated cargos. Autophagy, however, plays a key role in the control of p62 levels as it is constantly degraded via non-selective autophagy through its LIR domain that binds to LC3 on autophagosomes membranes (Moscat J. and Diaz-Meco M.T., 2009). New data demonstrated that p62 activates mTORC1 dependent nutrient sensing, which can upregulate c-Myc (Valencia et al., 2014); NF-κB-mediated inflammatory responses, and the NRF2-activated antioxidant defense. Thus, oxidative stress and inflammation induce p62 through NRF2 and NF-κB to promote selective autophagy and cell detoxification.

BNIP3/NIX(BNIP3L):

Several studies indicate that there may exist a crosstalk between PINK1-PARK2-dependent mitophagy and mitophagy receptor-mediated pathways. (Feng D, et al., 2013). Indeed, BNIP3L/NIX (BCL2/adenovirus E1B interacting protein 3-like) appears to promote CCCP-induced mitochondrial depolarization and PARK2 translocation to mediate mitophagy in mouse embryonic fibroblasts (Ding WX et al., 2010); this suggests that identification of more cofactors and coregulators are implicated in mitophagy. Indeed, BNIP3 interacts with LC3 via its LIR for autophagic degradation of mitochondria. Generally, there are three models for the mechanism of BNIP3- or BNIP3L-dependent mitophagy. First, BNIP3 or BNIP3L triggers mitochondrial depolarization and initiates mitophagy. Second, BNIP3 or BNIP3L functions as a receptor protein to recruit autophagic machinery to mitochondria. Third, BNIP3 or BNIP3L can compete with BECN1 for the binding to BCL2 or BCL2L1. The increased expression of BNIP3 or BNIP3L will release BECN1 from BCL2 or BCL2L1 to activate mitophagy. In response to hypoxia or oxidative stress, cells will undergo rapid mitophagy to enable cell survival, or undergo apoptotic or necrotic cell death. Both BNIP3 and BNIP3L are able to induce mitochondrial depolarization, cytochrome c release, and apoptosis when overexpressed (Thomas RL. et al., 2011).

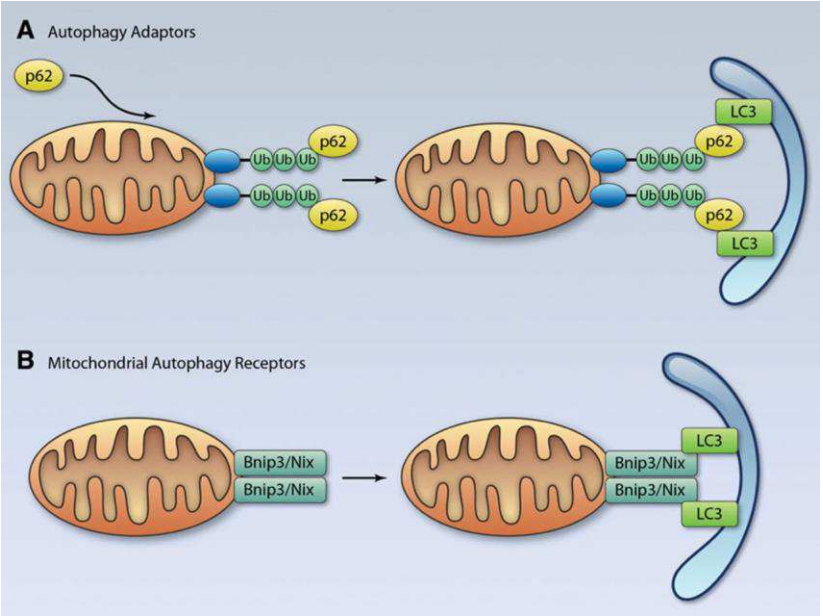


Fig.16: Removal of mitochondria via autophagy adaptors or autophagy receptors (Dieter A. Kubli and Åsa B. Gustafsson, 2012).

5. MITOCHONDRIAL ROLE IN CANCER CELLS

Mitochondrial dynamic is essential for the function of this organelle, thus, it is possible that mitochondrial dynamics alterations could participate in tumorigenesis by contributing to the accumulation of damaged mitochondria in cells (Fig. 17).

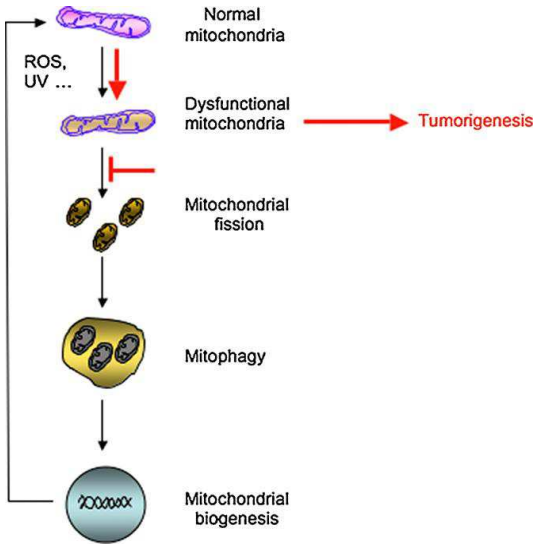


Fig. 17. Model linking a mitochondrial fission defect, accumulation of dysfunctional mitochondria and tumorigenesis. The dysfunctional mitochondria accumulate within cells, causing additional damages to mtDNA that could contribute to tumorigenesis. (Grandemange S. et al., 2008)

Mitochondrial DNA mutations deletions and depletions have been identified in cancer cells (Higuchi M. et al., 2007). These mutations could be the consequence of ROS damages accumulated, in fact mtDNA is subject to mutations due to its proximity with the respiratory chain and due to a not efficient DNA repair system. Yu and colleagues have demonstrated that reduced mitochondrial DNA copy number could be involved in breast cell transformation or progression (Yu M, et al. 2007).

Mitochondrial fusion activity is necessary for mtDNA maintenance, thus, it is possible that an impairment of this process could contribute to the loss of mtDNA observed in some cancers. Moreover, as mitochondrial fission is also important for elimination of damaged mitochondria, it is possible that an impairment of this process may contribute to the accumulation of damaged mitochondria (Fig. 17). However, it remains to determine whether and how dysfunctional mitochondria could contribute to tumorigenesis (Frezza C. and Gottlieb E., 2009).

5.1 MITOCHONDIAL METABOLISM IN CANCER

Hanahan et al. proposed six hallmarks to define a cancer cell: independence for growth signals, insensitivity to antigrowth signals, apoptotic resistance, acute replicative potential, sustained angiogenesis and invasive potential (Hanahan D. and Weinberg R.A. 2000). In many cancers, a common characteristic is dysfunctional mitochondria, explaining why cancer cells mainly rely on glycolysis to generate ATP (Warburg effect). Usually the healthy cells use the oxidative phosphorylation to produce energy, when they have the oxygen, which also inhibits the process of glycolysis ("Pasteaur effect") (Racker E., 1974). Instead, various studies on tumor cells have hightlighted the "effect Warburg", the use of the aerobic glycolysis to produce energy by the tumor cells, even in the presence of sufficient oxygen (Warburg O. et al., 1927). Initially, this phenomenon was thought to be a consequence of decreased availability of oxygen molecules for the mass in expansion, but subsequent observations have shown how this metabolism is more than a simple adaptation to hypoxia, and it could confers a proliferative advantage to cells in evolution. A number of mitochondrial protein defects have been described in the Warburg effect, such as alterations of the subunit of the ATP synthase (Lopez-Rios F. et al., 2007), or mutations of the succinate dehydrogenase and fumarate hydratase (King A. et al., 2006).

Compared to normal cells, tumor cells increase their bioenergetic requests, for which the glucose and glutamine plays a pivotal role in the progression of cancer influencing energy metabolism. To this end, the most human solid tumors including breast tumors, require a

supplement of glutamine to support the growth of the cells. In particular glutamine is a non-essential amino acid and serves as a precursor for the synthesis of many amino acids, proteins and nucleotides. It also participates in gluconeogenesis and helps provide oxidative fuel (NADPH and NADH) for the cells in rapid proliferation and fabrics as well as for the synthesis of glutathione (Mohammed R. et al., 2013). In fact most tumors, including the more aggressive forms of breast cancer, require a constant supply of glutamine to support growth and cell proliferation. Growing evidence suggests that specific alterations in the metabolism of glutamine, in tumor cells, provide possible methods for the treatment of tumors. Due to its role anaplerotic in TCA cycle, glutamine replenishes the necessary intermediate for the synthesis of macromolecules (Chen H. et al., 2015). Therefore, a reduction of the metabolism of glutamine can limit the proliferation of tumor cells and thereby serve as a point of metabolic control that is activated in response to the genotoxic stress (Jeong SM et al., 2013). Moreover, glutamine is metabolized to produce NADPH and GSH, that are necessary to maintain the oxidative homeostasis within the cell. Thus glutamine deprivation is sufficient to reduce levels of GSH (Lora J. et al., 2004) and can cause oxidative stress and sensitize the cells to chemotherapeutic agents.

Other studies have suggested that the lactic acid produced during glycolysis could activate the metal-proteinase enzymes and the remodeling of the matrix, thus promoting the cellular invasion and metastasis (Berardi M.J. and Fantin V.R., 2011; DeBerardinis R.J. et al., 2008). What remains controversial of the Warburg effect is the reason why the amount of lactate produced is very high, when most of this could be oxidized by increasing the production of ATP. Evidence explains the phenomenon by observing how the process of glycolysis exceeds the maximum speed of oxidation of pyruvate and this leads the cells to use mechanisms at high flow for the oxidation of pyruvate (Curi R. et al., 1988). It is further assumed that, in the face of the defects of the mitochondrial activity, aerobic glycolysis is necessary to decrease the production of ATP by oxidative phosphorylation (Frezza C. and Gottlieb E., 2009).

However, some studies have led to a reassessment of these knowledge: not all tumors are associated with an increase in the glycolysis, but it is found that the tumor cells both use the mechanisms for the production of energy, also the oxidative phosphorylation is responsible for the production of 50-80% energy for the cell (Guppy M. et al., 2002). Then, there are evidences that show how both the mitochondrial activity, such as the oxidative phosphorylation, may help the growth of the tumor (Yan H. et al., 2009).

5.2 MITOCHONDIAL DYNAMIC IN CANCER

The mitochondrial fusion and fission are involved in many cellular processes such as apoptosis, cell proliferation and the consumption of ATP, the signaling pathways that regulate these mechanisms may be altered by the carcinogenic process. The pathways respond to several stress and are able to coordinate the mitochondrial dynamics with other cell physiological aspects; some drugs that inhibit protein synthesis, are able to promote the mitochondrial stress-induced hyperfusion, in which are implicated OPA1 and MFN1 (Tondera D. et al., 2009). This process leads to an increased production of ATP (Mitra K. et al., 2009) and inhibits the mitophagy and apoptosis (Gomes L.C. et al., 2011; Rambold A.S. et al., 2011). A prolonged mitochondrial fusion can cause mitochondrial bound between daughter cells, resulting in a unequal distribution of mitochondria and an alteration of the chromosomes that can lead to aneuploidy. These processes can cause alteration of the organelle division and mitochondrial dysfunction that promotes cell growth (Kashatus D.F. et al., 2011). Evaluating the mitochondrial impairment in the progression of lung cancer, it has been observed that the tumor cells exhibit a fragmented mitochondrial network and show a decrease of the protein MFN2, and therefore of the fusion, opposed to an increase of the DRP1 and then of the fission process (Rehman J. et al., 2012).

Cancer cells are characterized by a basal resistance to apoptosis and the release of cytochrome c in the cytosol is a key process for initiation of this event. In lung cancer cells, it has been observed that an high concentration of mitofusin 2 could lead to a greater concentration of cytochrome c in the cytosol and a decrease at the mitochondrial level. Therefore it has been shown the overexpression of Mfn2 and inhibition of protein of fission can reduce the tumor proliferation and inducing apoptosis in a spontaneous way (Rehman J. et al., 2012). Moreover Mfn2 depletion in different cell models modifies the cellular metabolic profile, leading to reduced mitochondrial membrane potential, cellular oxygen consumption, mitochondrial proton leak, and mitochondrial co-enzyme Q level, as well as decreased oxidation of glucose, pyruvate, and fatty acids (Bach E.A. et al., 2003; Chen H. et al., 2005; Mourier A. et al., 2015). To compensate for the reduced activity of the respiratory chain and Kreb's cycle, the cell increases glucose uptake, decreases glycogen synthesis, and shifts to anaerobic glycolysis to generate ATP. However, these alterations are not resulting from mitochondrial mass variations.

On the other hand, Mfn2 overexpression causes increased mitochondrial membrane potential and glucose oxidation (Pich L. et al., 2005). Moreover several studies showed that Mfn2 protein and mRNA levels are down-regulated in different types of malignancies, including colorectal

(Cheng H. et al., 2013), lung (Rehman S. et al., 2012), liver (Wang D. et al., 2010, 2015), gastric (Zhang G.E. et al., 2013), and urinary bladder cancers (Jin B. et al., 2011). Alterations of mitochondrial network dynamics, through fusion or fission imbalance, induces the impairment of mitochondrial energy production in mammalian cells, which suggests that mitochondrial dynamics can be involved in the control of mitochondrial metabolism. If mitochondrial fusion influences mitochondrial OXPHOS, the inverse is also true since fusion of mitochondria is dependent on the mitochondrial inner membrane potential (Mattenberger Y. et al., 2003) and the OXPHOS activity.

However, there are many examples of OXPHOS dysfunction that can cause mitochondrial fission. Pharmacological inhibition of complex I of the respiratory chain is associated with a decreased mitochondrial membrane potential, an increase in ROS production and mitochondrial fission (Benard G, et al., 2007).

5.3 MITOCHONDIAL DYNAMIC IN CISPLATIN RESISTANCE

The mitochondrial dynamic assumes an important role also in resistance of tumor cells to chemoterapeutic agents such as cisplatin. By comparing the percentage of cells with tubular mitochondria in the gynecological cancer cells sensitive and resistant to the chemotherapy, it has been observed a high level of mitochondrial fusion process in resistant cells as compare to the sensitive (Kong B. et al. 2015). This suggests that the mitochondria fusion can promote cell survival, due to a better mitochondrial activity, through an efficient production of ATP and its transport (Kong B. et al. 2015).

In addition, the inhibition of mitochondrial fragmentation reduces the release of cytochrome c and delays the cell death. A study conducted on lung adenocarcinoma cells has highlighted an over-expression of OPA1, significantly correlated with the gender of the patient, the cellular differentiation, the stage of the tumor, the histopathological subtype, the high incidence of relapse of the disease and the reduction of the sensitivity to cisplatin. The results have shown that the high expression of OPA1 increases the resistance to cisplatin, with a consequent effect on the survival of the patient: patients with a low level of the fusion protein had a better prognosis, while patients with a high level of OPA1 exhibited cells more resistant, with consequent less hope of healing (Fang H.Y. et al., 2012). This is possibly due to the down-regulation of OPA1 that increases the mitochondrial cristae deformation (Frezza C. et al., 2006), thus, this alteration can lead to an increase in the release of cytochrome c, inducing apoptosis; accordingly silencing the expression of OPA1 may decrease the cisplatin resistance (Fang H.Y. et al., 2012).

Some studies have reported a relationship between DRP1 and chemoresistance in gynecologic cancer cells. The Piceatannol, a compound present in some foods, by inducing the dephosphorylation of serine 637 of the DRP1 and thus promoting the mitochondrial fission and apoptosis, increases the sensitivity of the cells to cisplatin. These responses can be decreased by the inhibition of the fission (Kong B. et al., 2015). There are also studies in contrast with these result, for example it has been observed that a down-regulation of the DRP1 protein leads to a drug sensitivity, in ovarian carcinoma cells (Qian W. et al., 2014). Moreover the over-expression of DRP1 seems to lead a cisplatin resistance in lung cancer (Parone P.A. et al., 2008; Chiang C.R. and Blackstone C., 2010). Therefore, DRP1 can confer both resistance and sensitivity to this drug, stressing the importance of the therapeutic strategy targeted to the type of cancer and stage (Kong B. et al., 2015).

5.4 MITOPHAGY IN CANCER

Autophagy has always been supposed to play a double role in tumorigenesis; either supporting survival or promoting death, depending on the type of cancer and its stadium (in fact it appears to act as a tumor suppressor in the early stages of cancer and contributes instead to progression in more advanced stages); on the mutation involved and the tumor microenvironment (Rosenfeldt M.T., Ryan K.M., 2011).

Generally, basal autophagy remains at a low level to maintain cellular homeostasis and sustains prolonged survival by degradation of polyubiquitinated or aggregated proteins and damaged organelles. For example it has been observed that the basal autophagy is greater in the tumor regions where there is a deficiency of oxygen, where it becomes essential for cell survival (Degenhardt K. et al., 2006); moreover the autophagosomes are more present in hypoxic tumor cells and deletions of genes essential for the autophagy lead to cell death (Karantza-Wadsworth V. et al., 2007; Mathew R. et al., 2007). Nevertheless a low autophagic activity can cause the accumulation of damaged macromolecules and organelles (in particular the mitochondria) and consequently induce oxidative stress, activation of consecutive response to DNA damage and genomic instability (Levin B. and Klionsky V.J., 2004; Chen No Karantza and V., 2011), then the autophagic defect is associated with the accumulation of mutations of oncogenes and increases susceptibility to cancer (Karantza-Wadsworth V. et al., 2007; Vogelstein B. et al., 2013).

Similarly, mitophagy follows this double nature of autophagy. The mtDNA exhibits a high susceptibility to damage attributed to exposure to ROS, restricted repair systems, and lack of

histone protection. (Gredilla R. et al., 2012). Thus, mitophagy serves to remove dysfunctional mitochondria to counteract oxidative stress and prevent carcinogenesis. Conversely, mitophagy can protect cells from apoptosis or necrosis and promote tumor cell survival under some adverse conditions such as poor nutrient supply and hypoxic stresses. (Lisanti M.P. et al., 2010). Therefore, mitophagy emerges as a key quality control factor and decision maker in cancer cells.

5.5 TRAP1 IN CANCER

There is a relationship between the protein TRAP1 (tumor necrosis factor receptor associated protein 1) and the network: in fact, TRAP1 regulates the expression of the DRP1 and Mff (factor of mitochondrial fission, involved in the recruitment of the DRP1 on OMM during the fission process) (Otera H. et al., 2010) and controls the modifications of mitochondrial morphology.

TRAP1 is one chaperone, member of the family of heat shock protein 90, present at the level of the mitochondrial matrix, with GTPase activity (Felts S.J. et al., 2000); its expression is increased in response to the many stimuli such as oxidative stress, hydroperoxide stress, and deprivation of glucose (Lee A.S., 2001; Carette J. et al,. 2002; Mitsumoto A. et al., 2002). It is involved in preventing cell death caused by reactive oxygen species (ROS) (Masuda Y. et al., 2004; Montesano Gesualdi N. et al., 2007), by counteracting their production; it also decreases the lipid peroxidation and preserves the mitochondrial membrane potential, and prevents the unfolding of damaged proteins (Siegelin M.D. et al., 2011). The action of this protein at the mitochondrial level seems to be a consequence of the ability to control the morphology: it has been observed that silencing of TRAP1 leads the balance between the two fusion and fission processes toward the fusion, without altering the expression of proteins OPA1 and MFN 1 and 2.

Instead TRAP1 leads to a significant decrease of the DRP1 and Mff; it controls their mitochondrial localization or on their expression (Takamura H. et al., 2012). Indeed, TRAP1 can control the expression levels and the process of ubiquitination (step previous to the proteins degradation in the proteasome) of mitochondrial proteins and thus leads to the DRP1 and Mff degradation (Amoroso M.R. et al., 2012). The two opposite processes of fusion and fission are useful to evaluate the different stress levels: an increase of fusion and/or a decrease of the fission help to decrease the stress level, while the decrease of fusion and the increase of fission occurs when cells are stressed. The protein TRAP1 is highly expressed in several tumors such as breast, colon and lung cancers as compared with normal tissues (Kang B.H. et al., 2007), and it has been

observed that the increase in its expression leads to mitochondrial aberration with increase of ROS, destruction of membrane potential, decrease of mtDNA and mitochondrial mass.

Therefore, high levels of this protein may be a critical link between mitochondrial alterations and carcinogenesis, even if this theory needs further clarification (Chang N.I. and Blackstone C., 2014). TRAP1, through the cellular protection from oxidative stress, has an anti-apoptotic effect and is associated with the chemoresistance. In fact, this protein seems to block the apoptosis induced by the drug in various types of cancers such as prostate cancer, colon rectal, breast cancer and ovarian cancer (Leav I. et al., 2010; Aust S. et al., 2012; Maddalena F. et al., 2013). Of note, there are few TRAP1 antagonists, recently discovered, that lead to a mitochondrial activity collapse and cell death, suggesting TRAP1 as a new molecular target for improving the chemotherapies (Landriscina M. et al., 2010).

AIM

The cisplatin and its derivatives are widely used as frontline chemotherapy for the treatment of several solid tumors, including sarcomas and ovarian and breast carcinomas. Unfortunately the emergence of resistance is a formidable challenge for research because it limits the clinical efficacy of these compounds. The resistance phenomenon is multifactorial and the molecular mechanism is not yet fully understood. Recent studies have shown that the cisplatin may interact not only with the nuclear DNA, but also with a variety of cellular components, including cytosolic proteins and the mitochondrial DNA (mtDNA) (Arnesano F. et al., 2008). The mtDNA, unlike the nDNA, does not possess effective repair systems, consequently it is more susceptible to the onset of mutations and oncogenic transformations. In literature there are few works closely related to cisplatin resistance and mitochondria, and there are conflicting data that correlate the cisplatin resistance to mitochondrial alterations (Cullen KJ. et al., 2007).

