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METABOLIC AND MOLECULAR PROFILING OF OVARIAN CANCER STEM CELLS AND CANCER NON-STEM COUNTERPART

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RIASSUNTO

Il tumore ovarico di tipo epiteliale (EOC – Epithelial Ovarian Cancer) rappresenta la prima causa di morte per neoplasia ginecologica. Sebbene la chirurgia e la chemioterapia a base di carbo/cis-platino abbiano migliorato la prognosi delle pazienti affette da tale carcinoma, il tasso di sopravvivenza a 5 anni dalla diagnosi di EOC in stadio avanzato rimane inferiore al 30%. Tale elevata mortalità è riconducibile alla diagnosi in fase tardiva ed all'insorgenza di resistenza alla chemioterapia di prima linea, che porta a frequenti ricadute entro pochi mesi dalla conclusione dei trattamenti.

Le cause della comparsa di fenomeni di resistenza alla terapia, e della conseguente ricorrenza del tumore, è ancora incerta. Recenti studi hanno indicato che la crescita del carcinoma epiteliale dell'ovaio è sostenuta da una minima popolazione di cellule, dette cellule tumorali staminali (CSC - Cancer Stem Cells), probabilmente responsabili della ricomparsa del tumore al termine delle sedute farmacologiche. Un consenso generale supporta l'idea che l'eliminazione di questa popolazione rappresenti uno dei più importanti obiettivi della terapia anti-tumorale. Tuttavia, si ha ancora una scarsa conoscenza dei meccanismi che conferiscono un vantaggio di sopravvivenza alle CSC sulle cellule tumorali non staminali, rendendo così arduo lo sviluppo di terapie mirate anti-CSC.

Le CSC del carcinoma epiteliale ovarico sono caratterizzate dall'espressione di due marcatori di superficie cellulare: CD44 (recettore dell'acido ialuronico) e CD117 (c-kit o recettore del fattore cellulare staminale SCF). Recentemente, il nostro gruppo di ricerca ha dimostrato che le cellule ovariche co-esprimenti CD44 e CD117, che rappresentano l'1-2% delle cellule tumorali provenienti dall'ascite delle pazienti affette da EOC, possiedono le canoniche proprietà staminali e sono in grado di sopravvivere *in vitro* ed *in vivo* alla deprivazione di glucosio. Abbiamo inoltre osservato che tale resistenza all'assenza di glucosio è principalmente dovuta alla capacità delle CSC, contrariamente alle cellule tumorali non staminali, di privilegiare la fosforilazione ossidativa anziché la glicolisi aerobica (Warburg Effect). Tuttavia, indipendentemente dalla frazione delle cellule tumorali staminali, l'analisi comparativa tra i diversi campioni di EOC ha evidenziato che non tutti presentano la stessa dipendenza dai glucidi; per alcuni, infatti, pochi giorni *in vitro* senza glucosio sono sufficienti a ridurre significativamente la vitalità cellulare, mentre per altri lo stesso effetto è ottenibile solo dopo molte settimane di coltura nelle stesse condizioni.

Dunque, approfondire tale questione e gli aspetti metabolici ad essa correlati è il primo scopo di questo progetto. A tal proposito, sulla base della vitalità cellulare in condizioni di coltura senza glucosio, abbiamo potuto suddividere le cellule derivanti da asciti di pazienti con EOC in due categorie: sensibili alla deprivazione di glucosio (GA – Glucose-Addicted) e resistenti a tale deprivazione (GNA – Glucose Non-Addicted). Sebbene variazioni nella regolazione del metabolismo del glucosio siano state frequentemente osservate nei casi di neoplasia, non è ancora noto se questo diverso tratto metabolico influenzi la risposta dei pazienti alle terapie, o se sia da queste modulato. Pertanto, abbiamo deciso di ricercare una eventuale correlazione tra i diversi profili di dipendenza dal glucosio da noi riscontrati e la risposta dei pazienti al trattamento a base di carbo/cis-platino. Infatti, da un punto di vista clinico, i pazienti vengono categorizzati come platino-resistenti o platino-sensibili, a seconda che la neoplasia recidivi entro od oltre i 6 mesi, rispettivamente, dalla fine della chemioterapia di prima linea. I nostri esperimenti hanno rivelato che, quando le cellule di EOC vengono coltivate in assenza di glucosio, tutti i campioni provenienti

da pazienti platino-sensibili ricadono all'interno del gruppo GA; confrontati con i campioni GNA, i GA mostrano una maggiore produzione degli enzimi del metabolismo glucidico, un maggior tasso di proliferazione, e una minore espressione delle pompe cellulari per l'espulsione dei farmaci. Parallelamente, i campioni derivanti dai pazienti platino-resistenti rientrano nella categoria GNA. La stretta associazione tra la sensibilità ai chemioterapici e il profilo cellulare di utilizzo del glucosio è stata confermata in un modello murino di xenotrapianti, nel quale è stato identificato uno stringente parallelismo tra risposta al platino e metabolismo glucidico. Infine, in una coorte di pazienti non chemio-trattate affette da EOC, le quali erano state categorizzate come GA o GNA alla diagnosi, le curve di Kaplan Meier hanno messo in luce che il fenotipo GA, rispetto a quello GNA, è associato con un maggior periodo di sopravvivenza senza recidive, in modo statisticamente significativo. Nel complesso, questi dati suggeriscono che il grado di dipendenza dai glucidi delle cellule di EOC, osservabile *in vitro*, può rappresentare un valido marcatore per predire la risposta dei pazienti alla chemioterapia a base di platino.