Previously we have demonstrated that ovarian cancer cells resistant to cisplatin (C13) are characterized by a compensatory mechanisms that turn energetic metabolism with a reduced activity of the respiratory chain and a lower mitochondrial mass as compare to sensitive line (2008), as well as a different susceptibility to various metabolic stress and an altered mitochondrial network.

Our goal was to identify new pharmacological targets related to metabolic and mitochondrial changes useful to overcome cisplatin resistance. In order to increase the current understanding of the mitochondrial dynamics regulation and metabolism role in chemoresistance, we studied two *in vitro* models: cancer cell presenting acquired resistance and cancer cells with innate resistance to cisplatin.

FIRST AIM: MITOCHONDRIAL SHAPE REMODELING IN CISPLATIN RESISTANCE

The overall hypothesis is that chemoresistance in cancer cells is determined by dysregulated mitochondrial dynamics, thus the purpose is to investigate the mitochondrial shape remodeling in cisplatin resistant cells to discover new targets useful for this form of acquired resistance.

In this scenario we analyzed other cell lines resistant and sensitive to cisplatin, in particular squamous epidermoid carcinoma of cervix and osteosarcoma. In these tumor cells we didn't find significant differences as regard the mitochondrial membrane potential ($\Delta\Psi$) and mass between sensitive and resistant lines; however it was clear a different mitochondrial morphology.

The mitochondrial shape is the result of the balance between fusion and fission processes.

The mitochondrial dynamic is a complex phenomenon and still not yet understood. Moreover several regulator proteins and signaling pathways are not completely clarified. Thus, we continued our study investigating the mitochondrial dynamics and proteins that have a key role in coordinating the mitochondrial processes of fusion and fission.

SECOND AIM: GLUCOSE AND GLUTAMINE METABOLISM IN CISPLATIN RESISTANCE

As regard the second aim, our study, performed in Professor Toker laboratory (Department of Pathology at the Beth Israel Deaconess Medical Center, Harvard Medical School), focused on the role of glucose and glutamine metabolism in breast carcinoma cells that have an innate resistance to cisplatin.

Reprogramming of the metabolic pathways is defined as one of the hallmarks of cancer (Hanahan et al., 2011). Compared to normal cells, tumor cells increase their bioenergetic needs; thus, glucose and glutamine plays a pivotal role in the progression of cancer, influencing energy metabolism. Most of cancer cells show a metabolic shift from oxidative phosphorylation to anaerobic glycolysis (Warburg effect), and consequently glutamine, due to its anaplerotic role in the tricarboxylic acid cycle (TCA), provides the intermediate for the synthesis of new macromolecules.

Previous data, obtained in Montopoli laboratory, have already shown a metabolic reprogramming in cancer cells that exhibit acquired resistance to cisplatin. Indeed, it has been demonstrated how ovarian cancer cells C13, resistant to cisplatin, increases their glucose dependency and are more sensitive to the glycolysis inhibition. Moreover, in the presence of an altered mitochondrial function, glutamine, becomes the privileged carbon resource, for the cisplatin-resistant line (Catanzaro et al.,2015).

Therefore, our goal was to explore the alterations of the metabolic pathways in breast cancer cells resistant to cisplatin and then identify possible targets to overcoming the chemoresistance. The transcription factor c-Myc controls the expression of several key genes involved in the regulation of metabolic pathways including glycolysis and glutaminolysis. However, the role of the glutamine metabolism and its association with c-Myc, in the development of cisplatin resistance, is not fully understood. Our studies show differences in glucose dependency between sensitive and cisplatin resistant cancer cells, and also an increased expression of the oncogene c-Myc in resistant cells. We have also identified a different expression of specific c-Myc target genes involved in the glycolysis and glutaminolysis regulation.

Thus, the modulation of the metabolic pathways may be an interesting approach to develop most effective treatments for breast cancer and to overcome the resistance to the chemotherapeutic drugs.

MATERIALS AND METHODS

First *in vitro* model of acquired cisplatin resistance

1. Cell lines

1.1 Human carcinoma cell lines

The 2008 cell line derived from human ovarian carcinoma, instead A431 cells derived from human cervix squamous carcinoma; their CDDP-resistant variant (C13; A431Pt) were selected by exposure to increasing CDDP concentrations for a period of 9 months. The cells were grown in RPMI 1640 medium *(Lonza)* and osteosarcoma cells, U2OS and U2OS-Pt, were grown in Mc Coy' 5A *(Lonza)* medium; both media were supplemented with 10% fetal bovine serum, 4 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin, in humidified condition at 5% CO_2 and 37° C. Cells were collected every 2 days with minimum amount of 0.25% trypsin-0.2% EDTA.

The A431 cells (epidermoid carcinoma) express the mutated form of the p53 gene with substitution at codon 273 (His273) (missense mutation). Instead ovarian carcinoma 2008 and osteosarcoma U2OS are wild type for p53.

1.2 206-ρ° cell line

206-ρ° cells, obtained from mtDNA depletion of 143B-TK$^-$ osteosarcoma cells, were cultured in high-glucose Dulbecco's modified Eagle's medium (Gibco), supplemented with 10% fetal bovine serum (FBS), mm L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin, 1mM sodium-pyruvate, 1 % of a solution of not essential aminoacid (NEAA) and 0.05 mg/ml uridine, in humidified condition at 5% CO_2 and 37° C.

1.3 Mouse embryonic fibroblasts (Mef's) OPA1 knock-out, Mfn1 knock-out, OPA1 transgenic, shTRAP1

All Mef's cells were grown in Dulbecco's modified Eagle's medium *(Lonza)*, supplemented with 10% fetal bovine serum (FBS), mm L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin, 1 % of a solution of not essential aminoacid (NEAA) and Mef's OPA1 knock-out also with 0.05 mg/ml uridine, in humidified condition at 5% CO_2 and 37° C. Mef's OPA1 KO, Mfn1 KO and OPA1tg were kindly provided from Scorrano Laboratory (University of Padova) (Cogliati et al., 2013) and Mef's shTRAp1 were kindly provided from Rasola Laboratory (University of Padova).

2. Cell viability assays

2.1. Trypan blue exclusion assay

This test measures the percentage of viable cells with the intact membrane thus excluding the blue dye, while dead cells take up the colouring agent. 12 wells plates were seeded with a constant number of cells and, following overnight incubation, were exposed to different treatments, according to experimental protocols. At the end of incubation the cells were washed, detached with 0.25% trypsin-0.2% EDTA and suspended in trypan blue (Sigma-Aldrich, St Louis, MO, USA), at 1:1 ratio in medium solution. Cell number was counted using a chamber Burker hemocytometer under the microscope.

3. Mitochondrial membrane potential (ΔΨ) and mitochondrial mass

3.1 Flow cytometry

Mitochondrial transmembrane potential (ΔΨm) was probed by the cationic lipophilic, green-fluorescent rhodamine-123 (Rh123) (Molecular Probes, Invitrogen, Carlsbad, CA, USA) that is readily sequestered by active mitochondria in a potential-dependent manner: a loss of ΔΨm is associated with a lack of Rh123 retention and a decrease in fluorescence. Mitochondrial mass was measured by Acridine Orange 10-Nonyl bromide (NAO) staining. NAO is a fluorescent probe that selectively binds to cardiolipin (CL) of mitochondrial membrane regardless of mitochondrial membrane potential determining mitochondrial mass. Cells were seeded and incubated for 48 hours, washed with phosphate buffer saline solution (PBS), detached with 0.25% trypsin-0.2% EDTA and centrifuged for 5 min at 1200xrpm. The cell pellet was then resuspended with Rh123 (10 µM) or NAO (25 nM) and incubated for 15 minutes in dark. Fluorescence intensity was analyzed using an Epics XL flow cytometer (Coulter Systems, Fullerton, CA, USA) equipped with a 488 Argon laser. The green emission signal of Rh123 was measured at 525 nm and the orange emission signal of NAO at 580 nm. Necrotic cells were excluded by electronically gating data on the basis of forward versus side scatter profiles; a minimum of 10^4 cells of interest were analyzed further. Mean fluorescence intensity (MFI) values were obtained using the EXPO 32 software (Coulter Systems, Fullerton, CA, USA).

4. Mitochondrial network

4.2. Confocal microscope

Cells were seeded at approximately 50% confluence on glass cover slips and incubated overnight in a 12 wells plate. After 48 hours, media were removed and cells were stained with 50 nM Mitotracker Orange (Invitrogen). Then cells were fixed with 4% formaldehyde (SIGMA St Louis, USA) for 15 min. After repeated washings, Mowiol 40-88 (Sigma, St Louis, MO) was added at a final concentration of 0.5 µg/ml and the slides were imaged using a fluorescence laser scanner microscope (Nikon Eclipse E600 confocal microscope and Nikon EZ-Cl software (version 3.91), magnification 60X).

5. Immunoblot assay

Cells were plated in 100 mm cell culture dish and allowed to attach overnight. After 24 hrs cells were washed 3 times with PBS and lysed with ice-cold lysis buffer [TRIS 25 mM pH 7,4; NaCl 150 mM; IGEPAL 1%; sodium deoxycholate 1%; SDS 0,1%; EDTA 1 mM] supplemented with the protease inhibitor cocktails (Protease Inhibitor cocktail tablets EDTA-free, Roche Molecular Biochemicals, Mannheim, Germany). Cell lysates were then centrifuged at 14000 g for 15 minutes at 4°C and the supernatant protein content was determined by Lowry procedure (Bio-rad DC Protein Assay) using bovine serum albumin as standard. Laemmli buffer 5X [250 mM Tris-HCl pH=6.8, 50% glycerol, 10% SDS, 500 mM β-mercaptoethanol, 0.004% bromophenol blue, H_2O q.b] was added (1/5 v/v) to protein lysates, and the samples were denatured for 5 min at 100°C.

Equal amounts of protein (30 µg) were loaded on a 10% polyacrylamide gel and electrophoretically separated in running buffer [25 Mm TRIS, 250 mM glycine (Applichem), 0.1% SDS, H_2O q.b.], for 1h at a constant current of 200 V (Bio-rad Mini-PROTEAN® Tetra System). After electrophoresis, the proteins were blotted onto an Hybond-P PVDF membrane (Amersham Biosciences), previously soaked in methanol, using a transfer buffer [25 mM Tris,192 mM glycine, H_2O q.b.]. A current of 250 mA was applied for 1h and 45min at 4°C. Non specific binding sites were blocked by immersing the membrane in TBS-Tween 20 solution containing 10% non-fat dried milk and shaking for 1h at room temperature. After 3x10 min washes with TBS-Tween 20 [10 mM TRIS, 150 mM NaCl, 0,1% Tween 20 (Sigma-Aldrich), H_2O q.b.] at room temperature, the membrane was exposed to the primary antibodies: MFN2 (1:1000; Rabbit, Sigma), MFN1 (1:1000; Mouse, Abcam), OPA1 (1:1000; Rabbit, Biosciences), LC3B (1:1000; Rabbit, Cell Signaling), p-DRP1

(1:1000; Rabbit, Cell Signaling), TRAP1 (1:1000; Cell Signaling) overnight at 4°C. After washing, the membrane was incubated with HRP-conjugated anti-rabbit secondary antibody (1:3500; PerkinElmer, MA,USA) and HRP-conjugated anti-mouse secondary antibody (1:10000, Dako) for 2h at room temperature.

The signal was visualized with enhanced chemiluminescent kit (Amersham Biosciences) according to the manufacturer's instructions and analyzed by Molecular Imager VersaDoc MP 4000 (Bio-rad). The integrated intensity was normalized to antibodies: Tom20 (1:2000; Rabbit, Santa Cruz Biotechnology), beta-actin (1:5000; AbCam), beta-tubulin (1:2000; Mouse, Sigma-Aldrich), calnexin (1:2000; Rabbit, Santa Cruz biotechnology), GAPDH (1:2000; Mouse, Santa Cruz Biotechnology).

6. qRT-PCR analysis

Cells were grown as indicated and total mRNA was extracted as per manufacturer's instructions using kit Direct-zol™ RNA MiniPrep (Zymo research) and measured with a NanoDrop 2000 (Thermo Fischer Scientific Inc.). The relative expression of each gene was determined by quantitative real-time PCR (Eco™Illumina, Real-Time PCR system, San Diego, CA, USA) using One Step SYBR PrimeScript RT-PCR Kit (Takara Bio, Inc., Otsu, Shiga, Japan) and the primers designed as follow:

BNIP3: F: 5'-GAATTTCTGAAAGTTTCCTTCCA-3',
R: 5'-TTGTCAGACGCCTTCCAATA-3';
H-FIS1: F: 5'-CTTGCTGTGTCCAAGTCCAA-3',
R: 5'-CCACAGCCCCGTTTTATTTA-3';
DRP1: F: 5'-CAGTGTGCCAAAGGCAGTAA-3',
R: 5'-GATGAGTCTCCCGGATTTCA-3'.

Linearity and efficiency of PCR amplifications were assessed using standard curves generated by serial dilution of complementary DNA; melt-curve analysis was used to confirm the specificity of amplification and absence of primer dimers. All genes were normalized to β-actin: F: 5'-CCAACCGCGAGAAGATGA-3', R: 5'-CCAGAGGCGTACAGGGATAG-3'for 2008-C13 cell lines; GAPDH: F:5'-CTGACTTCAACAGCGACACC-3', R: 5'-GTGGTCCAGGGGTCTTACTC-3' for A431-A431-Pt cell lines and CALNEXIN: F: 5'-GAAGGGAAGTGGTTGCTGTG-3'; R:5'-GATGAAGGAGGAGCAGTGGT-3' for U2OS-U2OS-Pt and SKOV3 WT-SKOV3 CDDP3. Expression levels of the indicated genes were calculated by the ΔΔCt method using Eco™ Software v4.0.7.0.

7. Transmission Electron Microscopy

12-multiwells plates were seeded with a constant number of cells and, following overnight incubation, cells were fixed with 2.5% glutaraldehyde in sodium cacodylate 0.1M pH 7.4 for 1 hour at 4°C, and then postfixed with 1% osmium tetroxide and 1% potassium ferrocyanide in the same buffer for 1 hour at 4°C. Samples were dehydrated through a graded series of ethanol, infiltrated and then embedded in epoxy embedding medium (Fluka). After being stained with uranyl acetate and lead citrate, the sections were observed under a Tecnai G2 (FEI) transmission electron microscope operating at 100 kV. Images were collected by a F114 (TVIPS) CCD camera.

The TEM images and experiment are performed from the University of Padua electron microscopy facility.

8. Statistical analyses

All data are expressed as mean ± SEM. Standard ANOVA procedures followed by multiple pairwise comparison adjusted with Bonferroni corrections were performed for cell viability assays. Unpaired Student's t-tests were used to analyse all the other results. Significance was considered at $p < 0.05$.

Second *in vitro* model of innate cisplatin resistance

All the experiments were performed in Toker Laboratory, BIDMC, Harvard Medical School, Boston.

1. Cell lines

1.1 Human Triple Negative breast cancer, basal like, cell lines.

Breast cancer cells MDA-MB-468, SUM 149, HCC1143 and HCC1937 were obtained from the American Type Culture Collection (ATCC) and authenticated using short tandem repeat (STR) profiling. All cell lines were maintained in RPMI 1640 (CellGro) supplemented with 10% FBS (Gemini), in humidified condition at 5% CO_2 and 37° C. DMEM lacking glucose, glutamine and pyruvate was obtained from CellGro. Cells were passaged for no more than 6 months and routinely assayed for mycoplasma contamination.

2. Cell viability assays

2.1. Trypan blue exclusion assay

12 wells plates were seeded with a constant number of cells and, following overnight incubation, were exposed to different treatments, according to experimental protocols. At the end of incubation the cells were washed, detached with 0.25% trypsin - 0.2% EDTA and suspended in trypan blue (Sigma-Aldrich, St Louis, MO, USA), at 1:1 ratio in medium solution. Cell number was counted using countess slide and cell Countess (Invitrogen).

2.2. Propidium Iodide assay

Cell viability was assayed with a propidium iodide-based plate reader assay, as previously described (Zhang, L. et al., 1999). Cells were seeded in 96-well plates and exposed to different treatments, according to experimental protocols. Then cells were treated with a final concentration of 30 µM propidium iodide for 20 min at 37°C. The initial fluorescence intensity was measured in a SpectraMax M5 (Molecular Devices) at 530nm excitation/620nm emission. Digitonin was then added to each well at a final concentration of 600 µM. After incubating for 20 min at 37°C, the final fluorescence intensity was measured. The fraction of dead cells was calculated by dividing the background-corrected initial fluorescence intensity by the final fluorescence intensity. Viability was calculated by this formula: 1-fraction of dead cells.

3. Immunoblot assay

Cells were plated in 60 mm cell culture dish and allowed to attach overnight. After 48 hrs cells were washed with PBS at 4˚C and lysed in radioimmunoprecipitation assay buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7.5), proteinase inhibitor cocktail, 50 nmol/L calyculin, 1 mmol/L sodium pyrophosphate, and 20 mmol/L sodium fluoride) for 15 minutes at 4˚C. Cell extracts were pre-cleared by centrifugation at 14,000 rpm for 10 minutes at 4˚C, and protein concentration was measured with the Bio-Rad DC protein assay. Lysates were then resolved on 10% acrylamide gels by SDS-PAGE and transferred electrophoretically to nitrocellulose membrane (Bio-Rad) at 100 V for 90 minutes. The blots were blocked in Tris-buffered saline (TBST) buffer (10 mmol/L Tris-HCl, pH 8, 150 mmol/L NaCl, and 0.2% Tween 20) containing 5% (w/v) nonfat dry milk for 1 hour, and then incubated with the specific primary antibody diluted in blocking buffer at 4˚C overnight. Membranes were washed three times in TBST and incubated with HRP-conjugated secondary antibody for 1 hour at room

temperature. Membranes were washed three times and developed using enhanced chemiluminescence substrate (EMD Millipore). Antibodies used: PARP (1.2000; rabbit), glutamine sinthetase (1:1000; mouse) and c-Myc (1:1000; rabbit) purchased by Cell Signaling Technology. The integrated intensity was normalized to beta-actin (1:5000) purchased from Cell Signaling Technology.

4. Quantitative Real-Time PCR

Cells were grown as indicated and total mRNA was isolated as manufacturer's instructions using Clonotech NucleoSpin RNA plus and measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc. Wilmington, DE, USA). 1 μg of total RNA was reverse-transcribed to complementary DNA using TaqMan Reverse Transcription Reagents Kit. Relative expression of each gene was determined by quantitative real-time PCR (ABI Prism 7700 sequence detector) using SYBR Green and the primers designed as follow:

c-MYC 5': AACACACAACGTCTTGGAGC;3': GCACAAGAGTTCCGTAGCTG

GOT1 5': GGGTAGGAGGTGTGCAATCT; 3': TGCATCCCAGTAGCGATAGG

GOT2 5': TGCGGTTTTGACTTCACAGG; 3': CCCAGGCATCCTTATCACCA

GTP2 5': GCAATTCAGCCGAGAGAAGG; 3': TTGGCAGGAATGAAGATCCG

GDH1 5': GCTATGGCCGTTTGACCTTC; 3': GTGTATGCCAAGCCAGAGTG

GDH2 5': TGGTGGCCTCAGGTGAAAAT ; 3': GGATCAGACGTTCGCAATCC

GLS1 5': AGAAGGAAACAGGGGATCGG ; 3': GCCATGACACTGCCTGATTC

GLS2 5': AGTGTGCAGTGGTTGATGTG ; 3': CGCTCACCTGTGTTCATGTC

ASCT2 5': GTGGGTTTACTCTTTGCCCG ; 3': TCCTCCACGCACTTCATCAT

LAT1 5': AACTATCACCTGGGCGTCAT ; 3': TAGAGCAGCGTCATCACACA

PC 5': GGACTTCACTGCCACCTTTG; 3': AGCTCAAAGAAGACCTGCCT

GLUT1 5': CCCAGAAGGTGATCGAGGAG ; 3': CCAGCAGGTTCATCATCAGC

GLUT4 5': CGAGCCATCCTTCAGTCTCT ; 3': TGTCGGTAGCTGGAATTGGT

PFKM 5': CCGTTCTGAGTGGAGTGACT ; 3': TCTGGGCAGTGGTAGTGATG

PGK1 5’: ACTCGGGCTAAGCAGATTGT ; 3’: GTGCTCACATGGCTGACTTT

LDHA 5’: CAGCCCGATTCCGTTACCTA ; 3’: CTGGGTGCAGAGTCTTCAGA

PKLR 5’: TCGTCTTTGCCTCCTTTGTG ; 3’: GATCTCGATGCCTAGGTCCC.

qRT-PCR was performed in triplicate using 2 µl of complementary DNA template in a 15-µl reaction. Linearity and efficiency of PCR amplifications were assessed using standard curves generated by serial dilution of complementary DNA; melt-curve analysis was used to confirm the specificity of amplification and absence of primer dimers. Expression levels of the indicated genes were calculated by the ΔΔCt method with *18S* rRNA as the reference gene.

5. Statistical analyses

All data are expressed as mean ± SEM. Standard ANOVA procedures followed by multiple pairwise comparison adjusted with Bonferroni corrections were performed for cell viability assays. Unpaired Student’s t-tests were used to analyse all the other results. Significance was considered at $p < 0.05$.

6. Multivariate analysis

To identify the most significant differences between groups, univariate statistical analysis was used. Filtering procedures, such as fold-change analysis, t-test (for paired and unpaired data) and ANOVA were applied, as provided by MetaboAnalyst web-based software (Xia J.et al., 2009; Xia J.and Wishart D.S., 2011). Significantly different data at the probability level of $p<0.05$ were used for further procedures of multivariate analysis, to obtain identification of relevant biomarkers. Tentatively, the entire data set was submitted to multivariate analysis by means of the procedures provided by MetaboAnalyst (Xia J. et al., 2009; Xia J. and Wishart D.S., 2011) and meta-P server (Kastenmüller G. et al., 2011).

Principal Component Analysis (PCA). PCA is an unsupervised method to detect the directions which best explain the variance in a data set, transforming a number of possibly correlated variables into a smaller number of uncorrelated variables defined as principal components, which are linear combinations of the original variables. The first principal component explains as much of the variability in the data as possible, and each following component accounts for the remaining variability. The data are represented in a dimensional space of n variables, which are reduced into a few principal components; these are descriptive dimensions indicating the maximum variation

within the data. After the principal components scores have been obtained, they can be graphically plotted to observe any groupings in the data set. PCA computation was obtained with MetaboAnalyst based on R prcomp package, using singular value decomposition algorithm. The covariance matrix and standardized principal component score were selected. The scores of the first two principal components were graphically plotted to observe any groupings in the data set.

Cluster analysis. Cluster analysis is a multivariate procedure of exploratory data analysis for detecting natural groupings in data. Data classification consists of placing samples into more or less homogeneous groups, in order to reveal any relationship among groups. Ward's method (minimizing the sum of squares of any two clusters), provided by MetaboAnalyst, was used. Distance indices were determined by Pearson's method. Hierarchical clustering was performed with the hclust function provided by R package stat. Results were presented as dendrograms and heat maps.

RESULTS

MITOCHONDRIAL SHAPE REMODELING IN CISPLATIN RESISTANCE

1. Cisplatin resistance

Even if the benefits of cisplatin are largely recognized, the therapeutic effectiveness of the drug is limited by the onset of cisplatin resistance (Koberle B. et al., 2010). In this project cisplatin-sensitive and cisplatin-resistant cancer cells, whose CDDP IC_{50} (24hrs) are shown in **Table 1**, have been characterized in order to define mechanisms of resistance not yet fully understood. **Tab.1** presents IC_{50} values for each cell line, obtained from concentration-response curves after cisplatin treatment. As expected wild type cells are more sensitive to cisplatin as compare to resistant counterpart.

	A431	A431-Pt	2008	C13	U2OS	U2OS-Pt
IC_{50} CDDP(μM)	7.019 2.789 - 17.67	20.88 13.29 - 32.81	1.376 0.3854 - 4.910	10.44 1.555 - 70.16	7.899 4.438 - 14.06	22.78 10.23 - 50.72

Table 1: CDDP cytotoxic effect, expressed as IC_{50} of cisplatin-sensitive (A431,2008,U2OS) and cisplatin-resistant cancer cells (A431-Pt,C13,U2OS-Pt). Data represent 3-4 indipendent experiments.

2. Phenotyping sensitive and cisplatin resistant cell: acquired resistance model

2.1 Mitochondrial potential and mass

Mitochondrial mass and mitochondrial membrane potential (ΔΨm), which is generated by the proton gradient across the inner mitochondrial membrane, were both analysed by flow cytometer (**Fig. 1**), using specific different fluorescent probes: 10-Nonyl bromide Acridine Orange and potential-dependent rhodamine-123(Rh123). Flow cytometer assay shows that mitochondrial potential and mass is significantly lower in C13 than in 2008 cells (**Fig. 1B**) (Catanzaro et al. 2015) but there are no differences as regard the other cell lines (**Fig. 1A and 1C**).

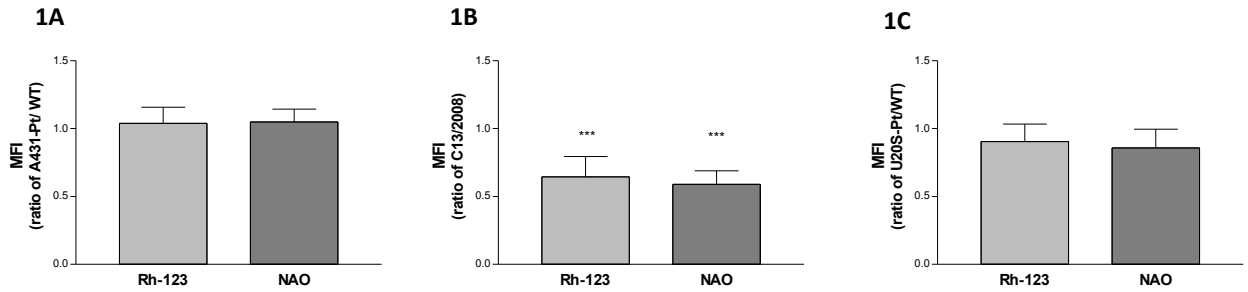


Figure 1: Mitochondrial mass and potential measured by flow cytometry and expressed as ratio of mean fluorescent intensity [MFI] of NAO (25 nM) and Rh123 (10 µM) between resistant cells to sensitive counterpart. **1A**. A431-Pt vs A431; **1B**. C13 vs 2008; **1C.** U2OS-Pt vs U2OS. Each bar represents the mean ± SEM of 4 independent experiments. ***p<0.001, C13 *vs* 2008.

2.2 Effect of galactose and rotenone

To test the mitochondrial functionality of cancer cells and to understand their dependency from glycolysis and OXPHOS for energy production, cell viability was measured after exposure to different experimental tool causing mitochondrial stress. The first experimental strategy was to treat cancer cells with glucose-free/galactose medium. Galactose is very slowly metabolized through the glycolytic pathway, therefore glycolysis dependent cells are not able to survive for a long time unlike those with an efficient oxidative phosphorylation (Reitzer et al., 1979). **Fig. 2** shows the cell viability in DMEM or in DMEM glucose-free and added with 5 mM galactose. It is evident that in galactose stressed metabolic condition, the cell proliferation is lower than in DMEM. The effect is more significantly pronounced in the cisplatin-resistant C13 cell line. This data suggests a major glucose-dependence of C13 whose oxidative phosphorylation is not so efficient to counteract the block of the glycolysis (data published, Catanzaro et al. 2015).

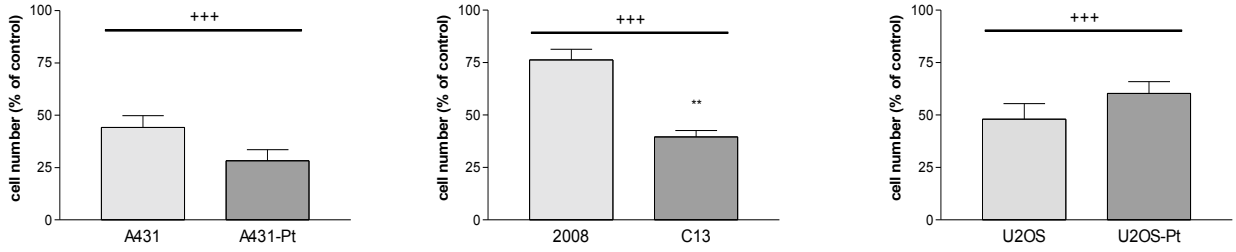


Figure 2: Effect of galactose 5mM on A431/A431-Pt, 2008/C13, U2OS/U2OS-Pt cell viability incubated for 24 hours in DMEM or in glucose free/galactose medium. Data are expressed as % of cell number compared to the control. Data are the mean±SEM of 3 different experiments. ** p<0.01, C13 *vs* 2008; +p <0.05, +++p <0.001, galactose vs control.

The second experimental strategy was to incubate cells with rotenone, an inhibitor of NADH-CoQ oxidoreductase (complex I) in the mitochondrial respiratory chain (Pitkänen S. and Robinson B.H., 1996). The results clearly demonstrate that CDDP-resistant cells C13 and A431-Pt are less sensitive than wild-type cells 2008 and A431 to the complex I inhibitor (**Fig. 3**).

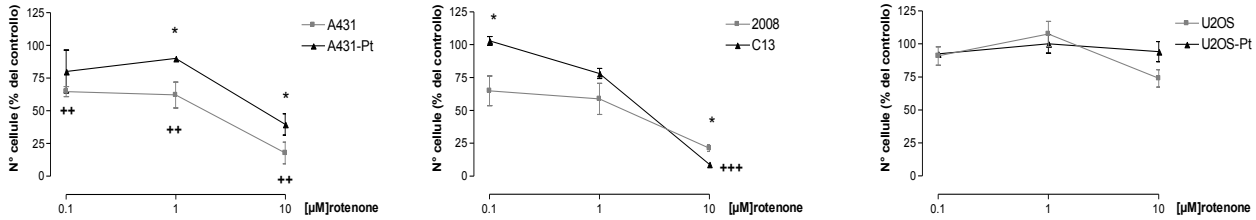
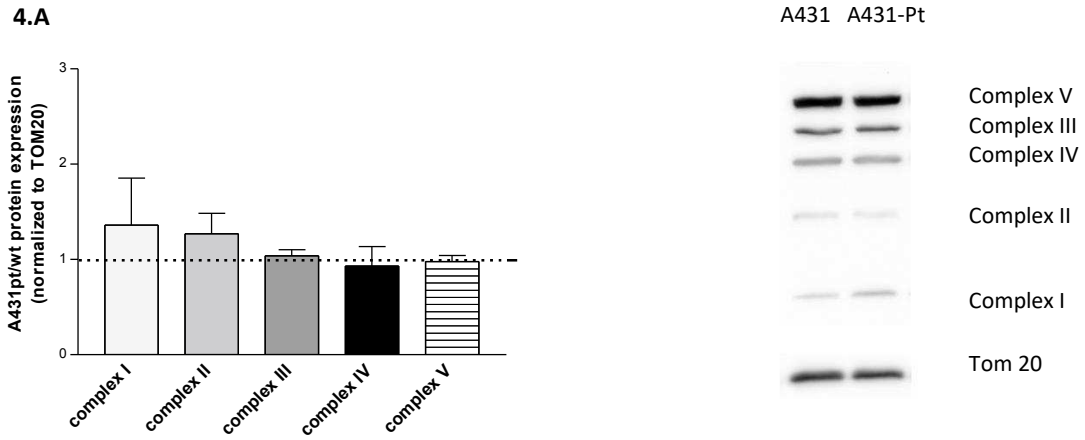


Figure 3: Effect of rotenone (0.1-10 µM) on A431/A431-Pt, 2008/C13, U2OS/U2OS-Pt cell viability after 24 hours of treatment. Data are expressed as % of cell number compared to the respective control. Data are the mean ± SEM of 3 different experiments. *p <0.05, C13 and A431-Pt vs 2008 and A431; +++ p. < 0.001 rotenone vs control.

2.3 OXPHOS expression

In order to characterize the oxidative phosphorilation, we measured the individual complexes expression of mitochondrial respiratory chain by Western Blotting. Data shown in **Figure 4** demonstrate that there are no significant differences in complexes expression; only resistant cell line U2OS-Pt (**Fig.4 c**) has a decreased expression of complex II and IV as compare to sensitive cells U2OS.



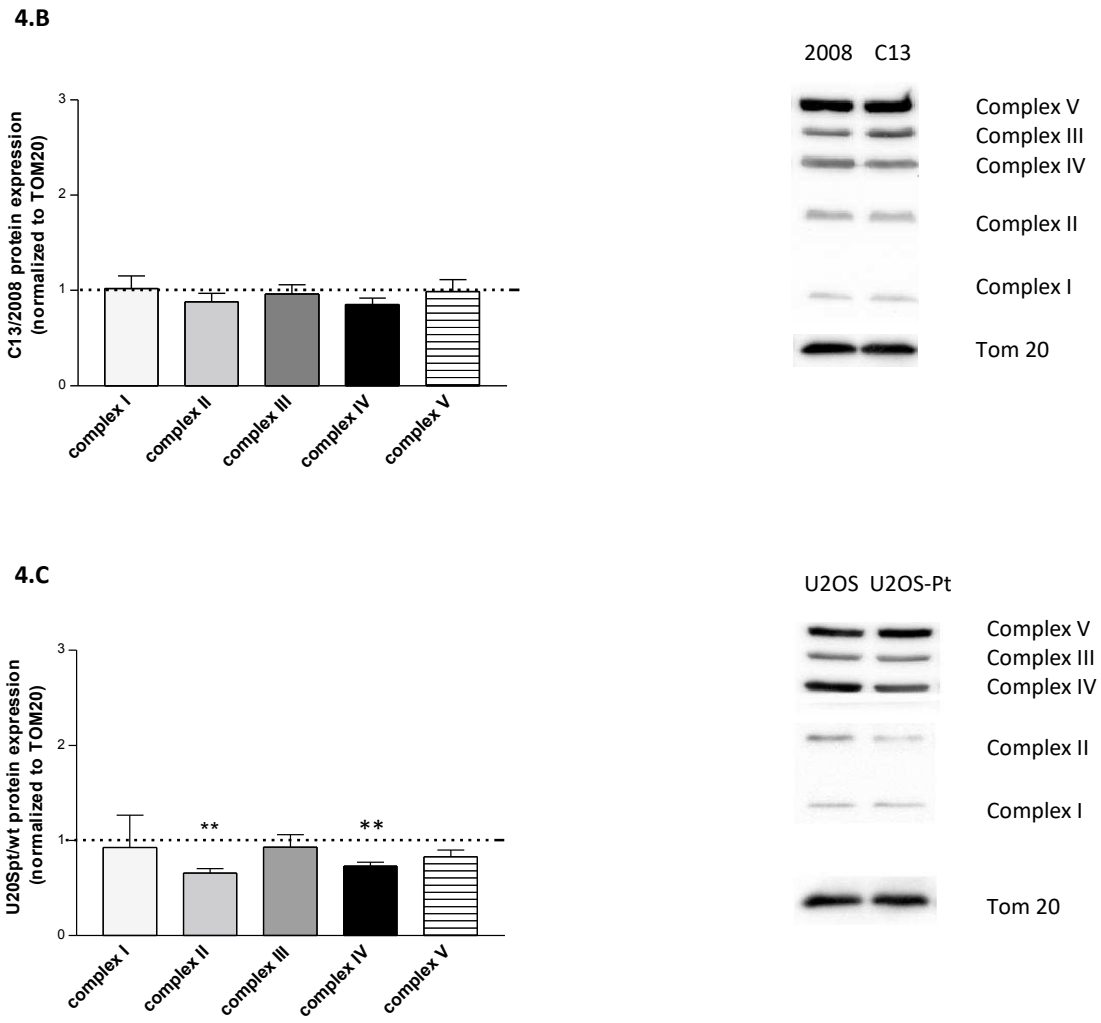


Figure 4: OXPHOS complexes protein expression. Optical density (O.D.) was normalized to TOM20 and is expressed as ratio between resistant to sensitive cell lines. Data are the mean ± SEM of four determinations. **p < 0.01 U2OS-Pt *vs* U2OS.

2.4 Mitochondrial DNA is a cisplatin target

We demonstrated that there are no difference as regard mitochondrial function between cisplatin-resistant and sensitive cancer cell lines. Thus, we would verify if mitochondrial DNA is involved in mechanism of cisplatin resistance.

Recent studies have shown that the density of mitochondria might affect the chemoresistance suggesting that mitochondria are a potential target for cisplatin (Quian W. et al. 2005). Results from early research in our laboratory demonstrated that in cisplatin-resistant cells (C13) the respiratory chain activity is lower and the dependency on glucose is higher than in cisplatin-sensitive cells 2008 (Montopoli M. et al., 2011). It was also observed that cisplatin is equipotent in both mtDNA-deprived 2008-ρ^0 and C13-ρ^0 cells, demonstrating that mtDNA lack decreases drug

sensitivity. Moreover, the results obtained in this study (**Fig.5**) confirm that mtDNA is a cisplatin target, indeed Rho 0 cell line is less sensitive to cisplatin as compare 143B TK$^-$, but not to taxol. In particular, the cisplatin IC_{50} values, obtained from concentration-response curves, are 2.84 (0.91-8.869) for 143B Tk$^-$ cells and 7.843 (3.270-18.81) for Rho0 cells. Thus, mtDNA is specifically involved in cisplatin mechanism of resistance.

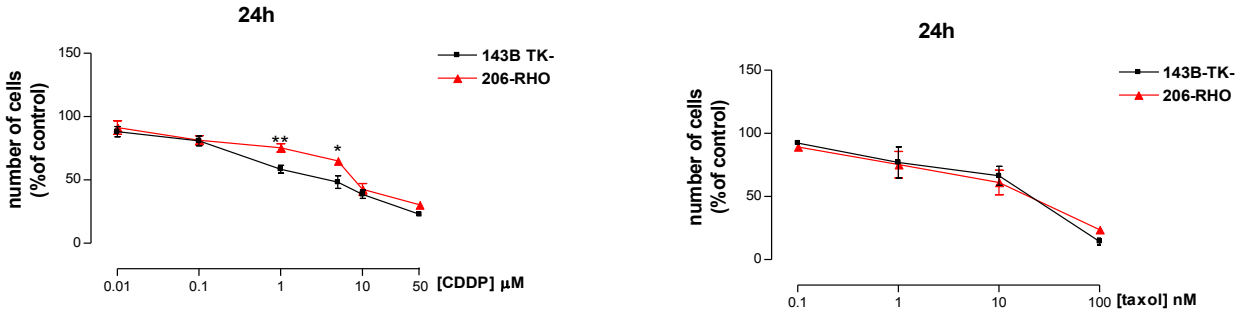


Figure 5: Effect of CDDP (0.01-0.1-1-10-50 µM) and Taxol (0.1-1-10-100 nM) on 143 TK$^-$ and 206-Rho0 cell viability after 24 hours of treatment. Data are expressed as % of cell number of treated cells compared to the respective control. Data are the mean ± SEM of 3 different experiments. *p < 0.05, **p<0.01 206-Rho0 vs 143 TK$^-$.

2.5 Mitochondrial network

Mitochondria form a dynamic structure called network whose structure is close related with the functionality of these organelles. Images (**Fig. 6**), acquired with confocal microscopy, using Mitotracker orange probe, showed a different mitochondrial network organization, common in all lines resistant to CDDP compared to their WT counterpart. In particular, in the uterine cervix cells it is possible to observe a network more condensed and tubular in the resistant clone as compare WT line in which the network is more filamentous. Instead, as regards the resistant lines of ovarian carcinoma C13 and osteosarcoma U2OS Pt, the mitochondrial network appears scattered and less structured than the lines 2008 and U2OS.

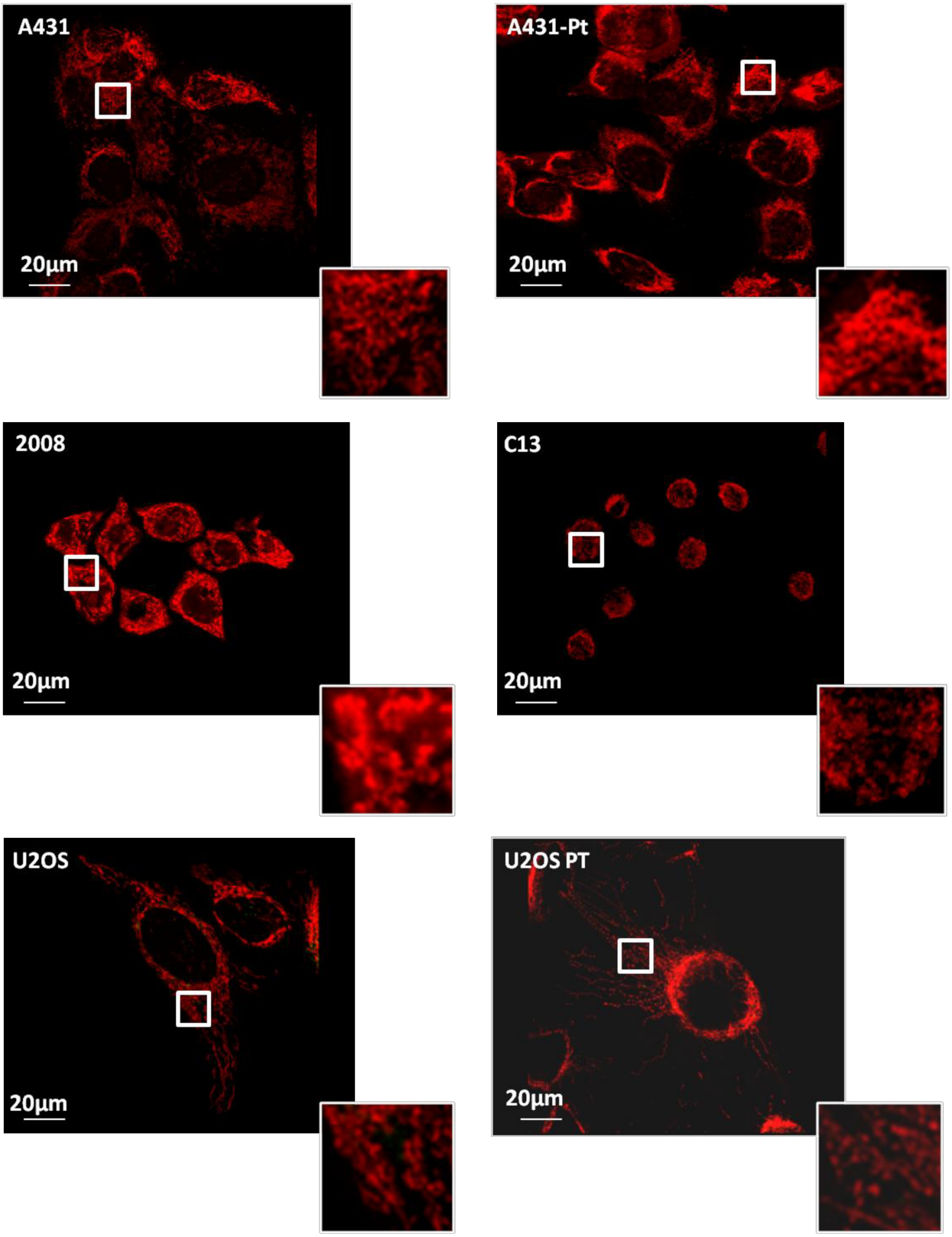


Figure 6: Images of mitochondrial network in wild type cells (A431, 2008, U2OS) and resistant (A431-Pt, C13, U2OS-Pt) acquired by confocal microscopy Nikon Eclipse E600 (60X) using Mitotracker Orange probe (λec / em: 554 / 576nm). Data represent 3 different experiments.