Analizzare il tratto molecolare che determina il peculiare metabolismo glucidico dei campioni di EOC costituisce il secondo obiettivo del nostro progetto di ricerca. A tal riguardo, i microRNA (miRNA), ossia piccole molecole di RNA non codificante, rappresentano un promettente settore di studio, in qualità della proprietà di queste strutture molecolari di regolare molti geni e vie di segnale. Inoltre, il loro coinvolgimento nello sviluppo e nella progressione tumorale è già stato dimostrato per il carcinoma ovarico, e recentemente i miRNA sono risultati essere importanti modulatori del metabolismo cellulare in molti tessuti normali e neoplastici. Pertanto, il nostro gruppo di ricerca ha prodotto un profilo di espressione di miRNA su cellule derivanti da pazienti affette da EOC, confrontando sia campioni GA contro GNA, sia CSC contro non-CSC; il nostro fine è stabilire se il pattern di miRNA alla base delle differenze metaboliche tra i campioni di EOC sia associato alla totale massa tumorale o piuttosto ad una specifica frazione cellulare neoplastica. Questi dati non hanno rivelato alcun miRNA differentemente espresso tra cellule GA e GNA; tuttavia, molti miRNA sono risultati deregolati nelle CSC rispetto alle non-CSC. Noi ci siamo focalizzati sul mir-602, up-regolato delle CSC; infatti, nonostante la scarsa conoscenza su questo miRNA, il suo target chinasi Caseina 1 Delta (CSNK1D), che detiene un ruolo chiave nella proliferazione cellulare e nella divisione asimmetrica, ci è parso molto interessante in virtù del suo già dimostrato coinvolgimento nella progressione del carcinoma mammario. In questo contesto, i nostri esperimenti hanno dimostrato che CSNK1D è down-espressa nelle CSC, in accordo alla up-modulazione del mir-602. Inoltre, l'inibizione *in vitro* del mir-602 riduce nelle CSC l'espressione della maggior parte dei geni associati alle proprietà staminali, suggerendo che il mir-602 potrebbe controllare alcune delle vie di segnale correlate alla staminalità. Dato che nessuno dei geni di staminalità analizzati si lega direttamente al mir-602, appare ragionevole supporre che suddetto miRNA possa indirettamente attivare l'espressione di tali geni tramite la sua funzione inibitoria su CSNK1D. Dunque, secondo la nostra ipotesi, le caratteristiche staminali sarebbero inibite dalla chinasi Caseina, la quale sarebbe a sua volta repressa dal mir-602. Nonostante molti altri esperimenti siano necessari per confermare questa nostra teoria, il progetto qui presentato mette in rilievo la possibile esistenza di un meccanismo di regolazione, mediato dal mir-602, responsabile delle proprietà di staminalità delle cellule del carcinoma epiteliale ovarico.

ABSTRACT

Epithelial Ovarian Cancer (EOC) is a very malignant neoplasm, accounting for 5% of cancer mortality in women. Although progress has been made in EOC treatments by improved debulking surgery and platinum-taxane regimens, the 5-year survival rate of advanced-stage EOC remains below 30%. This poor prognosis relies on the one hand on the late diagnosis and on the other on the chemo-resistance occurring after only a few months from the completion of treatment.

The reasons for recurrence and cancer drug resistance remain uncertain. Recent evidence suggests that EOC, akin most tumors, contains a tiny population of cells, named cancer stem cells (CSC), probably responsible for chemotherapy resistance and tumor recurrence. Ovarian CSC are characterized by the co-expression of two surface markers: CD44 (hyaluronic acid receptor) and CD117 (stem cell factor receptor or c-kit). Recently, our research group demonstrated that ovarian CD44/CD117 co-expressing cells, which represent 1-2% of cancer cells from ascitic effusions of EOC-bearing patients, are endowed with canonical stemness properties and are able to resist *in vitro* and *in vivo* glucose starvation. We reported that this glucose deprivation resistance is mostly due to the ability of ovarian CSC to privilege oxidative phosphorylation, rather than the aerobic glycolysis (Warburg effect) exploited by the non-stem tumor bulk. However, independently of CSC fraction, our experiments also highlighted that not all the analyzed EOC samples presented a similar glucose addiction.

Thus, investigating this issue and its related metabolic aspects is the first aim of the current project. Concerning this aim, we showed that tumor cells from EOC patients can be categorized, according to their *in vitro* viability under glucose starvation, into glucose deprivation-sensitive (glucose-addicted, GA) and glucose deprivation-resistant (glucose non-addicted, GNA). Although deregulated glucose metabolism is usually observed in cancer, whether this metabolic trait influences response to or is modulated by cytotoxic drugs is unknown; therefore, we addressed the possible correlation of these glucose addiction profiles with the patient response to platinum (PLT) regimens. In this regard, when EOC cells were cultured in the absence of glucose, all samples from PLT-sensitive patients felt into the GA group; compared to GNA samples, they disclosed higher expression of glucose metabolic enzymes, higher proliferation rates and *in vitro* sensitivity to PLT, as well as reduced multi-drug resistance pump expression. On the other hand, the samples derived from PLT-resistant patients felt into the GNA category. The close association between PLT sensitivity and glucose metabolic profile was confirmed in a xenograft model, where a stringent parallelism between PLT sensitivity/resistance and glucose metabolism was identified. Finally, in a cohort of naïve EOC patients categorized as GA or GNA at diagnosis, Kaplan Meier curves showed that the GA phenotype was associated with significantly better progression-free survival, compared to GNA patients. Overall, these results suggest that *in vitro* glucose addiction of EOC cells could be regarded as a reliable marker to predict the patient response to platinum regimens.

Investigating the molecular traits leading to the distinctive glucose metabolism of EOC samples is the second aim of this project. In this regard, microRNA (miRNA), that are small non-coding RNA molecules, represent a promising field, in view of their ability to modulate many genes and pathways. Moreover, their involvement in cancer development and progression has already been reported in ovarian cancer, and recently miRNA also emerged to regulate cell metabolism in several normal and cancer tissues. Thus, we performed a miRNA profile on EOC patient-derived samples,

comparing both GA versus GNA samples and CSC versus non-CSC, in order to establish whether the miRNA signature behind the metabolic differences of EOC samples is associated with the total tumor bulk or rather with a specific cell fraction. The data did not reveal any miRNA differentially expressed in GA compared to GNA cells, but several miRNA resulted significantly deregulated in CSC versus non-CSC. We focused our attention on mir-602, which was found up-regulated in CSC; indeed, despite little information about this miRNA, its target Casein Kinase 1 Delta (CSNK1D), which displays a key role in cell proliferation and asymmetric division, seemed very interesting in view of its involvement in breast cancer progression. We demonstrated that CSNK1D is down-regulated in CSC, according to mir-602 over-expression. Moreover, the *in vitro* inhibition of mir-602 reduced the expression of the major stemness-associated genes in CSC, suggesting that mir-602 could regulate some cell stemness pathways. Since none of the analyzed stemness genes is directly targeted by mir-602, it is reasonable to advance that mir-602 could indirectly activate the expression of such genes through its inhibitory function on CSNK1D; thus, we propose that cell stemness signaling is inhibited by casein kinase, whose translation is in turn repressed by mir-602. Although a few further experiments are needed to validate our idea, this project highlights a possible miRNA-mediated regulatory mechanism of cell stemness features in EOC.

1. INTRODUCTION

The term cancer (or tumor, or neoplasia) is used for diseases caused by the unlimited proliferation of abnormal cells, which become able to invade the surrounding tissues and distant organs, through the blood and lymph system. Cancer is the major cause of death in economically developed countries, and the second cause of death in developing countries.