2.6 Mitochondrial morphology

To better investigate the mitochondria morphology, mitochondrial shape was analyzed with transmission electron microscopy (TEM). TEM images (**Fig.7**) reflect the different mitochondrial morphology obtained with confocal microscopy: there is a different mitochondrial phenotype between ciplatin-resistant and sensitive cell lines. Indeed, cisplatin-resistant cells A431-Pt exhert longer mitochondria as compared to wild type cells (A431) and the cristae are well defined; on the other hand, in cisplatin resistant cells C13 and U2OS-Pt, mitochondria appear smaller and the cristae are less defined as compared to those of sensitive lines (2008 and U2OS respectively).

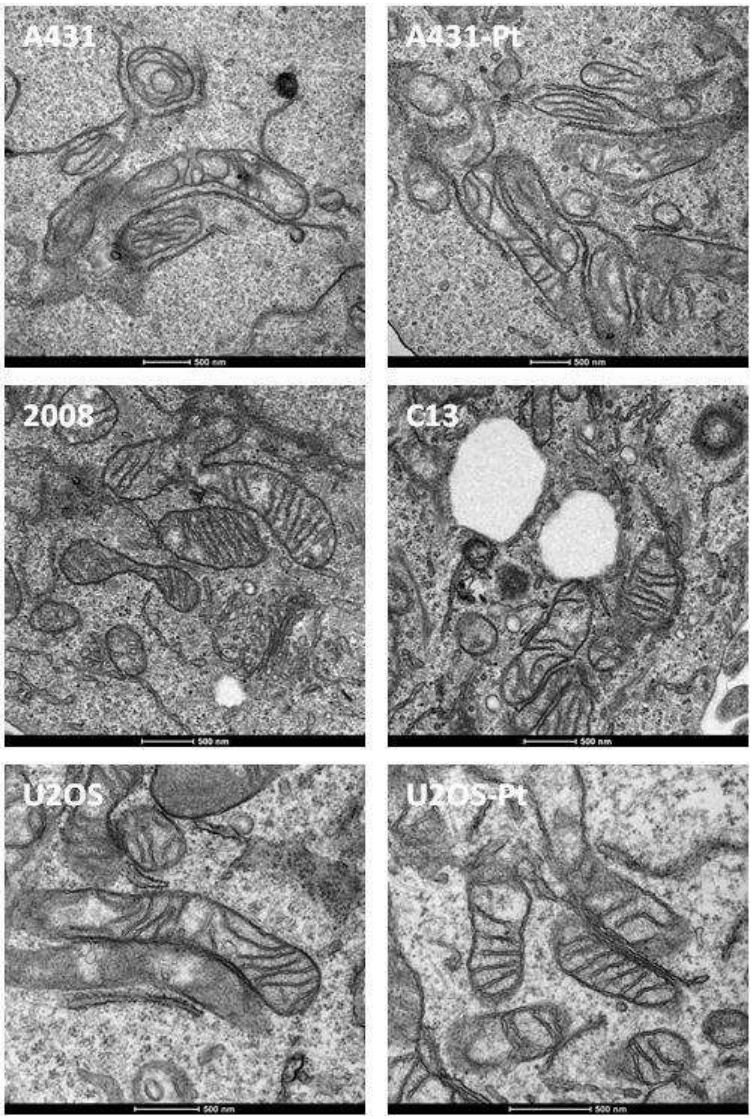


Figure 7: Images of mitochondrial morphology in wild type cells (A431, 2008, U2OS) and resistant (A431-Pt, C13, U2OS-Pt) acquired by Tecnai G2 (FEI) transmission electron microscope operating at 100 kV; images were collected by a F114 (TVIPS) CCD camera. The TEM images and experiment are performed from the University of Padua electron microscopy facility.

2.7 Mitochondrial fusion and fission

The network organization is the result of the balance between two opposite processes, fusion and fission, and the mitochondrial morphology can change in response to metabolic and pathogenetic conditions of these organelles and their intracellular environment.

2.7.1 Key regulator protein expression in fusion process

In order to understand the differences in mitochondria network organization, the expression of key proteins of this processes was evaluated: specifically the mitofusins 1 and 2, GTPases localized in the outer membrane, and OPA1 localized in the inner membrane that modulate the fusion process and cristae remodeling.

In the epidermoid carcinoma of cervix line (A431-A431 PT) (Fig. 2), it can be noted a significant increase of 40% in the OPA1 expression and 20% about MFN1 in resistant line.

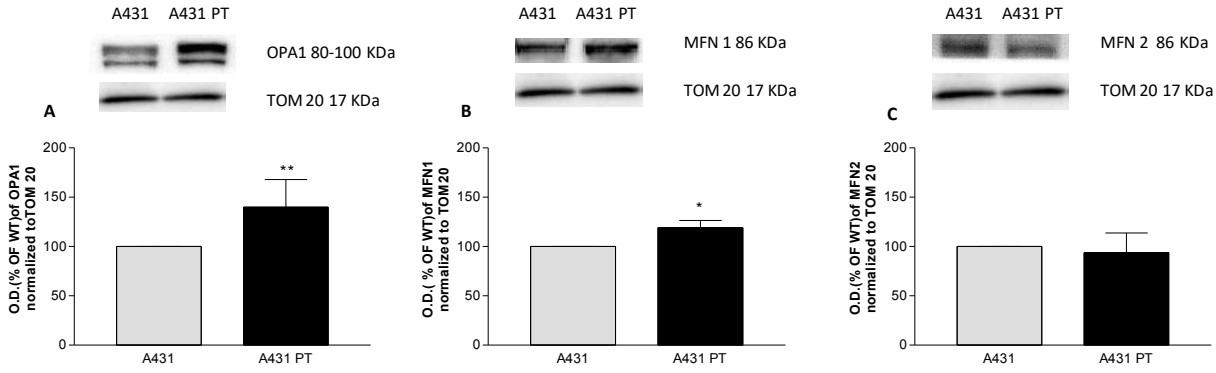


Figure 8: Expression of OPA1 (A), MFN1 (B), MFN2 (C), the optical density was normalized on TOM 20 in cancer cells wild type and resistant (A431, A431 PT). The data are expressed in percentage respect to wild-type; *p<0.05; **p<0.01; resistant vs WT. Data are the mean±SEM of 3 different experiments.

Instead in ovarian carcinoma 2008-C13 cancer cells (**Fig. 9**) there is an opposite situation in which the expression of OPA1 is 30% less in resistant clone while the expression of two mitofusins does not change significantly.

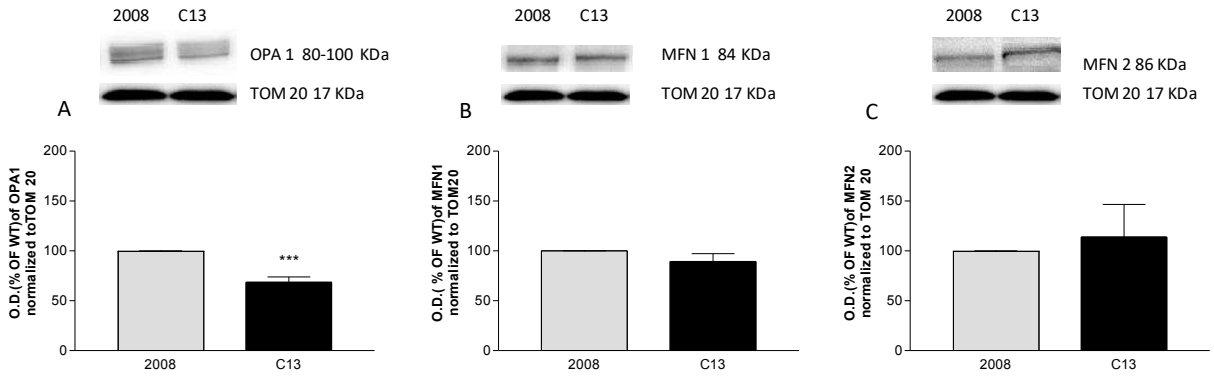


Figure 9: Expression of OPA1 (A), MFN1 (B), MFN2 (C), the optical density was normalized on TOM 20 in cancer cells wild type and resistant (2008, C13). The data are expressed in percentage respect to wild-type; ***p<0.001; resistant vs WT. Data are the mean±SEM of 3 different experiments.

Also in the resistant line U2OS PT (**Fig. 10**) there is a significant decrease of the OPA1 expression about 40% and an increase of the expression of MFN2.

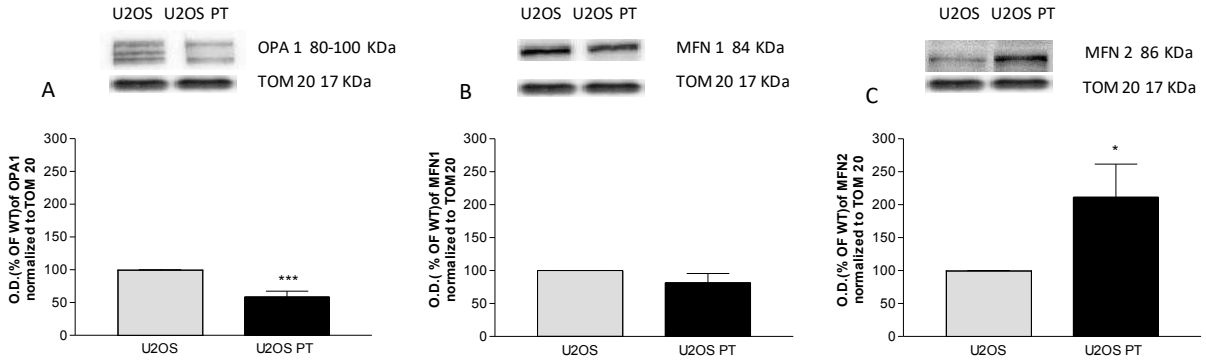


Figure 10: Expression of OPA1 (A), MFN1 (B), MFN2 (C), the optical density was normalized on TOM 20 in cancer cells wild type and resistant (U2OS, U2OS-Pt). The data are expressed in percentage respect to wild-type; *p<0.05; ***p<0.001; resistant vs WT. Data are the mean±SEM of 3 different experiments.

2.7.2 Effect of cisplatin on fusion protein

As shown in **Fig. 11**, the treatment with cisplatin (1-10 µM) for 24h induces opposite effects in resistant clone as compared to sensitive. In fact, in the line A431-Pt, OPA1 and MFN2 decrease, specially at higher concentration; in the sensitive line, instead, cisplatin increases the expression of both proteins by approximately 20%.

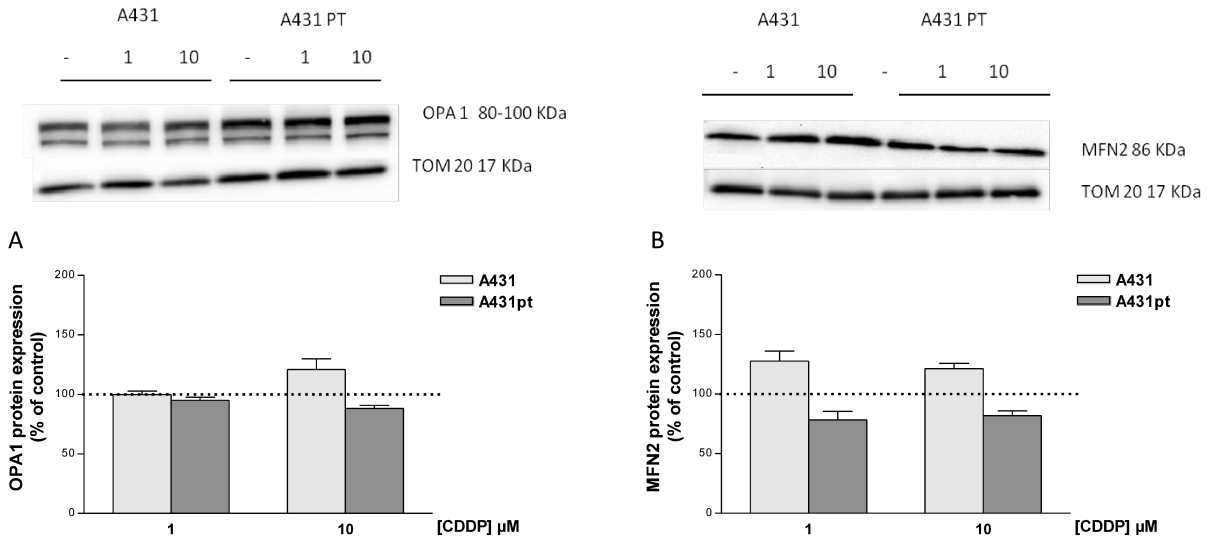


Figure 11: Expression of OPA1 (A), and MFN2 (B) after CDDP treatment (1µM-10µM), the optical density was normalized on TOM 20 in cancer cells wild type and resistant (A431, A431-Pt). The data are expressed in percentage respect to control. Data are the mean±SEM of 3 different experiments.

The lines 2008 and C13 (**Fig. 12**) have a similar trend to A431 and A431-Pt lines: in fact the cisplatin treatment induces an increase in the proteins expression in the sensitive line at 10µM concentration, with an increase of about 90%, while there is a statistically significant reduction of 10 % for OPA1 and 20% for MFN2 in the resistant line.

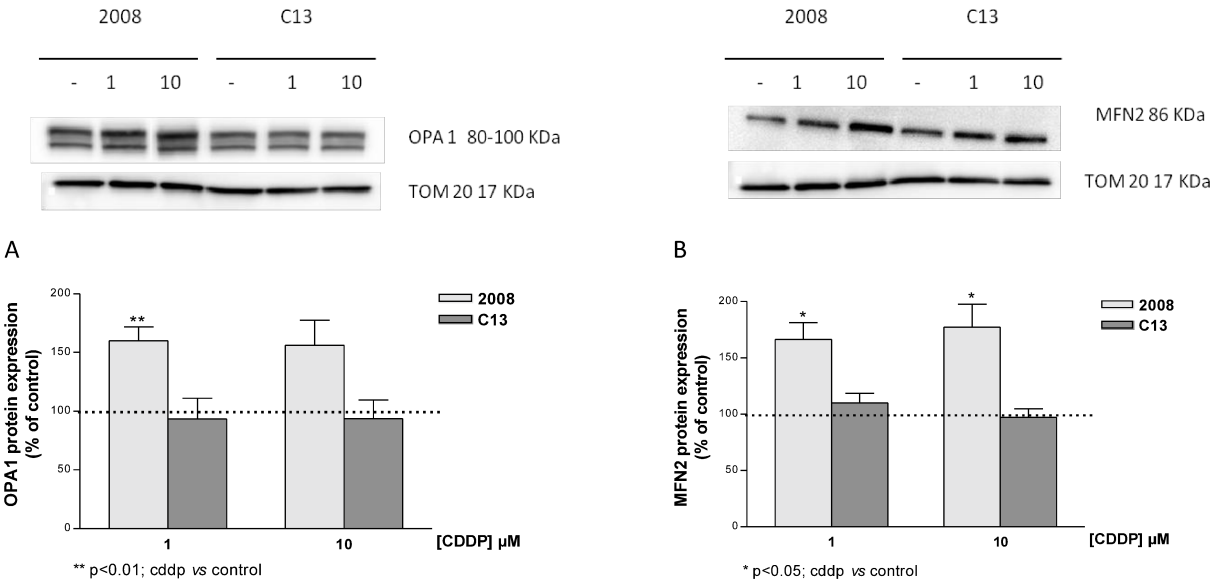


Figure 12: Expression of OPA1 (A), and MFN2 (B) after CDDP treatment (1µM-10µM), the optical density was normalized on TOM 20 in cancer cells wild type and resistant (2008, C13). The data are expressed in percentage respect to control. * p<0.05, ** p<0.01; treated vs control. Data are the mean±SEM of 3 different experiments.

Also the line of osteosarcoma U2OS and U2OS-Pt show a similar trend (Fig. 13): in the sensitive line, after cisplatin treatment, there is an increase of OPA1 (60%) and MFN2 (40%) while in resistant clone, decreases the expression of both proteins by approximately 20% for OPA1 and 10% for MFN2.

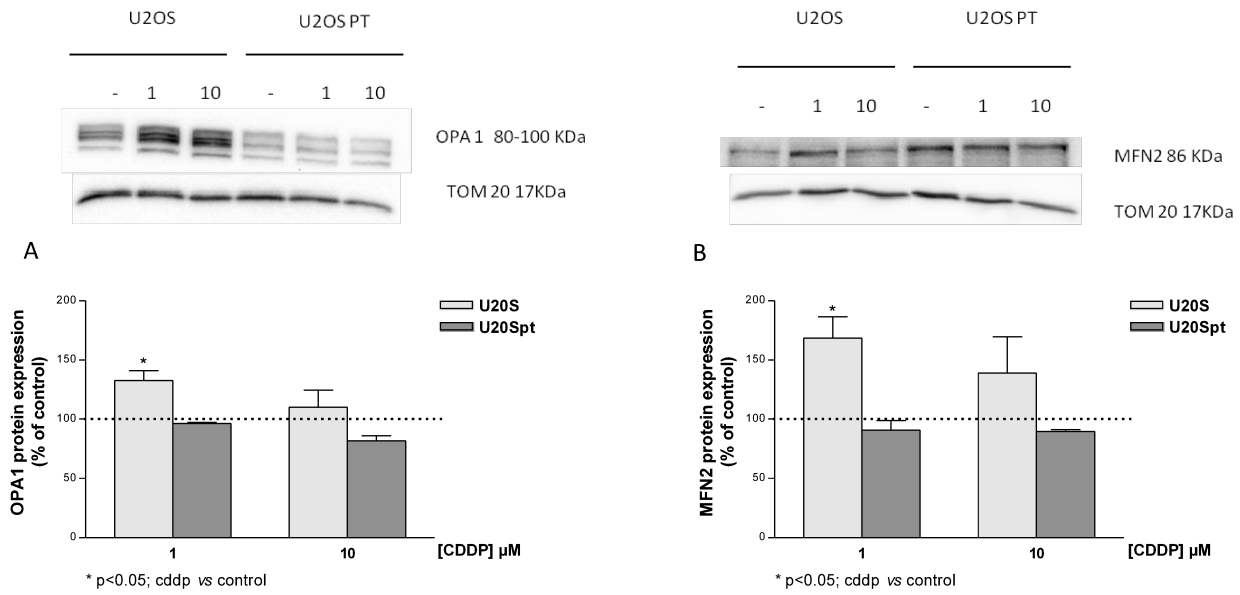


Figure 13: Expression of OPA1 (A), and MFN2 (B) after CDDP treatment (1µM-10µM), the optical density was normalized on TOM 20 in cancer cells wild type and resistant (U2OS, U2OS-Pt). The data are expressed in percentage respect to control. * p<0.05, treated vs control. Data are the mean±SEM of 3 different experiments.

2.7.3 Cisplatin response of OPA1, Mfn1 knockout and Opa1 over-expressed Mef's cells

Then, in order to check how the mitochondrial dynamics influence the cisplatin response, viability test was performed by Trypan Blue Assay, using Mouse Embryonic Fibroblast (Mef) cell lines: WT, Mef OPA1$^{-/-}$ knockout, Mef MFN1$^{-/-}$ knockout and OPA1 transgenic (over-expressed) kindly provided by prof. Scorrano Lab. The results show that the lines with an altered expression of proteins involved in mitochondrial fusion are less sensitive to cisplatin as compared to WT line and suggest a mitochondrial dynamics involvement in cisplatin activity.

Mef's cells	WT	OPA1 KO	MFN1 KO	OPA1 tg
IC_{50} CDDP(µM)	1.583 0.2154 to 11.63	2.214 0.7484 to 6.549	14.43 4.304 to 48.35	3.998 2.480 to 6.444

Table 2: CDDP cytotoxic effect, expressed as IC_{50} of Mouse Embryonic Fibroblast (Mef) cell lines: WT, Mef OPA1$^{-/-}$ knockout, Mef MFN1$^{-/-}$ knockout and OPA1 transgenic (over-expressed). Data represent 3-4 indipendent experiments.

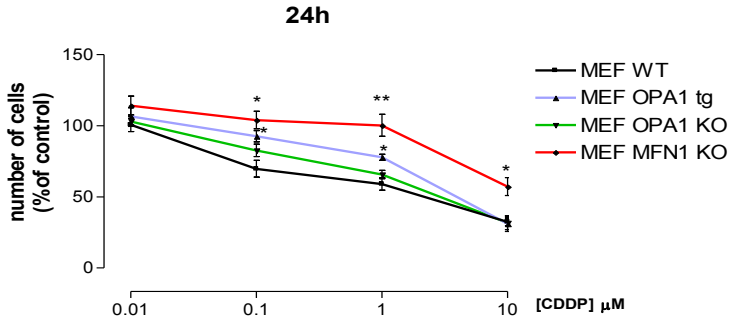


Figure 14: Effect of CDDP (0.01-0.1-1-10 µM) on Mef WT, Mef OPA1- / - knockout, Mef MFN1- / - knockout and OPA1 transgenic cell viability after 24 hours of treatment. Data are expressed as % of cell number of treated cells compared to the control. Data are the mean ± SEM of 3 different experiments. ** $p<0.01$, mef MFN1 KO vs mef WT, * $p<0.05$, mef MFN1 KO vs mef WT and mef OPA1 tg vs mef WT .

2.7.4 Key regulator protein expression in fission process

Fission is a division event that produces one or more daughter mitochondria and requires the coordination of several proteins: Drp1, HFis-1, MFF. Drp1 is a GTPases involved in the mitochondrial membrane fission; multiple receptors can recruit DRP1 to mitochondria, such as HFis1, localized in the outer mitochondrial membrane and MFF, mitochondrial fission factor. Drp1 undergoes to post-transcriptional modifications during the mitochondrial fission. The first of these modifications is the phosphorylation that promotes the distribution of Drp1 in the mitochondria and therefore their fragmentation.

By western blotting and real-time PCR, the protein expression and mRNA levels involved in the process of fission were evaluated.

In the line of the uterine cervix (A431-A431 PT) (**Fig. 15**), there is no differences between p-DRP1 protein expression, the phosphorylated isoform localized at the mitochondrial level, and total DRP1, but there is a significant decrease of the HFIS-1 gene expression about 20%.

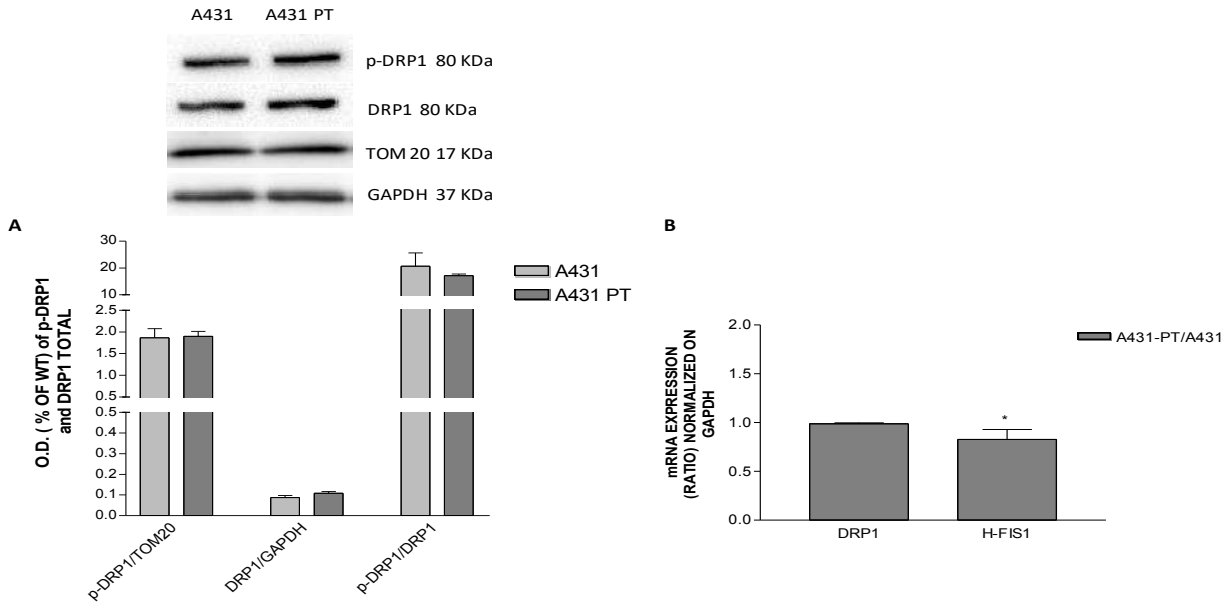


Figure 15: Expression of pDRP1 and total DRP1 (A), the optical density was normalized respectively to TOM 20 and GAPDH, in wild type and resistant (A431, A431 PT) cells. The In (B) mRNA expression of genes DRP1 and H-FIS1 normalized on GAPDH. The data are expressed as a ratio of resistant cells to wild-type set to 1. Data are the mean±SEM of 3 different experiments; *p<0.05; resistant vs WT.

Also in ovarian carcinoma lines 2008-C13 (**Fig. 16**) the p-DRP1 protein expression is similar to total Drp1. The HFIS-1 gene expression is higher in resistant clone then sensitive cells.

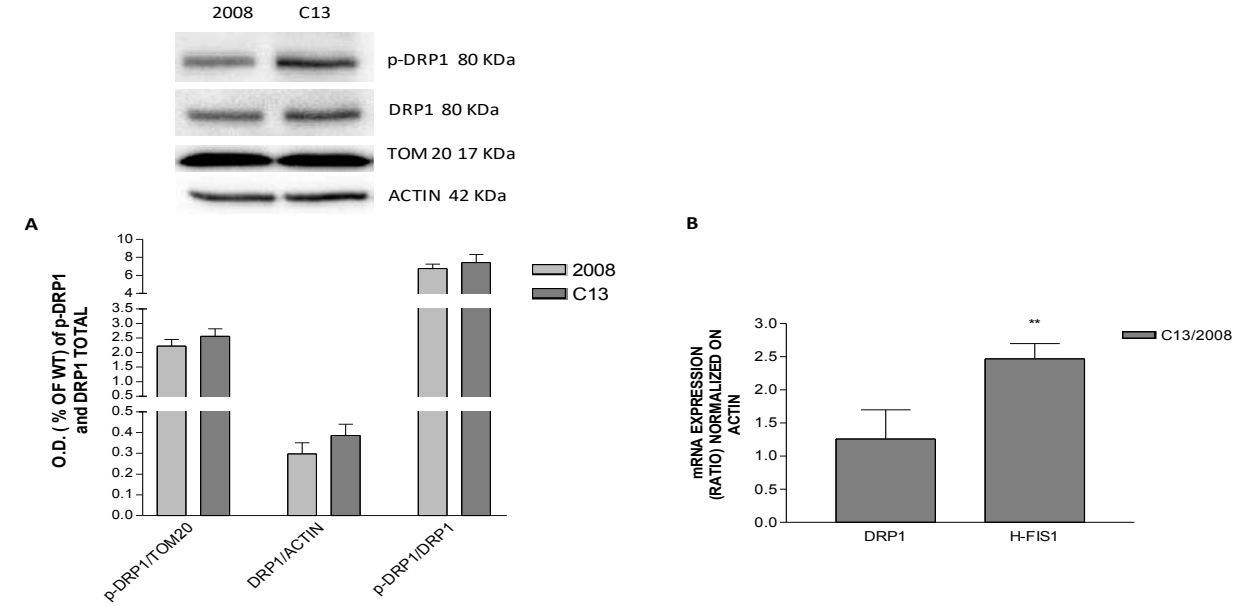


Figure 16: Expression of pDRP1 and total DRP1 (A), the optical density was normalized respectively to TOM 20 and actin, in wild type and resistant (2008, C13)cells. In (B) mRNA expression of genes DRP1 and H-FIS1 normalized on actin. The data are expressed as a ratio of resistant cells to wild-type set to 1. Data are the mean±SEM of 3 different experiments; **p<0.01; resistant vs WT.

In resistant line U2OS PT (**Fig. 17**) there is an increased expression of p-DRP1 and total DRP1 and also the gene expression of HFIS1 (50%).

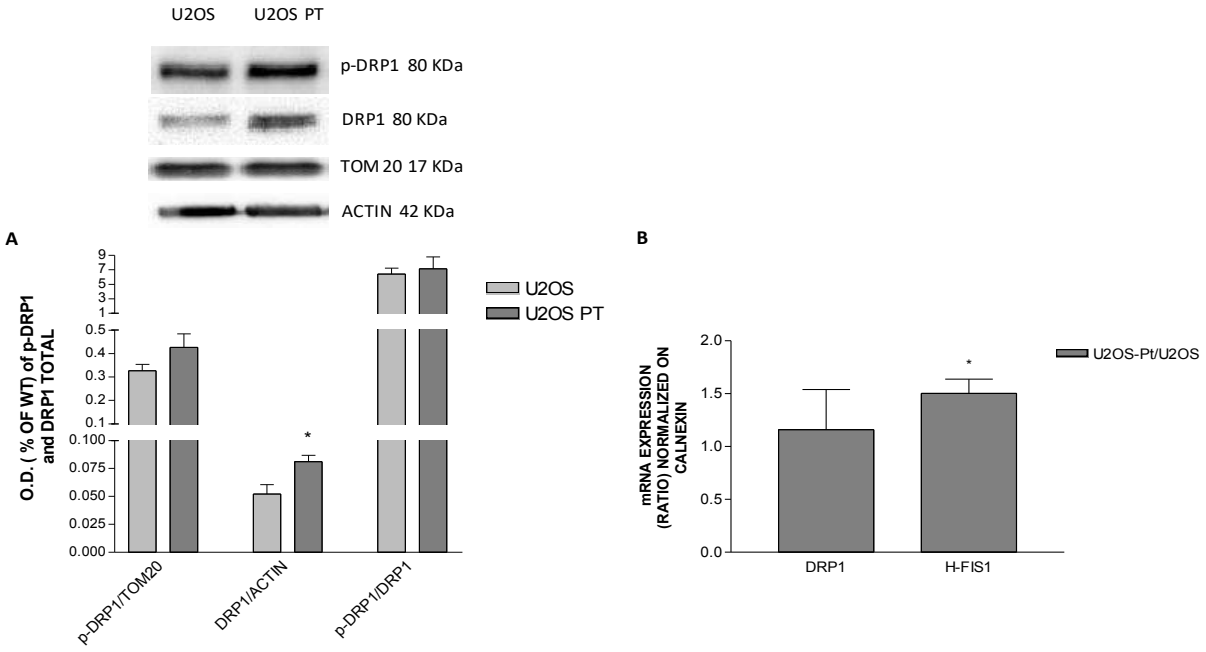


Figure 17: Expression of pDRP1 and total DRP1 (A), the optical density was normalized respectively to TOM 20 and actin, in wild type and resistant (U2OS, U2OS-Pt) cells. *p<0.01; resistant vs WT. In (B) mRNA expression of genes DRP1 and H-FIS1 normalized on calnexin. Data are the mean±SEM of 3 different experiments. The data are expressed as a ratio of resistant cells to wild-type set to 1; *p<0.05; resistant vs WT.

Even if variations in the ratio between active form p-Drp1 and total Drp1 are not observed, it is evident the higher expression of both isoform of Drp1 in resistant clones C13 and U2OS-Pt as compare to sensitive counterpart, suggesting an involvement of Drp1 protein in the modulation of fission process in ovarian carcinoma and osteosarcoma resistant cells.

To better investigate the fission process, also mitochondrial fission factor (Mff) protein expression was evaluated by Western Blot. All resistant clones have an higher expression of at least one of the two Mff isoforms (**Fig.18**), but the increased expression is significantly greater in C13 and U2OS-Pt resistant cell lines than A431-Pt.

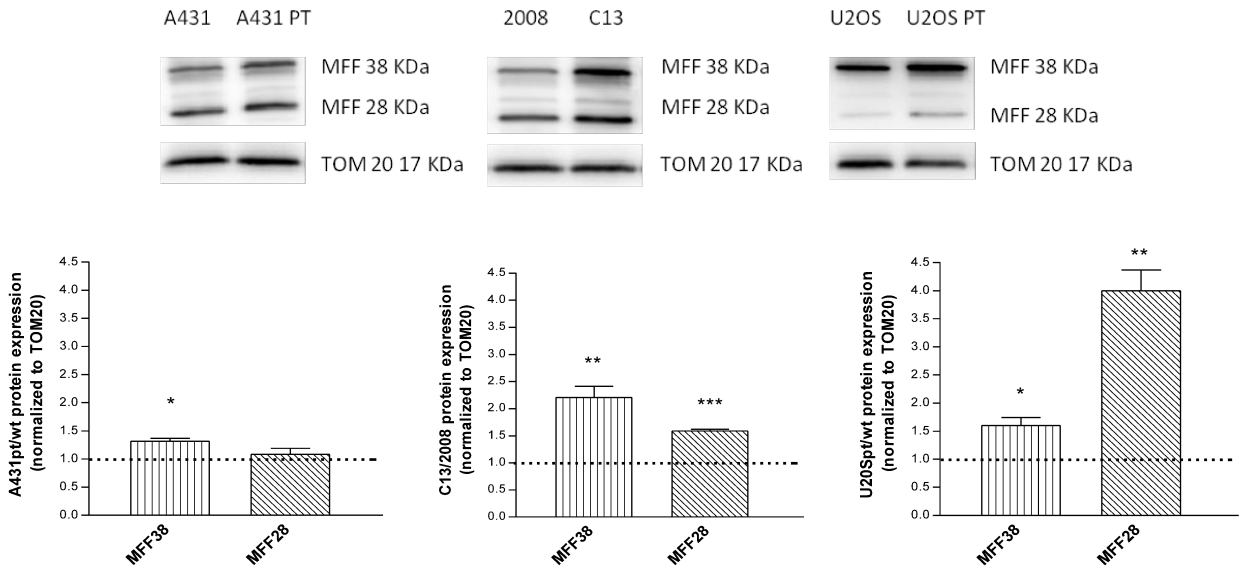


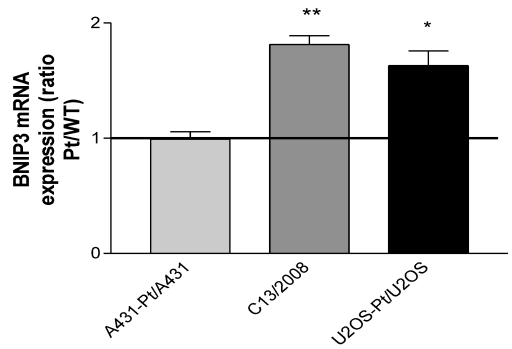
Figure 18: Mff protein expression , the optical density was normalized on TOM 20 in cancer cells wild type and resistant (U2OS, U2OS-Pt). Data are the mean±SEM of 3 different experiments. The data are expressed in percentage respect to wild-type. **p<0.001; resistant vs WT.

2.8 Imbalance toward fission leads to Mitophagy

The mitochondrial dynamic is closely correlated to selective mitochondria autophagy (mitophagy). In particular It has been suggested that fragmented mitochondria are more readily taken up by autophagosomes due to their smaller size; moreover Fis1 was found to be involved in mitophagy (Barsoum et al., 2006). Thus mitochondrial dysfunction, rather than mitochondrial fragmentation, is responsible for the mitophagy induction.

Data obtained by real time q-PCR (**Fig.19**) showed that the resistant clones C13 and 2008 have BNIP3 mRNA levels higher then WT lines. Furthermore resistant cancer cells C13 and U2OS-Pt show an increased BNIP3 protein expression (marker of Mitophagy) as compare to sensitive lines, but not A431-Pt line.

A



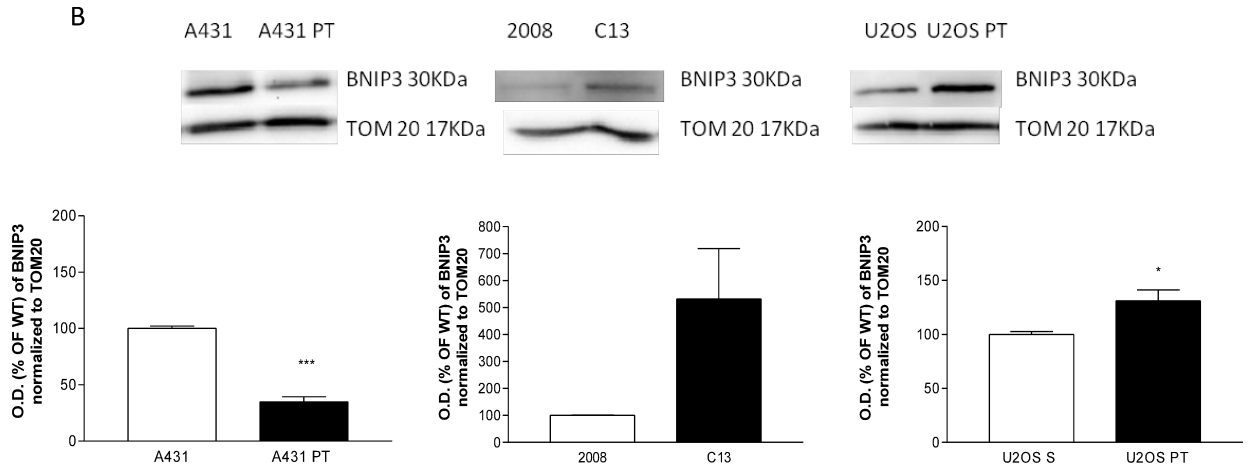


Figure 19: (A)mRNA expression of BNIP3 gene normalized on GAPDH (A431), beta-actin (2008-C13) and calnexin (U2OS). The data are expressed as a ratio of resistant cells to wild-type set to 1. (B) BNIP3 protein expression of A431/A431-Pt; 2008/C13; U2OS/U2OS-Pt normalized to TOM20. The data are expressed as percentage of resistant cells to wild-type. Data are the mean±SEM of 3-4 different experiments.; *p<0.05, ***p<0.001; resistant vs WT.

Moreover it has been evaluated the LC3 protein expression, involved in the autophagy flux, in order to check if autophagic process was involved in cisplatin response. The protein expression of LC3 I (cytoplasmic isoform) and II (product of proteolytic maturation, is located at the level of the membranes of autophagosome during the autophagy) is different between resistant lines and respective WT. In lines A431 and A431 PT cancer cells (**Fig.20**) was observed an increase in resistant clone of approximately 215% of LC3-B I, 35% of LC3-B II.

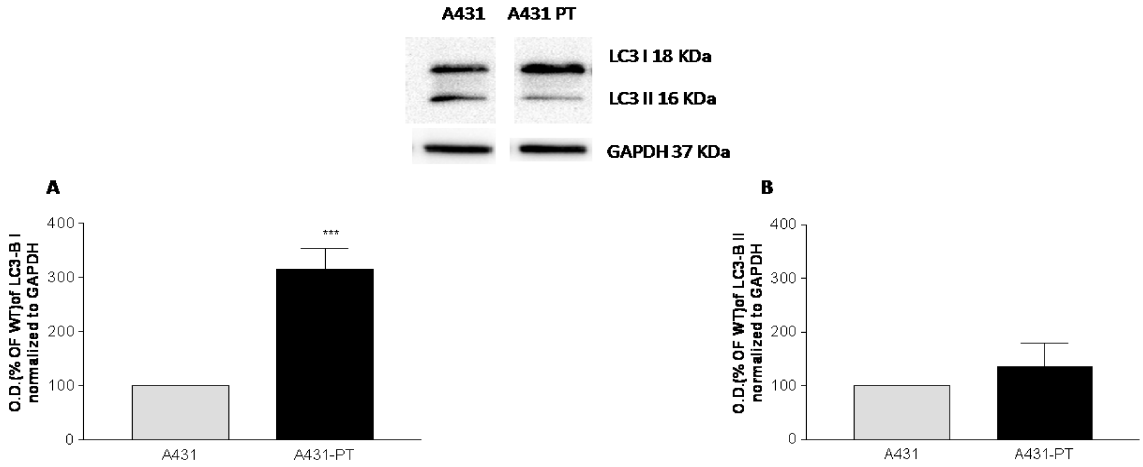


Figure 20: Expression of LC3-B I(A), LC3-B II (B), the optical density was normalized on GAPDH in cancer cells wild type and resistant (A431, A431 PT). Data are the mean±SEM of 3 different experiments. The data are expressed in percentage respect to wild-type. *** p<0.001 resistant *vs* WT.

In basal conditions, as can be seen in **Fig. 21**, the C13 line, compared to 2008, expressed more the isoform LC3-B I (about 194%).

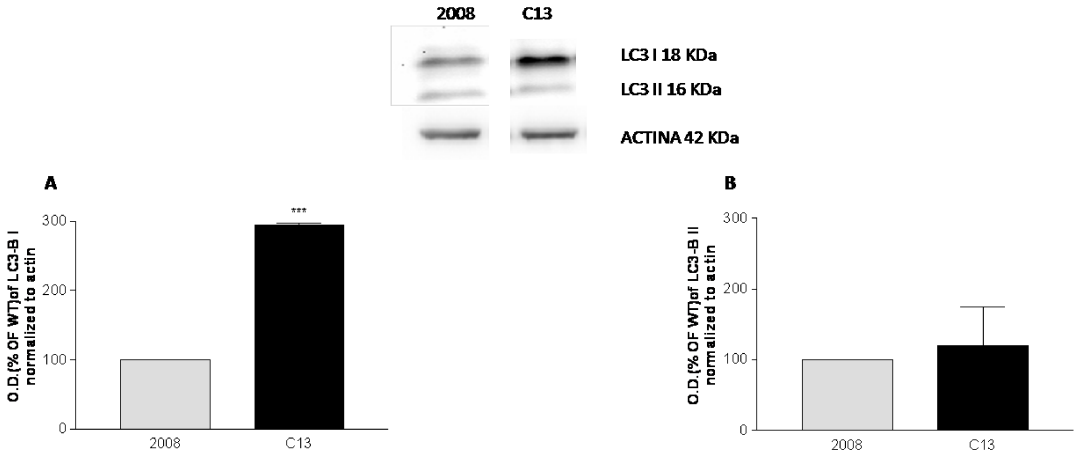


Figure 21: Expression of LC3-B I(A), LC3-B II (B), the optical density was normalized on beta-actin in cancer cells wild type and resistant (2008, C13). Data are the mean±SEM of 3 different experiments. The data are expressed in percentage respect to wild-type. *** p<0.001 resistant *vs* WT.