The initial step to cancer development is the uncontrolled growth of a cell in a part of the body, which becomes the primary site of the neoplasia. Unlike normal cells, which die after a limit number of divisions, cancer cells continue to proliferate and generate new tumor cells, and stop responding to canonical growth inhibition signals. Therefore, the carcinogenesis process involves DNA damages, which impair the function of genes associated to cell growth and division. Some types of cancer are inherited from parents, but in most cases cancer is caused by mutations which occur in specific genes during a person's lifetime. DNA damage can derive from both intrinsic factors (inherited mutations, hormones, immune conditions and mutations that occur from metabolism) and extrinsic factors (such as tobacco smoke, infectious organisms, chemicals and radiations).

The canonical approaches clinically used to remove tumor masses or reduce their growth are surgery, radiation therapy and systemic treatments such as chemotherapy or hormonal therapy. However, all these methods very often result not to be useful to eradicate the neoplasia; indeed, at the end of the treatments, some types of cancer, like ovarian cancer, are frequently characterized by tumor relapse [1].

The hypothesis widely accepted by the scientific community is that tumor recurrence could be related to the presence within the tumor mass of a rare population of cells, which are characterized by stemness properties and are called Cancer Stem Cells (CSC).

1.1. Anatomy of ovary and ovarian cancer

The ovaries are two small glands which play a key role in the female reproductive system. Indeed, they are responsible for the production of both the female sex hormones controlling the reproductive cycle and the gametes which can be fertilized to form embryos.

Each ovary is a tiny glandular organ similar for shape and size to an almond. The ovaries are localized in the pelvic cavity, and are attached to opposite sides of the uterus by several pairs of ligaments which connect the uterus and the ovary. The rear portion of the broad ligament forms the mesovarium, which sustains the ovary. The mesovarium is connected to the pelvic sidewall by the suspensory ligament of the ovary (infundibular pelvic ligament) (Figure 1). The large structure formed by these two ligaments holds the ovarian artery and vein, as well as nerve supply to the ovary.

Ovarian tissues are organized into the following layers:

- The most external layer is formed by simple epithelium, known as the germinal epithelium, which creates a smooth covering of the ovary.
- The tunica albuginea, localized just below the germinal epithelium, is a thick layer of fibrous connective tissue, which maintains and preserves the underlying tissues.

- The ovarian cortex, below the tunica albuginea, contains follicles and the connective tissues which support them. The follicles cover the oocytes that mature into ova during the woman's reproductive period.
- Finally, the most internal layer, that is the ovarian medulla, holds most of the vascular tissues supporting the other layers of the ovary.

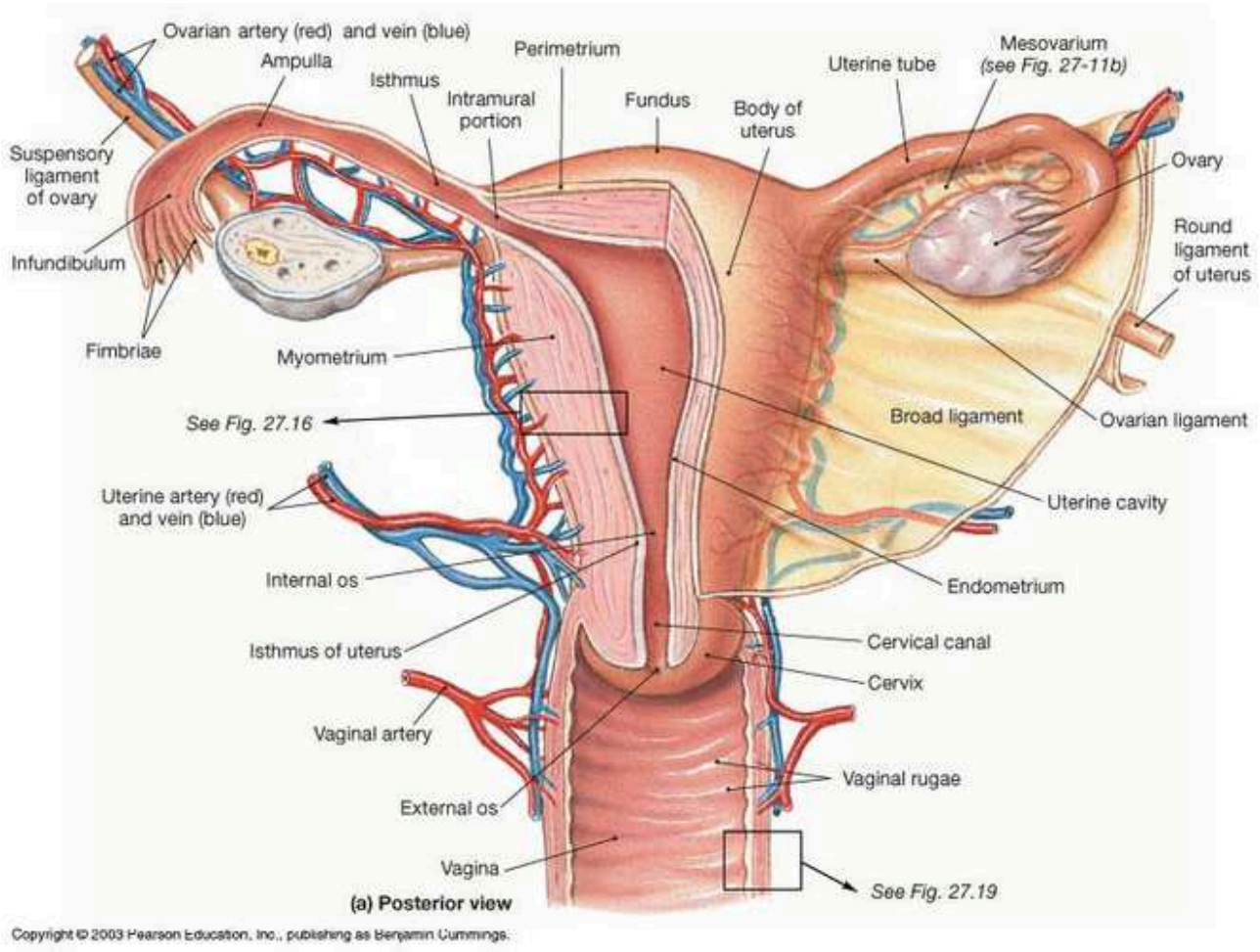

Figure 1. Anatomy of ovary. *Animated Dissection of Anatomy for Medicine (A.D.A.M.) (www.adameducation.com) and Human Anatomy and Physiology by Benjamin Cummings.*

The ovaries act as both glands and gonads, so playing a key role in the female reproductive physiology. As glands, the ovaries are responsible for the production and release of several female sex hormones, namely estrogen and progesterone. Estrogen regulates the development of the mammary gland and uterus during puberty and controls the phases of the menstrual cycle, including the regeneration of the uterine lining. Progesterone is strictly involved in the uterine processes which act during pregnancy to allow the embryo to implant and develop in the womb.