The two osteosarcoma lines didn't show significant differences in the expression of LC3-B II, while there is a decrease of LC3-B I (about 25%) in resistant cancer cells (**Fig.22**)

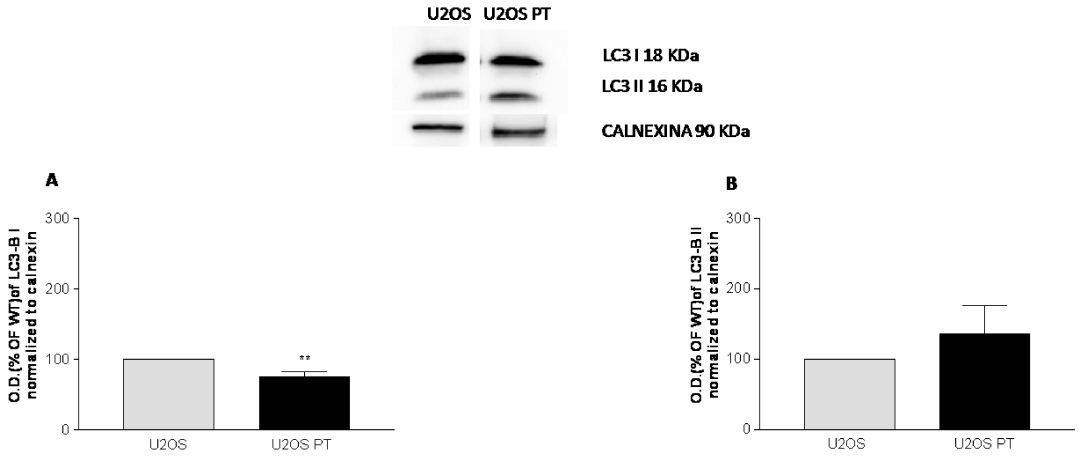


Figure 22: Expression of LC3-B I(A), LC3-B II (B), the optical density was normalized on beta-actin in cancer cells wild type and resistant (U2OS, U2OS-Pt). Data are the mean±SEM of 3 different experiments. The data are expressed in percentage respect to wild-type. ** p<0.01 resistant *vs* WT.

2.9 TRAP1 role in the regulation of mitochondrial dynamics

The results reported in the literature (Takamura et al. 2013) show TRAP1, (Tumor Necrosis Factor Receptor Associated Protein 1), the chaperonine localized in the mitochondrial matrix with specific function in the regulation of mitochondrial function, as a possible regulator factor in the balance between the fusion and fission processes.

In collaboration with the professor Rasola (University of Padova) the expression of TRAP1 was evaluated. In the lines A431 and A431 PT of the protein expression is different: in **Fig. 23** shows that resistant cancer cells presents a reduced expression of TRAP1 as compare to the sensitive line (approximately 30%).

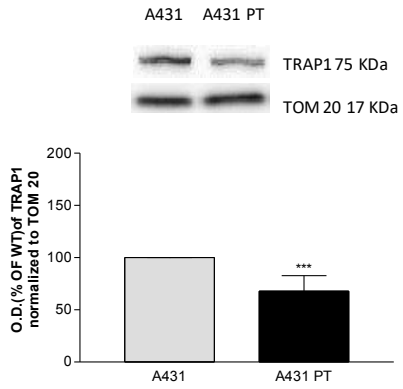


Figure 23: Expression of TRAP1, the optical density was normalized on TOM 20 in cancer cells wild type and resistant (A431, A431-Pt). Data are the mean±SEM of 3 different experiments. The data are expressed in percentage respect to wild-type. **p<0.01; resistant *vs* WT.

Instead, the protein profile of TRAP1, does not differ in 2008 and C13 cancer cell lines (**Fig. 24**).

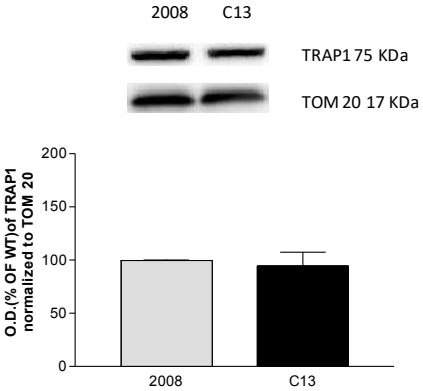


Figure 24: Expression of TRAP1, the optical density was normalized on TOM 20 in cancer cells wild type and resistant (2008, C13). Data are the mean±SEM of 3 different experiments. The data are expressed in percentage respect to wild-type.

The osteosarcoma line (U2OS-U2OS-Pt) (**Fig. 25**) has a greater expression of TRAP1 in resistant clone of approximately 45%.

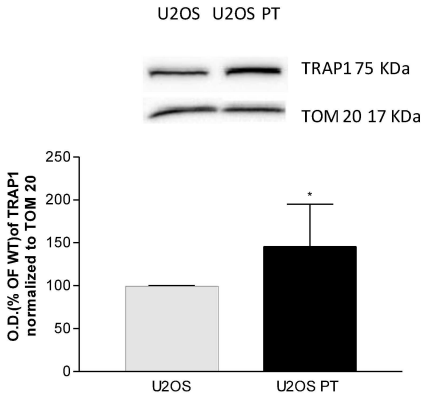
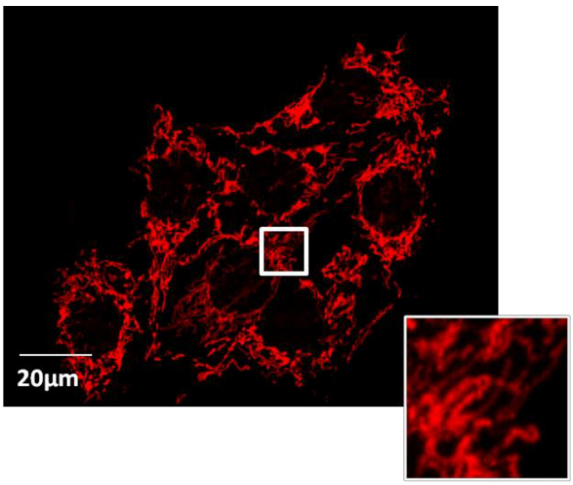


Figure 25: Expression of TRAP1, the optical density was normalized on TOM 20 in cancer cells wild type and resistant (2008, C13). Data are the mean±SEM of 3 different experiments. The data are expressed in percentage respect to wild-type. *p<0.05; resistant *vs* WT.

Thus, the resistant clones that have an imbalance to the fusion, have a decreased expression of TRAP1, and in the resistant clones that have an imbalance towards the fission there is an opposite situation. In order to understand if TRAP1 is involved in regulation of mitochondrial dynamic, it has been analyzed the network organization in Mef's lines with TRAP1 silenced. The confocal microscopy images shows a different mitochondrial network in the two shTRAP1 Mef's lines (sh70 70% of silencing; sh72 100% of silencing) as compared to the scrambled.

Mef scrambled



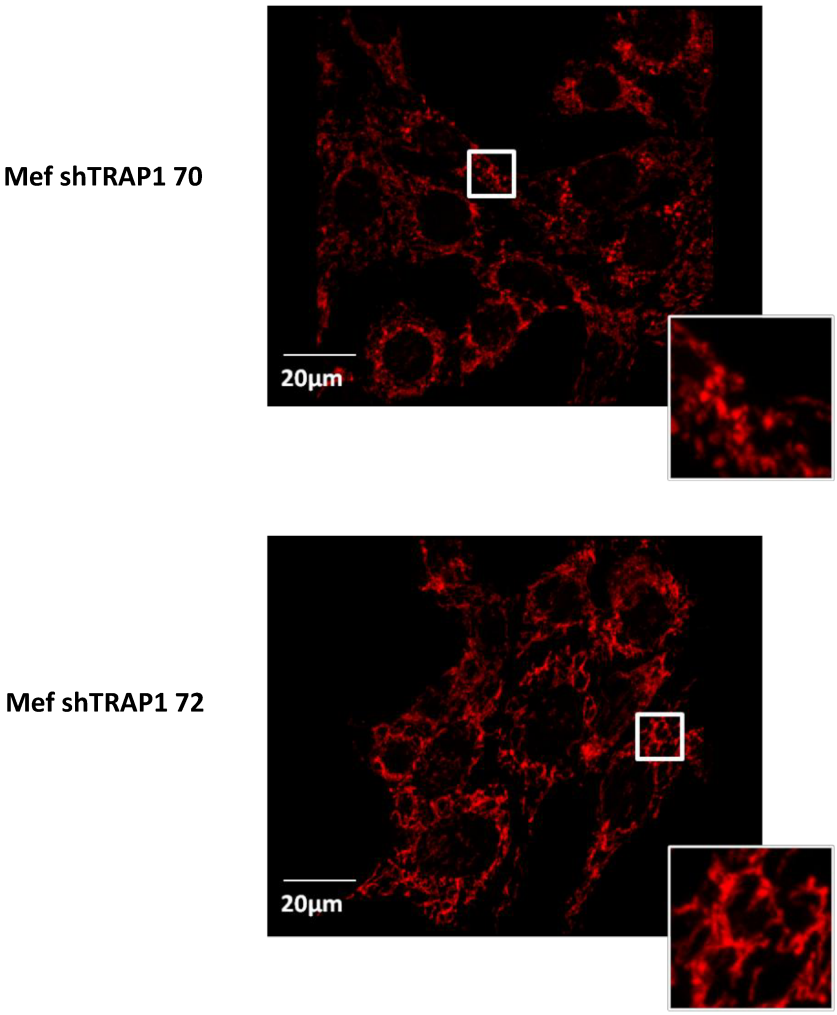


Figure 26: Mitochondrial Network in Mef 's scrambled, Mef's sh70TRAP1 and Mef's sh72TRAP1, measured by confocal microscopy Nikon Eclipse E600 (60X) using Mitotracker Orange (λec/em:554/576nm). Data represent 3 different experiments.

Finally, the expression of mitochondrial fission and fusion key proteins in the shTRAP1 Mef lines was measured. Indeed in the two shTRAP1 cell lines there is an increase of OPA1 and MFN1 expression and a decrease of DRP1 (**Fig.27**).

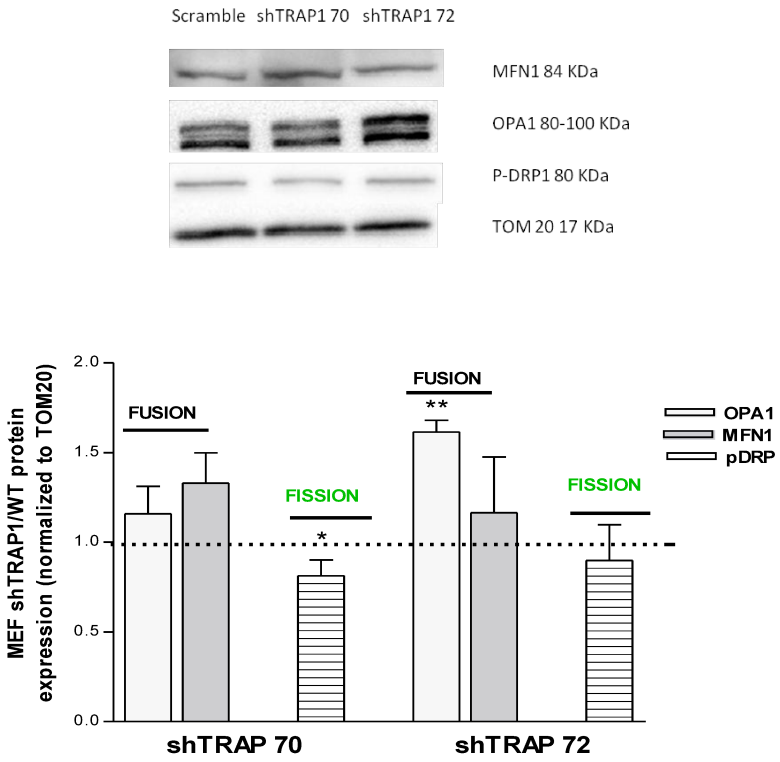


Figure 27: Expression of OPA1, MNF1 and p-DRP1 the optical density was normalized on TOM 20 in shTRAP1 and scambled Mef cells. Data are the mean±SEM of 3 different experiments. The data are expressed as ratio between shTRAP1 and scramble. *p<0.05, **p<0.01; resistant *vs* WT.

GLUCOSE AND GLUTAMINE METABOLISM IN CISPLATIN RESISTANCE

The metabolic reprogramming of the tumor cells is characterized by alteration of several oncogenes and oncosuppressor genes that can lead to important changes in energy metabolism such as the increase in the glycolytic and glutaminolytic flow, but also the up-regulation of amino acids and the lipids metabolism, induction of PPP pathway and macromolecules biosynthesis (Chen J.Q. and Russo J. 2012).

Cisplatin could be accumulate rapidly in mitochondria and deteriorate the mitochondrial DNA and metabolic function, leading to significant changes in the levels of metabolites involved in tricarboxylic acid cycle (TCA cycle) and glycolysis pathway. Due to its anaplerotic role in the TCA cycle, glutamine replenishes the intermediates needed by most cancer cells to synthesize macromolecules. Therefore, reduced glutamine metabolism may limit the proliferation of cancer cells and thereby serve as a metabolic checkpoint that becomes activated in response to genotoxic stress such as cisplatin (Jeong S.M. et al., 2013).

3. Phenotyping sensitive and cisplatin resistant cell: innate resistance model

The study of our laboratory of Padova have already shown a metabolic reprogramming in cancer cells that exhibit acquired resistance to cisplatin. In fact it has been shown how the ovarian cancer cells C13, resistant to cisplatin, increases its glucose dependency and is more sensitive to the glycolysis block. Moreover, in the presence of an altered mitochondrial function, glutamine, due to its anaplerotic role in the TCA cycle, becomes the privileged carbon resource, for the cisplatin-resistant line (Catanzaro et al.,2015).

In this scenario, our goal was to check if there was a energetic metabolic remodeling even in an innate resistance model. Thus, in Toker Laboratory (BIDMC, Harvard Medical School, Boston), glutamine and glucose metabolisms and their regulation controlled by the oncogene c-Myc were evaluated in breast cancer cells that present an intrinsic resistance to cisplatin.

3.1 Triple Negative Breast Cancer cells selection

Breast cancer cell lines that present different sensitivity to CDDP treatment were selected. Four lines were identified, which respond differently to CDDP treatment and to doxorubicin treatment (24 + 48 hours). These two chemotherapeutic agents are used in combination for the

treatment of breast cancer, in particular for TNBC (triple negative breast cancer) that does not respond to hormonal therapy; the cell lines studied are all TNBC cells, basal-like.

As shown in **Figure 28,** MDA-MB-468 and SUM 149 show an higher sensitivity to cisplatin and doxorubicin, while HCC1143 and HCC1937 are resistant to the treatment.

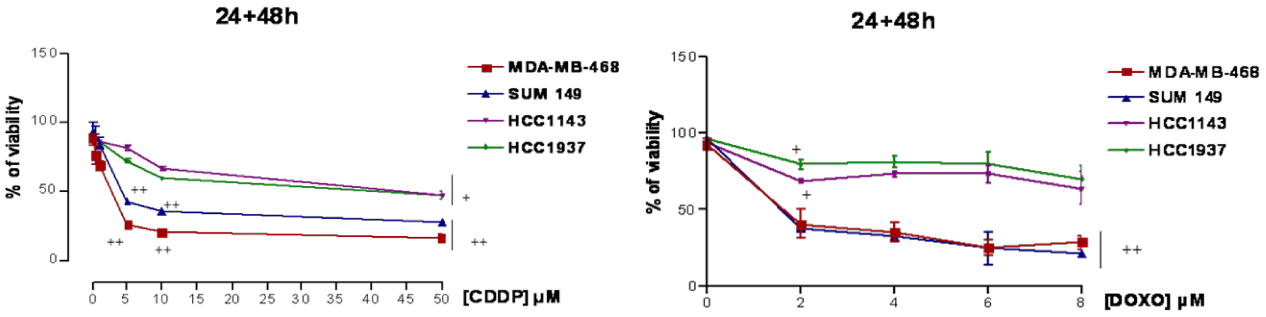


Figure 28: Effect of CDDP (0.1-1-5-10-50 µM) and doxorubicin (2-4-6-8 µM) on MDA-MB-468, SUM 149, HCC1143 and HCC1937 cell viability after 24+48 hours of treatment measured by Propidium Iodide Assay. Data are expressed as % of cell viability of treated cells compared to the respective control. Data are the mean ± SEM of 3 different experiments. +p<0.05, ++p<0.01; treated *vs* control.

Moreover the MDA-MB-468 and SUM 149 sensitive lines showed PARP cleavage after CDDP treatment (induction of apoptotis markers), but not the cisplatin resistant cells (HCC1143 and HCC1937) (**Fig. 29**).

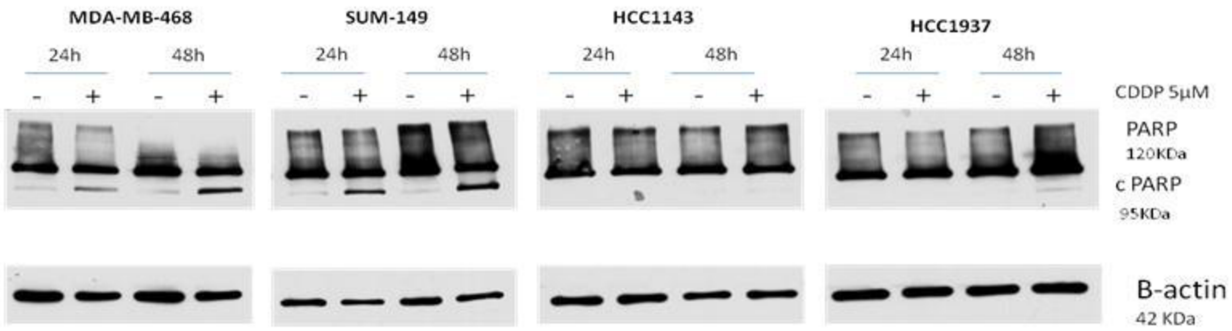


Figure 29: Protein expression of PARP and cPARP after treatment with CDDP 5µM (24 and 48 hours), the normalization protein used was β-actin. Data are representative of three independent experiments.

3.2 Effect of glutamine and glucose deprivation on cell viability

Several studies have proven that interfering with glutamine metabolism can inhibit the growth of various types of cancer cells, including breast cancer (Chen L. et al., 2015).

Therefore, cell viability was measured by Propidium Iodide assay and cell growth curves by Trypan Blue assay, following treatment in glutamine deprivation (with the dialyzed serum), and with medium containing a low glutamine concentration (650µM), concentration of physiological glutamine, (Tardito et al. 2014) as compare to the concentration of glutamine which is normally in the culture media (2055 µM). The **Figure 30** shows a greater sensitivity to glutamine deprivation in two cell lines: SUM149 and HCC1143, that is significant after seven days of treatment.

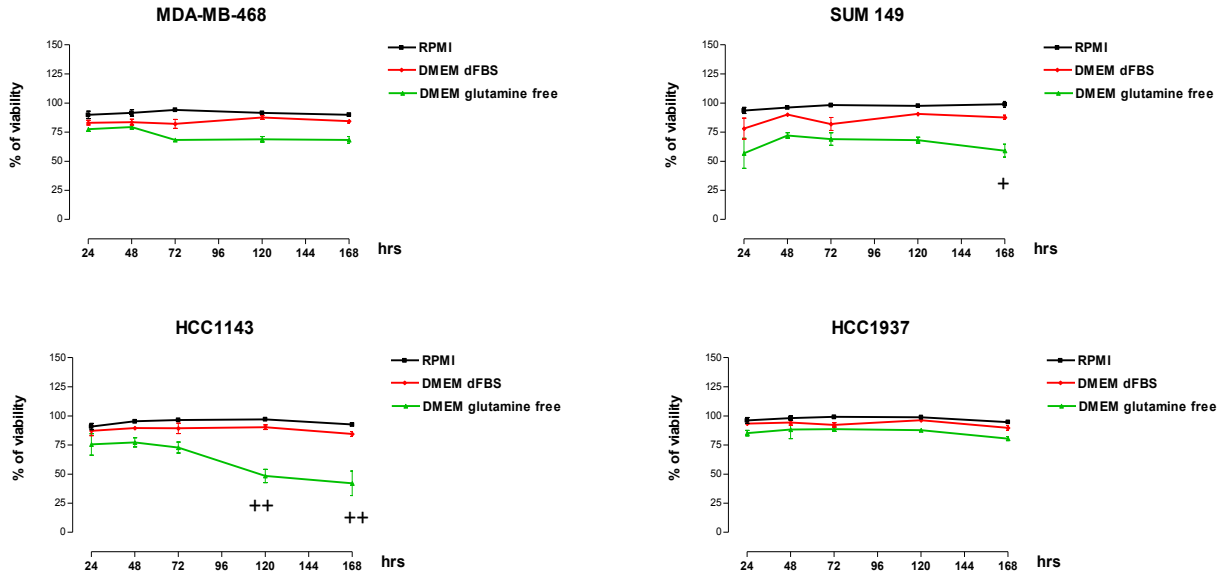


Figure 30: Effect of glutamine deprivation on MDA-MB-468, SUM 149, HCC1143 and HCC1937 cell viability after 24+48+72+120+168 hours of treatment measured by Propidium Iodide Assay. Data are expressed as % of cell viability of treated cells compared to the respective control. Data are the mean ± SEM of 3 different experiments. +p<0.05, ++p<0.01; treated *vs* control.