Ovarian cysts and ovarian cancer are the most important diseases of the ovary. Ovarian cysts are fluid-filled sacs which form in the ovaries because of two main disorders: either the failure of the

egg release or the failure of the dissolution of the follicle, the sac in which human egg cells form, after the egg is released. The presence of small cysts is common in the ovaries of healthy women, but some women are affected by an ovarian disorder, named polycystic ovary syndrome, characterized by the presence of more follicles than usual; consequently, in these women, the normal growth of the follicles is impaired causing cycle irregularities and the formation on many cysts. The majority of cysts is not malignant and will never become cancerous. Thus, the regular monitoring of the cysts, using ultrasound scan and blood tests, has replaced for most women the surgical resection of the cysts, which was the treatment of choice in the past years.

The analysis of the level of the protein CA125 (cancer antigen 125) in the blood is normally used to monitor women affected by ovarian cysts, because high levels are indicative of ovarian cancer. Other molecular markers analyzed to discriminate benign ovarian cysts from ovarian cancer are lactate dehydrogenase (LDH), alpha feto protein (AFP) and human chorionic gonadotropin (HCG).

Ovarian cancer is the fifth most common cause of death from cancer in women (Figure 2) and the seventh most common cause overall worldwide [2,3].

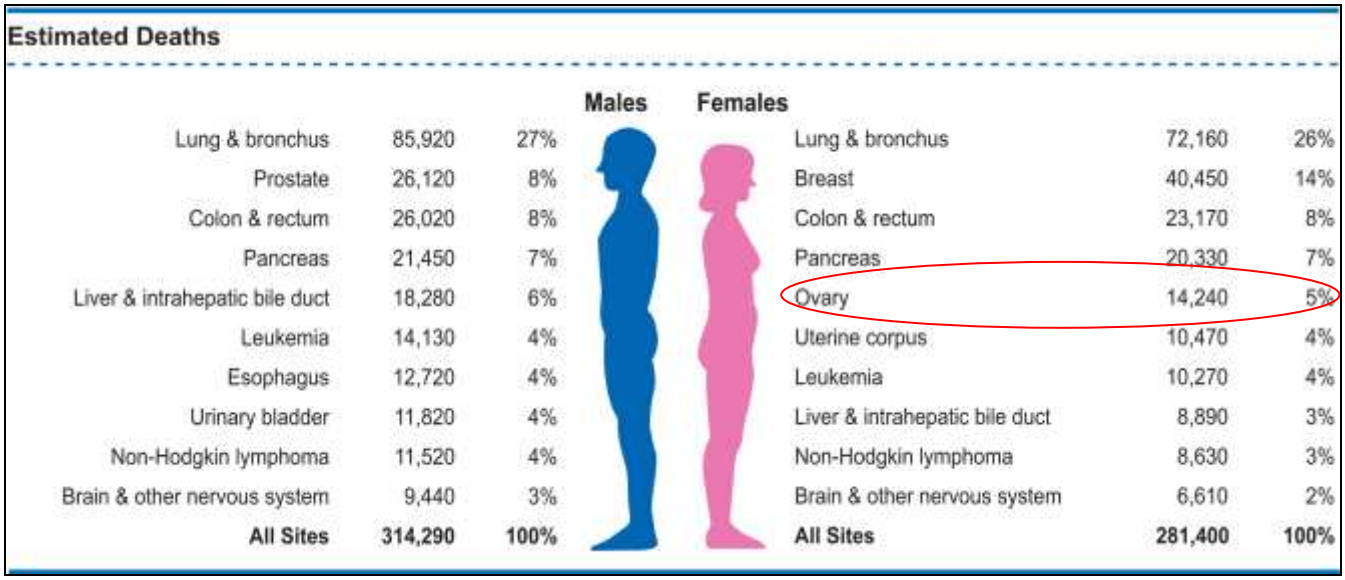

Figure 2. Cancer estimated deaths. *Ovarian cancer is the fifth most common cause of death for cancer in women. Siegel R., Miller K., Jemal A. (2016). "Cancer statistics, 2016". CA Cancer J Clin (1): 7-30.*

SEER (Surveillance, Epidemiology and End Results – Program of the National Cancer Institute, which works to provide information on cancer statistics) estimates that in 2016 about 22.280 new cases of ovarian cancer are diagnosed and 14.240 women died of ovarian cancer (Figure 3). Approximately 1.3 % of women will be diagnosed with ovarian cancer at some point during their lifetime.

The incidence of ovarian cancer is highest in women aged 55-64 years. The survival rate after five years from the diagnosis is commonly used to compare the aggressiveness of different cancers, and for ovarian cancer this rate is 46.2%. The time of diagnosis strictly influences the survival rate; however, only about 15% of ovarian cancer patients are diagnosed at an early stage of the neoplasia.

Indeed, more than 80% of the patients do not report any symptom when the malignancy is limited to the ovaries, and very often the symptoms which overcome during cancer progression are shared with many less serious gastrointestinal, genitourinary and gynecological diseases. For this reason, ovarian cancer is denominated "silent killer" [4,5], and is diagnosed at late stages when the efficacy of the therapy is low.

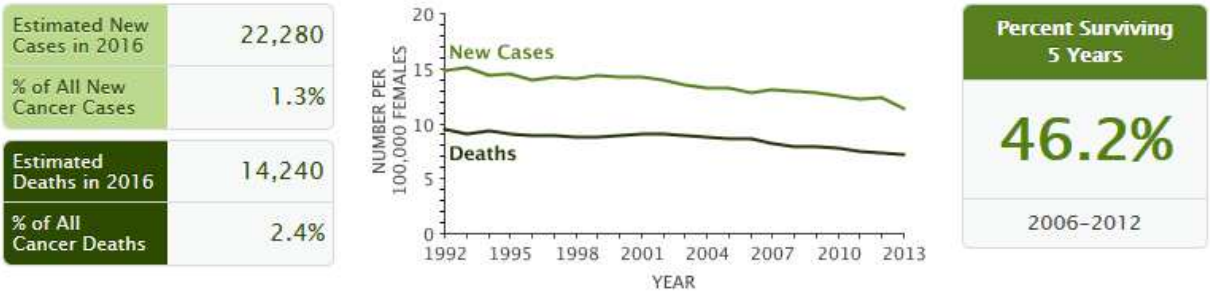

Figure 3. Statistics of ovarian cancer – SEER analysis. *National Institute (http://seer.cancer.gov/statfacts/html/ovary.html)*

The routine modalities to diagnose ovarian cancer are trans-vaginal sonogram (TVS) and the analysis of the serum levels of the protein CA125, widely accepted as the best marker for ovarian carcinoma. A clinical trial, carried out in United Kingdom in 200.000 subjects, reports that the combination of TVS and serum CA125 detection could increase the percentage of early stage diagnosis to 48%, with high sensitivity (89.4%) and specificity (99.8%). Generally, the histological analysis of a biopsy derived from the abnormal tissue is used to confirm the outcome of the previous clinical examinations, and ensures a reliable diagnosis of ovarian carcinoma.