Also the **Figure 31**, which represents the growth curves, confirms a greater sensitivity to glutamine deprivation in SUM149 and HCC1143 cell lines especially after 48 and 72 hours where it is significant; whereas as regards the treatment with low concentration of glutamine there are no significant differences.

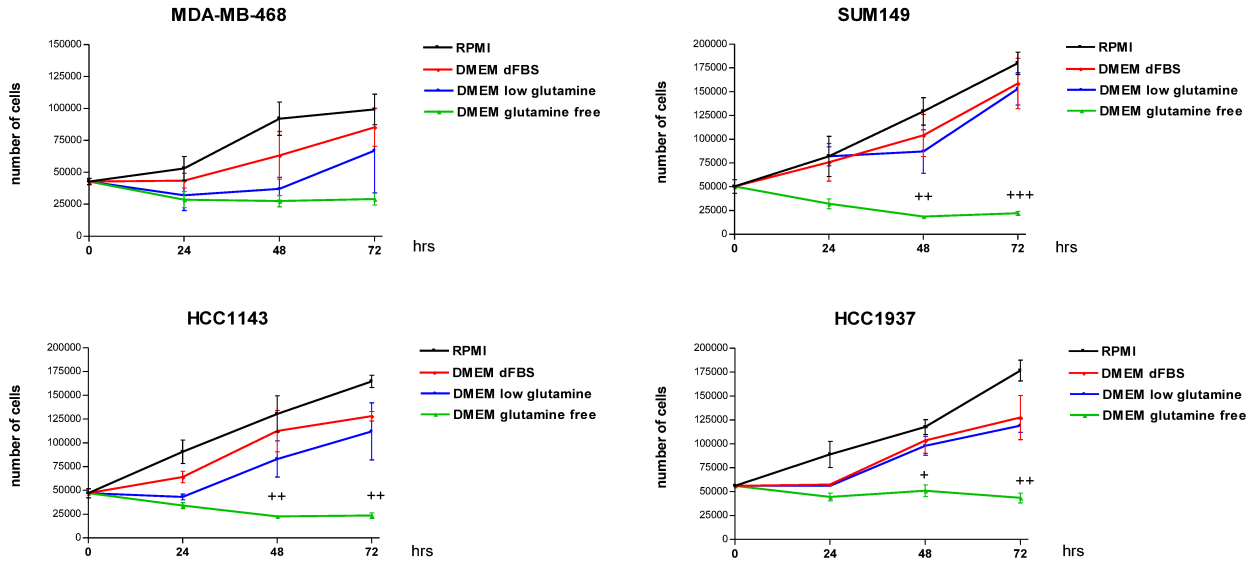


Figure 31: Effect of glutamine deprivation on MDA-MB-468, SUM 149, HCC1143 and HCC1937 cell viability after 24+48+72 hours of treatment measured by Trypan Blue Assay. Data are expressed as % of cell number of treated cells compared to the respective control. Data are the mean ± SEM of 3 different experiments. +p<0.05, ++p<0.01, +++p<0.001; treated *vs* control.

To further confirm the data obtained with the assays of cell viability, it has been performed Western blot analysis to evaluate the cleavage of PARP in these four lines, after treatment in glutamine deprivation at 24 and 48 hours. In **Figure 32** the SUM149 and HCC1143 TNBC lines, which demonstrate cleavage of PARP, present an activation of apoptotic mechanism following the deprivation of glutamine.

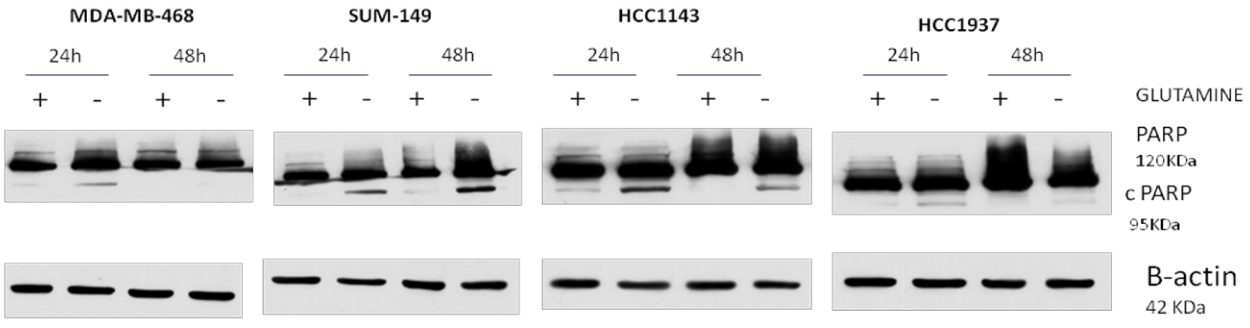


Figure 32: Protein expression of PARP and cPARP after treatment in glutamine-free medium, the normalization protein used was β-actin. Data are representative of three independent experiments.

Having already obtained interesting data in cisplatin resistant ovarian carcinoma cells about glucose metabolism, glucose dependency was evaluated in TNBC line to investigate if glucose is involved in mechanisms of intrinsic resistance. Cell viability assay was performed by Trypan Blue assay measuring cell growth curves in glucose free media and in media with low glucose (1g/L). As shown in **Figure 33**, the cisplatin resistant lines, HCC1143 and HCC1937, have a greater dependence on glucose.

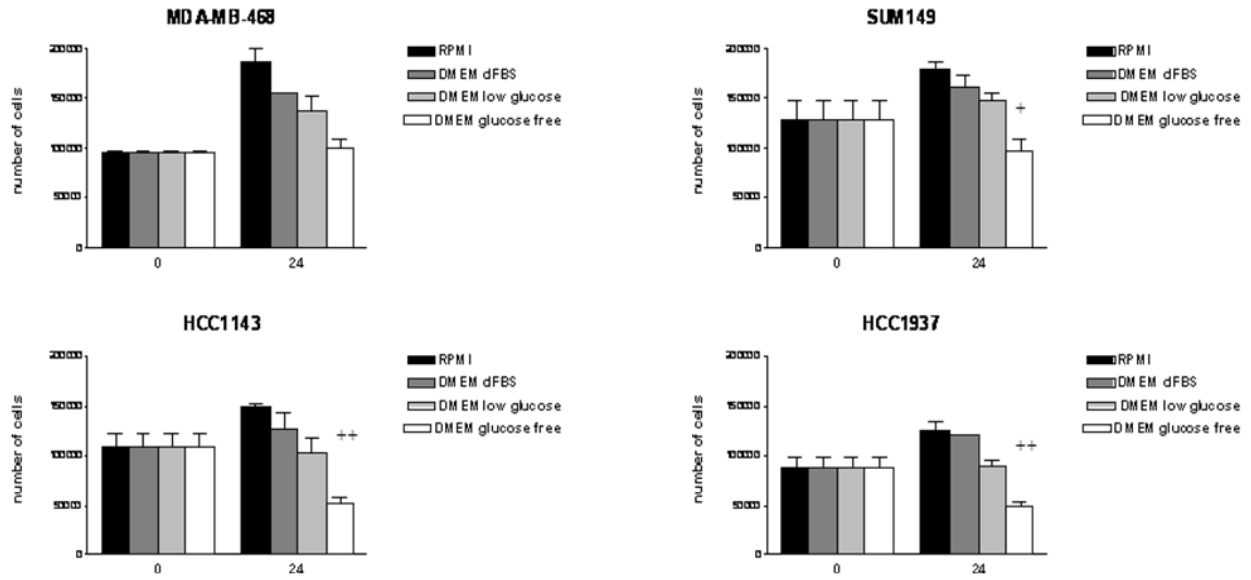


Figure 33: Effect of glucose deprivation or low glucose media (1g/L) on MDA-MB-468, SUM 149, HCC1143 and HCC1937 cell viability after 24 hours of treatment measured by Trypan Blue Assay. Data are expressed as % of cell number of treated cells compared to the respective control. Data are the mean ± SEM of 3 different experiments. +p<0.05, ++p<0.01; treated *vs* control.

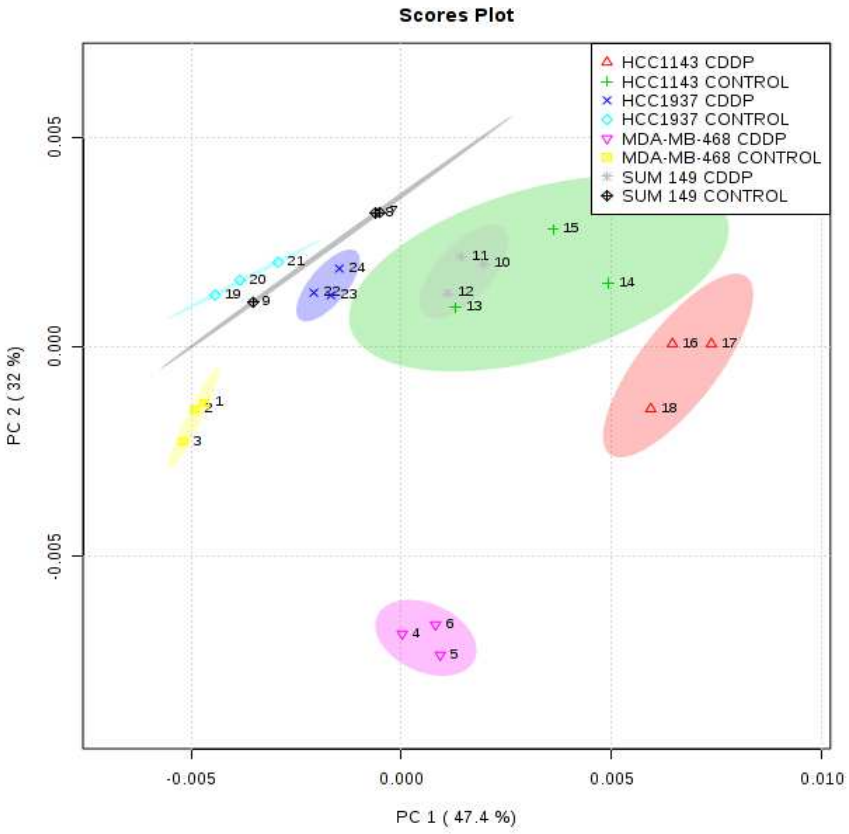
3.3 Screening of genes involved in glutamine metabolism

In order to check if cisplatin can modify mRNA levels of genes involved in glutamine metabolism, the mRNA expression of all genes involved in glutaminolysis was measured after 24 hrs with 5 µM CDDP treatment. In particular were measured the genes coding for: the two main glutamine transporter (LAT1 and ASCT2), the glutaminase, the enzyme which converts glutamine to glutamate, the glutamate dehydrogenase which converts glutamate to alpha-ketoglutarate which enters in TCA cycle inside the mitochondrion. Moreover mRNA of the genes coding for GOT and GTP2 were measured, which convert the glutamate to aspartate (which contributes to the nucleotides biosynthesis) and alanine respectively. Also glutamine synthetase mRNA were

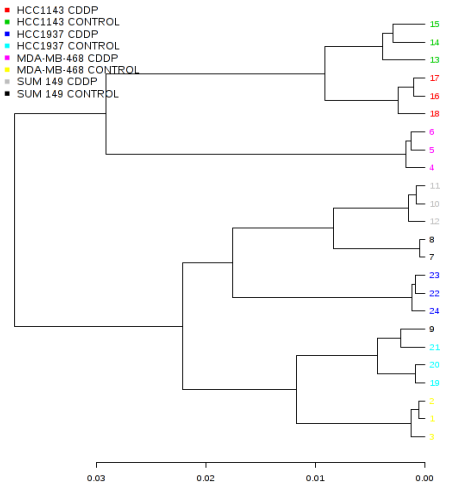
measured, coding for the enzyme that converts glutamate in glutamine. As shown in **Figure 34** cisplatin cause an overall increase of all the genes involved in glutaminolysis in all TNBC cell lines.

The multivariate analysis, Principal Component Analysis (PCA) and Clustering Analysis (Dendrogram and Heatmap) were performed by Professor Ragazzi (University of Padova).

34.A



34.B



34.C

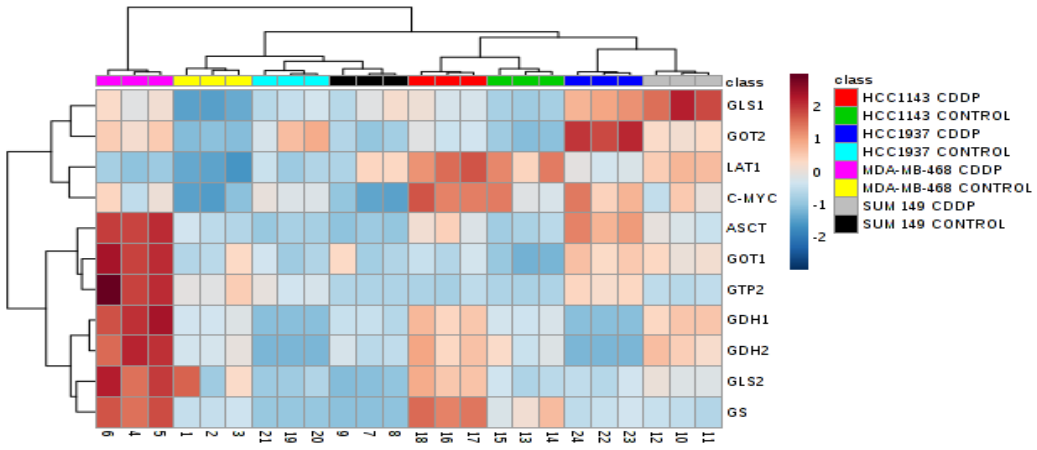
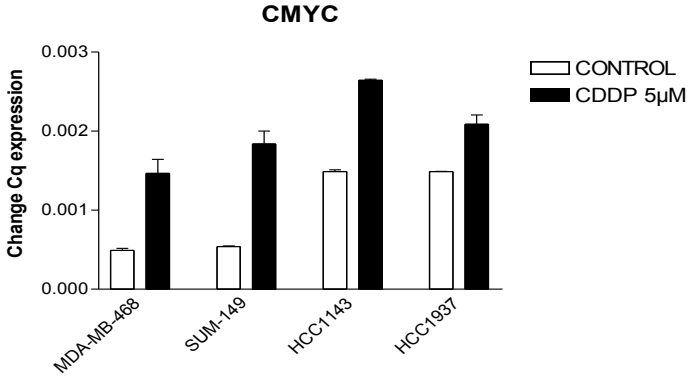


Figure 34: mRNA levels of ASCT2, LAT1, GLS1/2, GDH1/2, GOT1/2, GTP2, GS, C-MYC after treatment of 24 hours with CDDP 5µM. The mRNA was measured with Real Time q-PCR. Data are the mean±SEM of 3 different experiments. Expression levels of the indicated genes were calculated by the ΔΔCt method with *18S* rRNA as the reference gene. 34 (A) shows the 2-D scores plot between selected PCs, the PCA analysis is performed using the prcomp package. The calculation is based on singular value decomposition; 34 (B) shows the clustering result in the form of a dendrogram; 34 (C) shows the clustering result in the form of a heatmap (distance measure using euclidean, and clustering algorithm using ward.D).

Then, also C-MYC gene expression and c-Myc protein expression were evaluated after 24 and 48 hours of CDDP 5µM treatment. As shown in **Figure 35 B ,** in resistant lines HCC1143 and HCC1937 there is an increase in c-Myc expression after 48 hours treatment with CDDP.

35.A



35.B

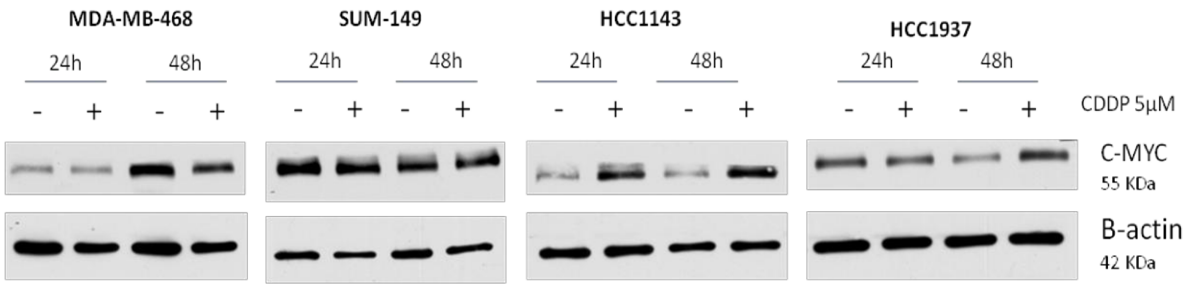


Figure 35: (A) mRNA levels of C-MYC, after treatment of 24 hours with CDDP 5µM. The mRNA was measured with Real Time q-PCR. Expression levels of the indicated gene was calculated by the ΔΔCt method with *18S* rRNA as the reference gene. Data are the mean ± SEM of 3 different experiments; (B) Protein expression of c-Myc after CDDP treatment, the normalization protein used was β-actin. Data are representative of three independent experiments.

3.3 Screening of genes involved in glucose metabolism

Results of glucose dependency clearly showed that the resistant cell growth is significantly reduced when coltured in deprivation of glucose. Data suggest that C13 cells have a major dependence from glucose as compare to sensitive cell lines.

Finally, in order to check if there was a different gene profile in lines resistant to cisplatin, was measured the gene expression, in basal conditions, of c-Myc target genes that encode for some enzymes involved in the glycolysis and glucose transporters. As shown in **Figure 36**, were examined mRNA levels of some genes that control the glycolytic flow: two glucose transporter GLUT1 and GLUT4, PFKM (Phosphofructokinase), PGK1 (phosphoglycerate kinase), PKLR (Pyruvate kinase), PC (piruvate carboxylase), LDHA (lactate dehydrogenase A). Only the LDHA gene is down-regulated in both resistant lines.

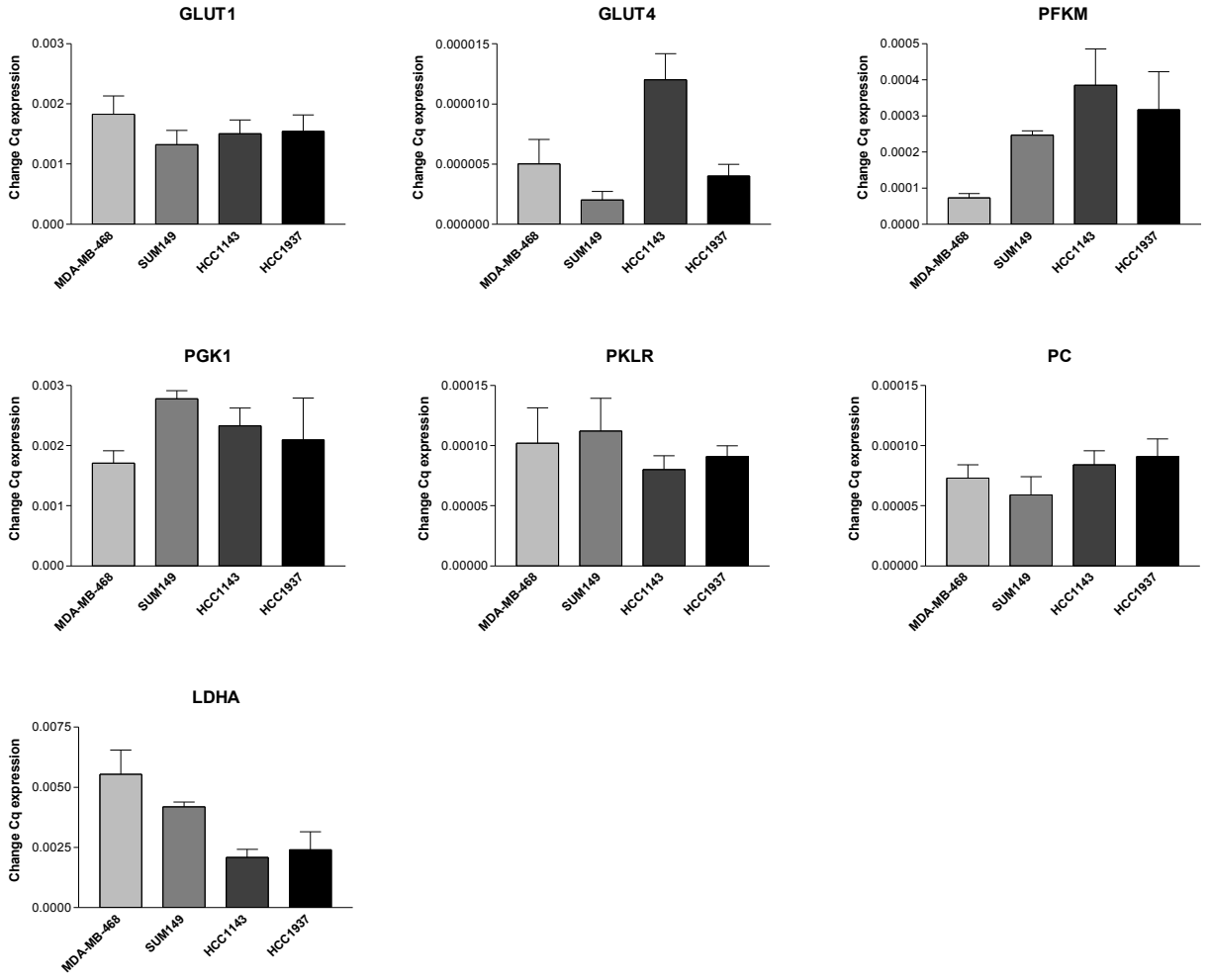


Figure 36: mRNA levels of glycolytic genes (GLUT1/4, PFKM, PGK1, PKLR, PC, LDHA), on basal conditions,. The mRNA was measured with Real Time q-PCR. Expression levels of the indicated gene was calculated by the ΔΔCt method with *18S* rRNA as the reference gene. Data are the mean ± SEM of 3 different experiments.