Ovarian cancer has a distinctive biology and behavior at the clinical, cellular and molecular levels.

The main risk factors related to this malignancy are the following:

- A family history of ovarian carcinoma. Indeed, while ovarian carcinoma is not considered a hereditary disease, it has been reported that mutations in specific genes, such as BRCA1 and 2, are related to a high risk of ovarian cancer development. Since these mutations can be inherited, women with two or more close relatives who have developed this malignancy have an increased risk for the development of ovarian cancer.

- A family and/or clinical history of breast or colon cancer. In some cases, breast and colon cancer are associated to mutations in the above genes; moreover, these two types of tumor can metastasize to the ovary. Thus, having some close relative with breast/colon cancer, or having a clinical history of these diseases, confers an increased risk of developing ovarian cancer.

- Childbearing and menstruation. The number of menstrual periods which a woman experienced in her lifetime seems to directly correlate with an increased risk for ovarian cancer development. According to this, it has been observed that women who began menstruating before age 12, those who had no children or had their first pregnancy after age

30, and those who experienced menopause after age 50 have a greater chance of developing ovarian cancer compared to the general population.

- Drugs. Some studies reported that women who have undergone hormone therapy, to increase the fertility or after menopause, may have a slightly increased risk for ovarian carcinoma. On the other hand, the consumption of oral contraceptives seems to decrease the chance of developing the disease.

Most ovarian tumors can be included into one of three major categories: epithelial-, sex cord-stromal-, and germ cell tumors, based on the anatomic structure which is the likely origin of the tumor. Several subtypes can be grouped into each categories (Figure 4).

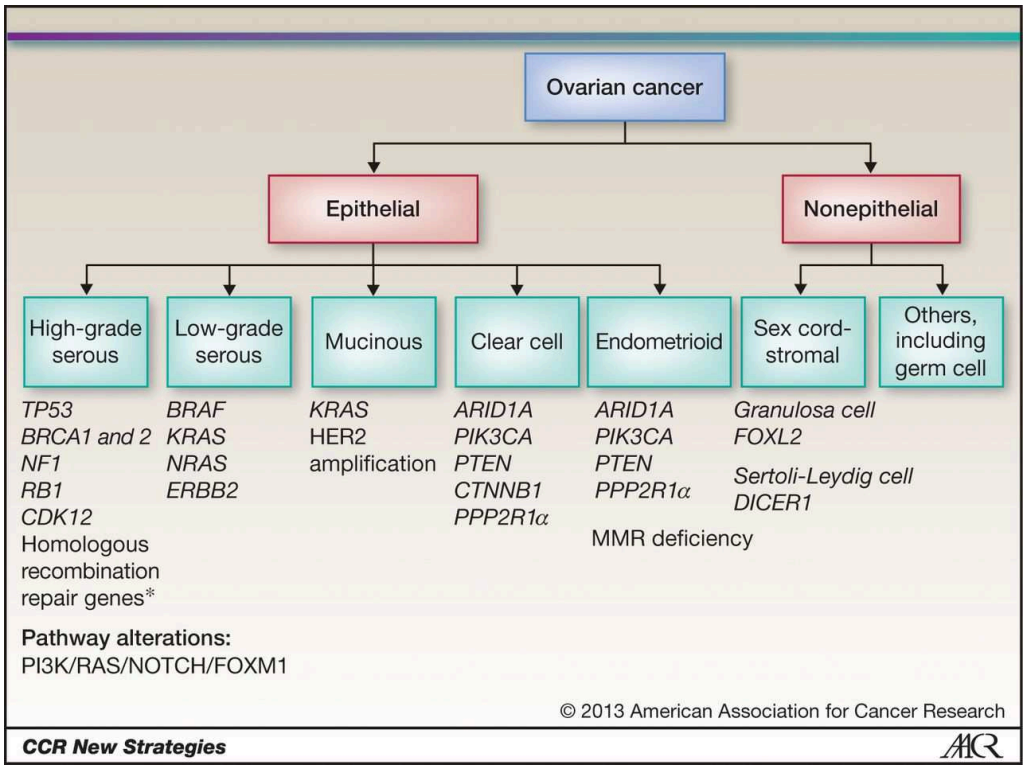

Figure 4. Ovarian carcinoma: clinical and molecular fetures of the major types and subtypes. *Epithelial ovarian carcinoma is the most frequent form of this malignancy and it is divided into five main types. Among the non-epithelial forms, two major types can be defined: sex cord-stromal and germ cell tumors. Banerjgee S. and Kaye S. "New Strategies in the Treatment of Ovarian Cancer: Current Clinical Perspectives and Future Potential". Clinical Cancer Research, 2013.*

The most common type is epithelial ovarian carcinoma (EOC - 95% of all the tumors); based on histopathology and molecular alterations, it is classified into five main types: high-grade serous (70%), endometrioid (10%), clear cell (10%), mucinous (3%), and low grade serous carcinomas (<5%). These subtypes are distinct malignancies, as suggested by the differences in epidemiological and genetic risk factors, precursor lesions, molecular events during oncogenesis, patterns of spread, response to chemotherapy and prognosis [6]. Thus, for a successful treatment, the histopathological diagnosis of the tumor type is critical. The cells of the five tumor types are morphologically different, and this simplifies tumor typing. Recent investigations have demonstrated that in some cases the tumor, traditionally thought to be a primary ovarian tumor (serous, endometrioid, or clear-

cell carcinomas), could instead originate in the fallopian tubes or in the endometrium and involve the ovary secondarily. Thus, the exact origin of ovarian cancer is hardly established, and probably this is the reason for the failures of early detection and of new therapeutic approaches to circumvent tumor recurrence and thus reduce the mortality rate [7].

The International Federation of Gynecology and Obstetrics (FIGO) has defined four stages for ovarian carcinoma [8].

- Stage 1: tumor is confined to ovaries;
- Stage 2: tumor involves one or both ovaries with pelvic extension (below the pelvic brim) or primary peritoneal cancer;
- Stage 3: tumor involves one or both ovaries with cytologically or histologically confirmed spread to the peritoneum outside the pelvis and/or metastasis to the retroperitoneal lymph nodes;
- Stage 4: distant metastasis, excluding peritoneal metastasis, can be detected.

Moreover, about 30% of ovarian cancer patients present ascites, an abnormal accumulation of exudative fluid in the abdominal cavity; this inflammatory fluid contains a cellular fraction consisting mainly of lymphocytes and mesothelial cells, and in most cases of ovarian cancer cells (Figure 5). The presence of neoplastic cells in the ascites is associated with a less favorable prognosis, since these cells may contribute to the spread of cancer to secondary sites [9].

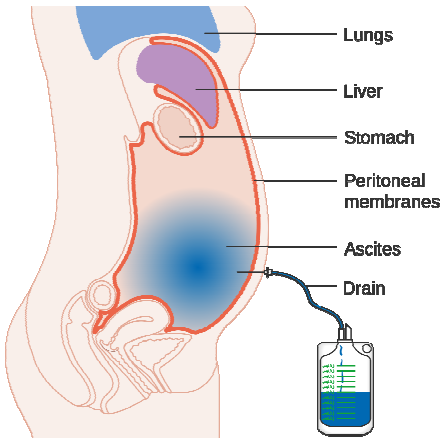

***Figure 5. Ovarian carcinoma ascitic fluid**. Cancer Research UK (www.cancerresearchuk.org).*

Clinical treatments commonly used to overcome ovarian carcinoma symptoms and spread rely on surgery and chemotherapy, sometimes in combination with radiotherapy. Chemotherapy include paclitaxel, cisplatin, topotecan and gemcitabine. Obviously, these anti-tumoral treatments are more efficient when the neoplasia is limited to the ovary. Indeed, in most cases of early-stage (stage I or II) ovarian carcinoma, surgery is enough to cure the disease, and the survival rate at five years from early-stage diagnosis is around 90%. Nevertheless, due to the lack of effective screening programs and of disease-specific symptoms, ovarian cancer is unfortunately diagnosed at advanced stages in more than 80% of the cases. Standard treatment for these patients with advanced ovarian cancer is maximal surgical cytoreduction followed by systemic platinum-based chemotherapy; the five-year

survival rate ranges from 10% to 30% for women diagnosed with ovarian cancer at stage 3 or 4. Despite in the initial phases chemotherapy is successful in giving a response rate up to 80% in first-line treatment, in most patients the tumor relapses in a few months. In fact, the anti-tumoral therapy against ovarian carcinoma is impaired by the onset of chemo-resistance; therefore, the study of ovarian cancer biology is essential to improve the treatment outcome [10].

Many authors have reported that chemo-resistant cancers are associated with the presence in the tumor mass of a tiny population of cells characterized by stemness properties, the Cancer Stem Cells (CSC) [11].

1.2. Stem Cells and stemness

Stem cells are the progenitors of all the cells in the human body, and they are characterized by the following features:

- Self-renewal: it is the capacity to divide generating many other stem cells identical to the precursor cell, so maintaining a stem cell pool in a non-differentiated state for lifetime. This process requires a tight regulation of cell cycle and a tight balance in the expression of proto-oncogenes (promoting self-renewal), gate-keeping tumor suppressors (limiting self-renewal), and care-taking tumor suppressors (maintaining genomic integrity). Self-renewal program is a cell-intrinsic mechanism involved in a complex network of cell-extrinsic signals from the "niche", that is the tissue microenvironment which substains stem cell survival and functions.
- Asymmetric division: it is the ability to originate, during cell division, two different daughter cells, one maintaining stem phenotype and the other already differentiated.
- Toti-, pluri-, multi-potency: it is the process by which stem cells differentiate into different cell types. Stem cells can be totipotent, pluripotent or multipotent depending on the type of cells deriving from them. Totipotent stem cells are able to generate all the cell types of the human body; pluripotent stem cells can form all the cell types except for extra-embryonic tissues; finally, multipotent stem cells are able to create only the specific cell types closely related to the tissue in which stem cells are localized.
- Resistance to stress condition: it means that stem cells are able to survive also to the critical conditions which generally kill normal cells, by exploiting their ability to enter in a quiescent state, to extrude drugs and natural toxins from the cell, to repair DNA damages and to escape apoptotic pathways. More in detail, quiescence is the property to block proliferation and many of the metabolic processes, remaining in a basal status which requires a very low energy consumption rate; while the extrusion of drugs and toxins from the cells is due to the expression on the cell surface of several ATP-binding cassette (APC) transporters.
- Unlimited proliferation: in the adult life, stem cells usually remain in a quiescent state, but they are able to restart a high proliferation rate and to rapidly expand and differentiate in response to particular situations which require a regeneration of the tissue. This potentially unlimited cell division is controlled by intrinsic and extrinsic regulatory mechanisms. For

instance, the intracellular protein p53 is strictly involved in cell cycle arrest at G1/S phase, thus playing an essential role in the intrinsic modulation of cell proliferation. On the other hand, since the extracellular microenvironment of stem cells consists of signaling molecules, as well as inter-cellular contacts and interactions between stem cells and the neighboring extracellular matrix (ECM), the signals from the external niche are also important in the regulation of stem cell survival and expansion. The microenvironment is considered to determine the stemness features of cells, such as self-renewal ability and development to committed cells. This is the reason why stem cells implanted in a totally different niche can potentially differentiate into cell types of the new environment.

The stem cell potential in tissue regeneration is much exploited in the clinical treatment of various diseases. For instance, regenerative medicine is a branch of medicine (Figure 6) in which stem cells can play an essential role, by virtue of their ability to join damaged tissue areas and to recreate new cells and tissues by driving repair and renewal processes, thus restoring functionality.