This study shows differences in the dependency of glucose between cisplatin-resistant and sensitive TNBC cell lines, and also an increased expression of the c-Myc oncogene in cisplatin-resistant cells. Of note, we also identified differential expression of specific c-Myc target genes involved in the regulation of glycolysis and glutaminolysis.

DISCUSSION

Cisplatin is employed for the treatment of a wide array of solid tumor including testicular, lung and ovarian cancer (Galanski M., 2006).

Even if the benefits of cisplatin are widely recognized, the main problem of its clinical effectiveness is the onset of resistance (Köberle B. et al., 2010), which appears to be multifactorial, for example through the decreased uptake of the drug, the increased export, the increased levels of intracellular glutathione and DNA repair mechanisms, or a decreased apoptosis (Rabik A.C. and Dolan M.E., 2007; Boulikas T. et al., 2007). However, the molecular mechanisms of resistance are not yet fully understood.

Cisplatin cytotoxicity has been originally ascribed to interactions between cisplatin and nuclear DNA, which lead to the formation of adducts that activate the apoptotic machinery (Wang D, and Lippard S., 2005). Now, instead, it is understood that the cytotoxicity mechanism is much more complex and shows different intracellular targets.

Recent studies have shown that the cisplatin intracellular concentration, binding to nuclear DNA, is about 5-10%, thus the drug concentration that can interact with other cellular components is significant, including the mitochondrial DNA (mtDNA) (Arnesano F. et al., 2008). MtDNA has no efficient repair systems like nuclear DNA and therefore, it is more sensitive to oncogenic mutations because of the nearness of mtDNA to the ROS production site that makes this genome vulnerable to oxidative damage (Marrache S. et al., 2014).

Our *in vitro* model of human ovarian carcinoma is composed by the wild type line 2008 and its derived clone C13 that is generated by *in vitro* selection in the presence of increasing concentrations of cisplatin (Andrews PA. and Albright K.D., 1992). The advantage of using isogenic cells lines, as an acquired resistance model, is the possibility to study the differences between chemosensitive and chemoresistant that are caused by cisplatin response and not due to the different genotype.

Our results from early research demonstrated that in cisplatin-resistant ovarian cancer cells (C13), the respiratory chain activity is lower and the dependency on glucose is higher than in cisplatin-sensitive cells 2008 (Montopoli M. et al., 2011).

It was also observed that cisplatin is equipotent in both mtDNA-deprived 2008-ρ0 and C13-ρ0 cells, demonstrating that mtDNA lack decreases drug sensitivity. Moreover, mtDNA is involved in metastatic capability, indeed recent studies have shown that also the density of mitochondria

might affect the chemoresistance (Quian W. et al., 2005), suggesting that mitochondria are a potential target for cisplatin.

Furthermore, in previous studies, oxygen consumption, cell viability in metabolic stress condition such as galactose and rotenone, mitochondrial potential/mass and mtDNA content were measured in 2008 and C13 cells; the results showed a significant reduction of all mitochondrial parameters in cisplatin resistant clones (Catanzaro D. et al., 2015).

Mitochondrial function is important for cancer cells because it is involved in bioenergetic activities, such as ATP production, in the regulation of programmed cell death, in the ROS production and trafficking of small metabolites. It is well known that cancer cells undergo a shift in their basal metabolic pathways, the "Warburg effect", whereby even under high oxygen tension they produce most of their ATP by glycolysis (Warburg O., 1956). This shift in metabolism has been reported to be accompanied by a change in mitochondrial morphology and size, although the molecular mechanism accompanying the morphological changes associated with the Warburg effect remain poorly understood.

Some evidence showed a link between mitochondrial bioenergetic functions and its architecture. In particular, besides its critical implication in mitochondrial quality control, mitochondrial dynamics has been linked to energy demand and supply balance (Molina, A.J., et al., 2009).

In this scenario, we proceeded studying CDDP resistance mechanism, by phenotyping other cancer cell lines sensitive and resistant, in particular: epidermoid carcinoma of the cervix and osteosarcoma to compare the data with those of 2008 and C13 lines.

The results obtained by confocal microscopy showed a mitochondrial network differently organized in resistant variants underlining a probable implication of mitochondrial dynamic in resistance mechanisms.

The network organization is the result of the balance between two opposite processes, fusion and fission, and the mitochondrial morphology can change in response to metabolic and pathogenetic conditions. Mitochondrial fission and fusion influence nearly all aspects of mitochondrial function, including respiration, calcium buffering and apoptosis (Cortassa M. et al., 2004; Jeong S.M. et al., 2004) and they are important processes for cell survival during a variety of stressors (Tondera D. et al., 2009). Indeed, fusion is important to mtDNA distribution within mitochondria population and to maintain these organelles active; instead, fission is necessary for the mitochondria distribution in all cell compartments to respond to different energetic needs.

Mitochondria dynamics are controlled by the activity of key regulating proteins, which include fission proteins and fusion proteins.

Mitochondrial fission requires the cytosolic dynamin related protein 1 (Drp1) and its mitochondrial receptors fission 1 (Fis1), mitochondrial fission factor (Mff) and Mitochondrial Division (Mid) 49 and 51. While the factors that control mitochondrial fusion are Optic Atrophy 1 (OPA1) and Mitofusin 1 and 2 (Mfn1/2).

Our results indicate an altered mitochondrial dynamic in chemoresistant cancer cells compared to their chemosensitive counterparts. Indeed, cisplatin-resistant cell line A431-Pt showed an imbalance toward fusion processes by an increased protein expression of OPA1 and a decreased expression of fission proteins, besides a tubular and hyperfused mitochondrial network. Moreover, TEM images demonstrated a well defined cristae structure in resistant line A431-Pt as compared to sensitive counterpart.

Interestingly, also Tondera et al. reported that mitochondrial hyperfusion could be induced by selective stresses and conferred cellular resistance to stress with higher cellular ATP level (Tondera et al. 2009). Together with our observations, these findings suggest that fused mitochondria, in A431-Pt resistant cells, promote cell survival and mtDNA protection to cisplatin-induced damage and that fusion is a mechanism by which intact mitochondria could complement a damaged unit and possibly recover its activity, thereby maintaining metabolic efficiency (Chan D.C., 2006).

A431 cells (epidermoid carcinoma) express the mutated form of the p53 gene with substitution at codon 273 (His273) (missense mutation). The tumor suppressor p53 exerts its biological function by regulating transcription of numerous downstream target genes involved in cell cycle arrest, apoptosis, DNA repair, senescence, and metabolism as a transcription factor (Levav-Cohen Y, et al., 2014). p53 is also directly recruited to the mitochondria and induces apoptosis independent of its function as a transcription factor (Vaseva A.V. and Moll U.M., 2009). When p53 activity is lost by gene deletion or mutations, it causes the inhability to control cell death in tumors (Muller P.A. and Vousden K.H., 2013).

Extensive cross-talks of the signaling pathways regulating mitochondrial dynamics and apoptosis proteins have been reported. Kong B. et al. suggested that chemoresistant gynecologic cancer cells lose their response to CDDP partly due to deactivated p53 and that p53 is involved in the regulation of mitochondrial dynamics by controlling the L-OPA1 processing (Kong B. et al., 2015).

Moreover, Patten et al. indicated that Opa1 complex through self-assembly is involved in the control of the cristae junctions (Patten D.A. et al., 2014), which are narrow tubular tunnels connecting cristae to the space between the outer and inner membranes (inter-membrane space), and it is essential for maintaining the mitochondrial cristae integrity and consequently the mtDNA as well as preventing the aberrant cell death through the cytocrome c relase and apoptosis. However, the loss of long form of OPA1 seems to destabilize Opa1 complexes that eventually lead to the loss of cristae structures, and cytochrome c release (Merkwirth, Dargazanli et al. 2008). Whether OPA1-regulated cristae structure is involved in chemoresistance is not known and needs to be further studied.

Taken together, these findings propose that resistant epidermoid carcinoma cells A431-Pt use mitochondrial fusion as a mechanism to counteract cisplatin induced cell death, and moreover these cells present an overexpression of OPA1, suggesting that stabilize OPA1 could prevent the cytocrome c release and apoptosis.

While fusion may recruit dysfunctional mitochondria into the active pool, autophagy targets depolarized mitochondria for digestion and elimination (Elmore S.P., et al., 2001); the mechanism that sorts mitochondria between the two fates is still unclear (Levine B., Yuan J., 2005). If fusion and autophagy are competing fates of the damaged mitochondria, fission might play a key role in allowing such competition to occur.

As regards the other two resistant ovarian carcinoma C13 and osteosarcoma U2OS-Pt cell lines, fission protein are more expressed (Drp1 and Mff protein expression and H-Fis gene expression) and OPA1 is down-regulated.

The mitochondrial dynamic is closely correlated to mitophagy, in particular it has been suggested that fragmented mitochondria are easily engulfed in the autophagosomes due to their smaller volume. Furthermore, Fis1 is involved in mitophagy; cells overexpressing hFis1 accumulate fragmented mitochondria, several markers of autophagy and autophagic vesicles (Gomes L.C. and Scorrano L., 2008). Thus mitochondrial dysfunction and mitochondrial fragmentation are responsible for the induction of mitophagy (Mao K. and Klionsky D.J., 2013).

Data obtained by real time q-PCR and western blotting showed that the resistant clones, presenting an imbalance toward fission process, have a more elevated BNIP3 mRNA level and protein expression then WT lines. BNIP3 expression is induced by stresses such as hypoxia and oxidative impairment. Recently, BNIP3 was reported to modulate autophagy as a mitochondrial receptor that tethers mitochondria to autophagosomes (Hanna R.A., et al.,2012).

These data suggest that cisplatin resistant cells present a faster mitochondrial turn-over, by mitophagy as a mitochondrial quality control mechanism. Indeed, the mitophagic process could avoid the mtDNA damage caused by cisplatin and make the cell able to survive despite the presence of cisplatin.

Then, in order to check how the mitochondrial dynamics influence the cisplatin response, a viability test was performed using Mouse Embryonic Fibroblast cell lines: WT, Mef OPA1 $^{-/-}$ knockout, Mef MFN1 $^{-/-}$ knockout and OPA1 trasgenic (over-expressed) kindly provided by Professor Scorrano laboratory.

The results show that the lines with an altered expression of proteins involved in mitochondrial fusion are less sensitive to cisplatin as compared to WT line and suggest a mitochondrial dynamics involvement in mechanisms of resistance to cisplatin.

This finding indicates a deregulation in the proteins that control mitochondrial dynamics, the "mitochondria-shaping" proteins, a process dependent on mitochondrial fusion and fission and inextricably linked to mitochondrial biogenesis, distribution, signaling and apoptosis (Frezza C., et al., 2006).

The mitochondrial dynamic is extremely complex and its regulation has not yet been elucidated. Some studies highlighted a link between mitochondrial bioenergetic functions and its dynamics (Schrepfer E. and Scorrano L., 2016). In particular, mitochondrial morphology could modulate ATP production capacity in response to alterations in energy demands. Moreover, in cancer cells, the interplay between deregulated signal transduction and changes in mitochondrial metabolism is multifarious.

Several papers demonstrated that TRAP1 (Tumor Necrosis Factor Receptor Associated Protein 1), the chaperonine localized in the mitochondrial matrix, is an important regulation factor of mitochondrial metabolism in tumor cells, favoring mitochondria integrity and cell survival (Rasola et al., 2014; Yoshida, S. et al., 2013; Felts S.J. et al., 2000). In addition, TRAP1 is involved in the induction of a chemoresistant phenotype in human colorectal carcinoma (Costantino E. et al., 2009) due to his role in ROS-adaptive responses in tumor cells.

The results reported in the literature show TRAP1 as a possible regulator factor in the balance between the processes of fusion and fission, during the stress stimuli (Takamura H. et al., 2013).

In collaboration with Professor Rasola laboratory, TRAP1 protein expression was investigated in order to verify if it could have a key role in the regulation of processes studied in this thesis. In stress conditions, the overexpression of TRAP1 decreases the production of ROS and lipid

peroxidation and preserves the potential mitochondrial ATP production and the activity of the complex IV (Butler E.K. et al., 2012; Voloboueva L.A. et al., 2008; Xu L. et al., 2009). Takamura et al. show that a decrease in stable expression of TRAP1 leads to an abnormality of the mitochondrial morphology, by the alteration of DRP1 expression.

Thus, the resistant clones that have an imbalance to the fusion, have a decreased expression of TRAP1, and there is an opposite situation in the resistant clones that have an imbalance towards the fission. In order to understand if TRAP1 is involved in the regulation of mitochondrial dynamic, it has been analyzed the network organization and the expression of OPA1, MFN1 and p-DRP1 in Mef's lines with TRAP1 silenced. The data obtained show an increase in fusion protein expression in shTRAP1 Mef's cells and a different mitochondrial network organization. Of note, there are few TRAP1 antagonists, recently discovered, that lead to a mitochondrial activity collapse and cell death, suggesting TRAP1 as a new molecular target to improving the chemotherapies (Landriscina M. et al., 2010).

As regards the project in collaboration with Professor Toker (Department of Pathology at the Beth Israel Deaconess Medical Center, Harvard Medical School), another important feature correlated with the cisplatin resistance, previously studied in our laboratory (Catanzaro D. et al., 2015) was evaluate: the metabolic reprogramming in resistant cancer cells.

In the last years, increasing evidences have suggested that growth signalling pathways directly control cell metabolism and proliferation through the regulation of metabolic enzymes (Cairns R.A. et al., 2011; Ward P.S. and Thompson C.B. 2012).

The metabolic reprogramming is an hallmark in neoplastic transformation, correlated with the degree of tumor invasiveness. It is characterized by an increase of the glucose uptake and lactate production, as well as a decrease in the capacity of mitochondrial respiration in the presence of oxygen (effect Warburg). This metabolic reprogramming is even more thrust in the cisplatin resistant phenotype as we have demonstrated in previous study (Catanzaro D. et al., 2015).

Indeed, cancer cells are characterized by alteration of several oncogenes and tumour suppressor genes that can lead to important modifications of bioenergetc and biosynthetic activities (Chen J.Q. and Russo J., 2012), allowing them to an high metabolic adaptability to the dynamic tumor microenvironment. The dysregulation of the main metabolic pathway is often associated with oncogenic signaling pathways involved in the bioenergetic capabilities of cancer cells. The hypoxia-inducible factor (HIF) and c-MYC are two of the main critical factors for tumorigenesis. Acting alone, HIF and c-Myc partially regulate the adaptation mechanisms that

cancer cells undergo in a low oxigen microenvironment. However, acting in concert, these transcription factors reprogram metabolism, protein synthesis, and cell cycle progression to support bioenergetics and cell survival (Gordan J.D. et al., 2007).

The study performed in Professor Toker laboratory is focused on metabolic reprogramming in breast cancer cells that have an innate resistance to cisplatin.

Breast cancer is the second most common cancer in the world and women who have this disease show a high rate of relapse (Mendes et al., 2015). Breast cancer triple negative TNBC is the strongest form of the disease and is the class treated only with chemotherapy (Hirshfield K.M. and Ganesan S., 2014). Moreover, there is currently no targeted therapy specific to the TNBC, and the patients that exhibit this phenotype have a poor prognosis (Timmerman et al., 2013).

Several oncogenes can modify important metabolic pathways, such as the glucose transport, tricarboxylic acid cycle (TCA), glutaminolysis, oxidative phosphorylation and pentose phosphates pathway (PPP) (Chen J.Q. and Russo J., 2012).

Both glucose and glutamine are fundamental metabolic substrates in tumor cells and are essential for the development of cancer invasion and metastasis. Thus, this scenario prompted us to explore c-Myc transcription factor and some of its target genes involved in glycolytic and glutaminolytic fluxes.

Data obtained showed that resistant lines HCC1143 and HCC1937 have a greater dependence on glucose and a down-regulation of the gene coding for lactate dehydrogenase A.

LDHA is a c-Myc target gene that converts pyruvate, derived from glucose through glycolysis, to lactate. Besides, resistant TNBC cells showed an increased expression of c-Myc.

c-Myc has an important role in cell proliferation in a large number of human tumors including breast cancer. C-Myc could transactivate genes involved in glycolysis under normoxia; however pyruvate, which is converted to lactate by LDHA, could also be converted to acetyl-CoA and oxidized by increased Myc-mediated mitochondrial biogenesis. (Li F. et al., 2005).

Thus, our results suggest that cisplatin resistant TNBC cells redirect pyruvate toward TCA cycle rather than lactate conversion.

Furthermore, lactate can be used as a metabolic fuel by oxidative cancer cells. Pérez-Esculedo et al., demostrated that HeLa cancer cells support the oxidative use of lactate to take advantage of intracellular lactate signaling to optimize glutamine metabolism, in particular oxidative glutaminolysis. Lactate can stimulates glutamine uptake and catabolism through c-Myc transactivation (Péerez-Escuredo J. et al., 2015).

The data of glutaminolysis genes showed that cisplatin causes a general increase of all the genes in all cell lines. Specifically, results showed an increase of two glutamine transporter: ASCT2 (Alanine, serine, cysteine-preferring transporter 2) and LAT1 (L-type amino acid transporter 1), c-myc target genes besides glutaminase 1 that converts glutamine in glutamate within mitochondria.

Glutamine is an amino acid that is abundant in human plasma and is present in high concentrations in the medium used for cell culture. Several oncogenes, including c-myc, have been identified to promote expression of metabolic enzymes and regulators that carry preferential use of glycolysis with respect to mitochondrial oxidative phosphorylation (OXPHOS). Due to its anaplerotic role in TCA cycle, glutamine reconstitutes the necessary intermediates for the synthesis of macromolecules (Chen et al., 2015). Moreover, glutamine serves as a precursor for glutathione (GSH) synthesis. Therefore, a reduction of the glutamine metabolism can limit the proliferation of tumor cells and thereby it serves as a point of metabolic control that is activated in response to the genotoxic stress such as chemotherapeutic drugs. Thus, the deprivation glutamine is sufficient to reduce levels of GSH (Lora J et al 2004) and can cause oxidative stress and sensitize the cells to cisplatin.

Even if the metabolic reprogramming is essential for the rapid proliferation of tumor cells, a systematic characterization of the metabolic pathways of transformed cells is not yet fully known. (Hsu P.P. and Sabatini D.M., 2008).

It will be our future interest to clarify the roles of glucose and glutamine in breast cancer cells resistant to cisplatin, in order to find possible pharmacological targets useful to overcome the resistance. Our future experiments will be focused on the investigation of glucose transporters (GLUT1 and 4) expression and their translocation on plasma membrane, with the purpose of pointing out the involvement of glycolytic flux in cisplatin resistance. Then, a possible cross-talk between lactate and glutamine metabolism will be considered for future analysis, using possible inhibitors and verifying the uptake of this two “oncometabolites”.

In conclusion, the data presented in this thesis show how the phenomenon of resistance is extremely complex and closely related to mitochondrial function, metabolism and dynamics. Therefore, the data obtained confirm that mitochondria is a cisplatin target and is involved in cisplatin cytotoxicity and resistance mechanism.

With the aim of verifying the dependence on mitophagy for survival in resistant ovarian cancer and osteosarcoma cells, the mitophagic flux will be investigated trough different

approaches. We will perform, through MitoKeima probe, a quantification of mitochondria degradation after different conditions. In particular, we will analyze the stimulation of the autophagic process with FCCP, the autophagy inhibition with chloroquine and finally cisplatin will be used to better understand the involvemet of mitophagy/autophagy in the resistance mechanism.

Then, since it is well known that BNIP3 can induce mitophagy and we verified that it is overexpressed in C13 and U2OS-Pt resistant lines, BNIP3 will be silenced in our cancer cell lines sensitive and resistant to cisplatin. Thus, in order to correlate mitophagy and cisplatin resistance, si-BNIP3 cells will be treated with cisplatin to verify whether the reduction of mitophagy levels could restore a chemosensitization to cisplatin.

Therefore, combining chemotherapeutic drugs, such as cisplatin, with mitophagic modulators may offers opportunities to counteract resistance in cells that exert the mitochondrial quality control process as mechanism of defense to cisplatin.

Acquiring knowledge in the mitochondrial remodelling and in energy metabolism, in particular in cisplatin-resistant cancer, helps to identify new targets useful to innovative pharmacological approaches. Moreover, these data could be useful to clarify the mechanism of action of cisplatin, which is not completely clear.

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