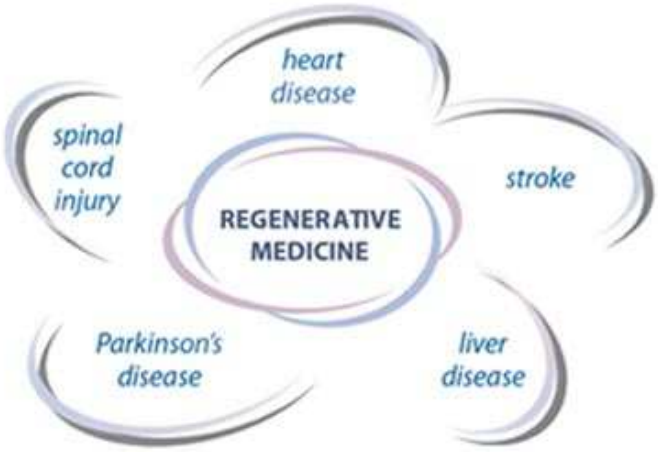

Figure 6. Application of stem cells in regenerative medicine. *REVIVA – Regenerative medicine center.*

To date, the most widely used stem cell therapy is the bone marrow transplant, a clinical procedure exploited in the treatment of blood tumors and diseases. Moreover, the suitability of stem cell treatment is also evident in other medicine branches, such as those of degenerative and heart diseases. For example, embryonic stem cells represent a promising strategy in the field of cardiac tissue regeneration; nevertheless, many ethical problems are related to this application, since it requires the destruction of embryos which is considered an unethical practice, unlike the use of adult stem cells which is ethically accepted. The finding that stem cells from one tissue implanted in a different organ can form cells of the latter provides an excellent starting point in the development of new innovative organ regeneration techniques. For instance, some studies have demonstrated that the Mesenchymal Stem Cells (MSC) isolated from adult tissues, such as bone marrow or adipose tissue, are able to form neural cells both *in vitro* and *in vivo*, thus representing a valid source suitable to organ regeneration treatment [12].

Stem cells are also used as a therapy against tumor development. For example, certain forms of cancer, such as leukemias and lymphomas, can be successfully treated with stem cells transplanted

from bone marrow or other sources. As a matter of fact, despite cancer cells effectively die under high doses of chemotherapy or radiation, these anti-tumor treatments have several side effects, first of all the death of bone marrow cells. Thus, a stem cell or bone marrow transplant has the purpose to replenish the body with healthy bone marrow cells after the end of chemotherapy and radiation. In other cases, the transplant can also have the benefit that the new blood cells may attack and kill any survived cancer cell [12].

Even though stem cells are endowed with many potential benefits, their unlimited proliferation and their ability to originate many other different cells are known to be involved in the onset of tumors, especially because of the accumulation of gene mutations in the pathways that control stem cell self-renewal.

1.3. Cancer Stem Cells (CSC)

CSC are the "dark side" of the stem cells, defined as "immortal tumor-initiating cells that can self-renew and have pluripotent capacity" [13]. Hans Clevers adds that "the CSC concept postulates that, similar to the growth of normal proliferative tissues such as bone marrow, skin or intestinal epithelium, the growth of tumors is fuelled by limited numbers of dedicated stem cells" [14].

Cancer cells endowed with stemness properties were discovered by Rudolf Virchow; in the mid – 19th century, he noticed that some cancer cells were similar to embryonic stem cells in terms of histological characteristics, proliferation rate and differentiation capacities. In the seventies, many authors discovered that the tumorigenic potential of cancer cells in leukemia was different from that in solid tumors. This concept suggested a new idea, that hematologic tumor is a "stem-cell disease", which starts from transformed stem cells and develops as a heterogeneous cancer tissue, containing cancer stem cells and differentiated tumor subpopulations [15]. The implementation of these discoveries with new technologies, such as the use of fluorescent antibodies against specific cell molecules in flow cytometry, allowed the characterization of cancer stem cells. In 1997, Bonnet and Dick provided the first evidence for CSC, demonstrating the existence of a rare population of leukemia-initiating cells with stem cell-like features [16]. In their study, they showed that, in cells derived from acute myeloid leukemia (AML) patients, only the $CD34^+/CD38^-$ cell fraction could originate hematopoietic malignancy when injected into immunodeficient (NOD-SCID) mice; as a matter of fact, a higher *in vivo* tumorigenic potential is regarded as the main CSC property in the following studies. Indeed, CSC have been then characterized in several solid malignancies, such as melanoma, breast, colon, brain, pancreatic, ovarian, prostate, gastric, hepatic and lung tumors [17-20]. The first evidence of CSC in solid tumors was the isolation of $CD44^+/CD24^-$ cells from breast cancer patients in 2003 by Al-Haij et al. [17].

Overall, these discoveries have suggested the hypothesis that cancer heterogeneity has a hierarchical structure. Many years ago, the scientific community assumed the existence of a stochastic model, according to which every cell within a tumor has the same potential in determining and maintaining tumor growth; in other words, every cancer cell has the same probability to be transformed in a tumor-initiating cell by a specific event in the tumor cell population [13,21]. To date, another model, defined hierarchical model, is widely accepted; this model assumes that only a pool of specific-tissue stem cells has all the biological characteristics to initiate and maintain the tumor growth, thus originating the heterogeneous tumor mass (Figure 7).

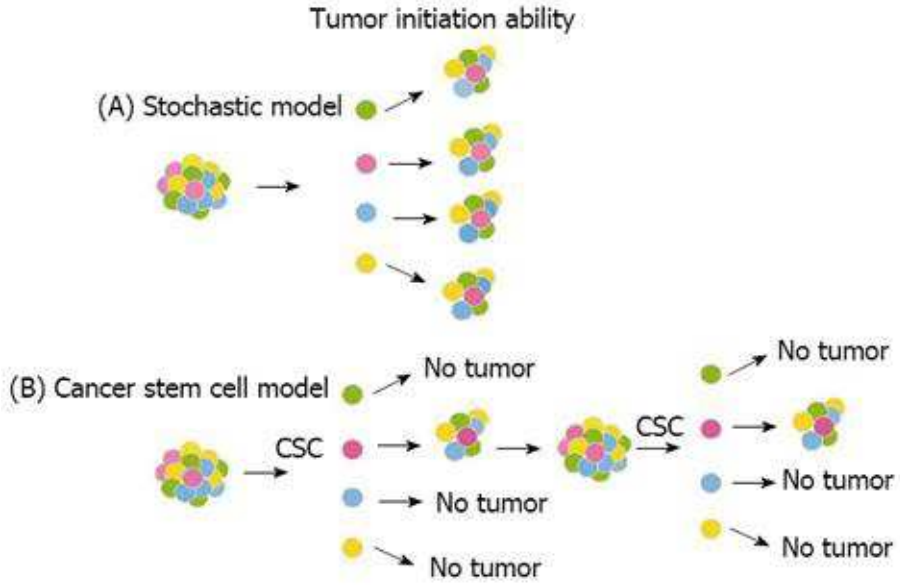

Figure 7. Stochastic model and hierarchical model (Cancer Stem Cell model) for cancer heterogeneity. *Fanali C., Lucchetta D., Farina M. et al.; "Cancer stem cells in colorectal cancer from pathogenesis to therapy: Controversies and perspectives". World J Gastroenterol. Jan 28, 2014; 20(4): 923-942*

The exact origin of CSC is still unknown, and several hypotheses have been proposed. One is that CSC could derive from mutations of normal stem cells, thus maintaining all the stemness features, such as self-renewal, asymmetric division, ability to indefinitely proliferate and to differentiate into all the tissue cell types. The other hypothesis is that CSC could arise from mutated progenitor and/or differentiated cells (Figure 8) [22]. Such cells may be characterized by different grades of proliferation ability; however, they cannot present the self-renewal capacity: thus, according to this hypothesis, a mutated progenitor or a differentiated cells needs to acquire specific mutations in proliferation-associated genes to become a cancer stem cell.

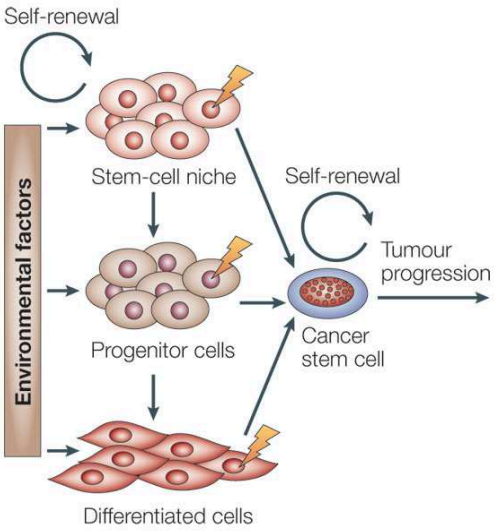

Figure 8. The origin of cancer stem cells. *Cancer stem cells arise from mutated normal stem cells, progenitor cells and differentiated cells, in response to a selective pressure given by environmental factors. Bjerkvig R, Tysnes BB, Aboody KS, Najbauer J and Terzis AJA. The origin of the cancer stem cell: current controversies and new insights. Nature Reviews 2005; 5:899-904*

CSC represent a rare population (<1% of tumor bulk) of tumor cells characterized by several peculiar properties:

- They are able to initiate and maintain tumor growth, by virtue of their self-renewal property, which allows them to maintain a sort of stemness reservoir and, at the same time, to generate committed cells responsible for tumor mass development.
- They can resist chemotherapy regimens, because of their ability to exclude or inactivate anti-tumoral drugs through mechanisms including ABC transporter expression, detoxifying enzyme activity, enhanced DNA damage response and activation of key survival pathways [23], such as anti-apoptotic cascade signaling.
- They are able to enter in a state of quiescence, which allow them to resist specific stress condition, such as nutrient and oxygen starvation, and thus to cause tumor relapse following chemo- or radiotherapy treatment [24].
- They are responsible for metastasis formation through the expression of some transcriptional factors, such as Snail, Slug, Zeb1 and Bmi-1, able to induce epithelial-to-mesenchymal transition (EMT), that is a key phenomenon responsible of epithelial junction degradation and tumor invasion during the early steps of metastasis [25-27].
- They are characterized by the expression of some specific surface markers, which can allow their isolation from the tumor bulk. Unfortunately, a universal marker for CSC in all types of tumors (Table 1) is not available.

Tumor type	Cell surface marker
Acute Myeloid Leukemia (AML)	$CD34^+/CD38^-$
Breast Cancer	$ESA^+/CD44^+/CD24^-/Lineage^-$
Ovarian Cancer	$CD133^+$ $CD44^+/CD117^+$
Glioblastoma	$CD133^+$ $CD15^+$
Medulloblastoma	$CD133^+$ $CD15^+$
small cell and non-small cell lung cancer	$CD133^+$
Hepatocellular carcinoma	$CD45^-/CD90^+$
Prostate cancer	$CD44^+/A2B1$ hi/$CD133^+$
Colon cancer	$CD133^+$ $CD44^+$
Melanoma	$CD20^+$ $ABCB5^+$
Pancreas adenocarcinoma	$CD44^+/CD24^+/EpCAM^+$
Renal carcinoma	CD133 enhances vascularization
Head and neck squamous cell carcinoma (HNSCC)	$CD44^+$
Cancer stem cells can be enriched from tumors located throughout the body using specific MicroBead-conjugated antibodies.	

Table 1. Cancer stem cells markers in different tumors. *Miltenyi Biotec antibody for CSC markers.*

1.3.1. Cancer Stem Cells in ovarian cancer

Recent evidence suggests that Epithelial Ovarian Cancer (EOC), akin other tumors [14], contains a tiny population of cancer stem cells probably responsible for tumor initiation, maintenance and growth, and for chemotherapy resistance and tumor recurrence. Moreover, the role of stem cell dysfunction in adult mammalian ovaries in ovarian cancer and polycystic ovary syndrome has already been reported [28].

The first study on the identification and isolation of stem-like cells in ovarian cancer was published by Bapat et al. [20]. In the same context, Zhang and colleagues have demonstrated that a specific cell population with stemness features is defined by the co-expression of two surface markers, named CD44 and CD117 [29]. CD44 is the receptor for hyaluronic acid, and CD117, also known as c-kit, is the tyrosine kinase receptor of the Stem Cell Factor (SCF) cytokine. More in detail, these authors generated spheroid from the ascites of EOC-bearing patients; after serial passages in a stem cell-based medium (without serum and enriched in growth factor), these cell cultures revealed that the surviving spheroid cells were highly enriched in CD44/CD117 co – expressing cells (Figure 9).

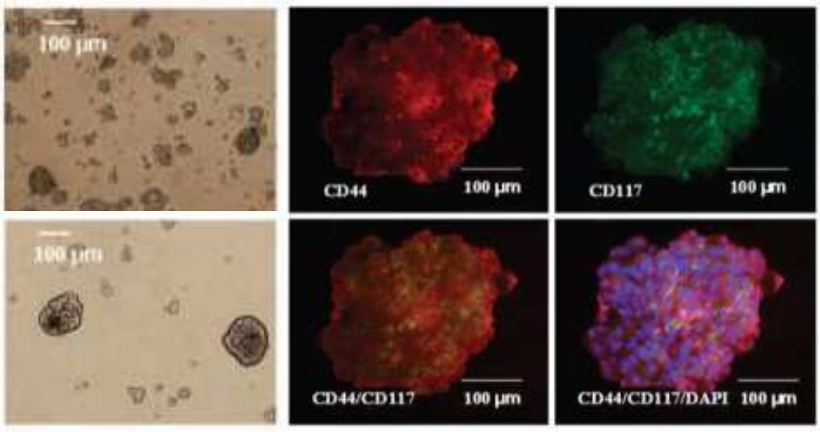

Figure 9. Spheroids of ovarian cancer stem cells*. (Left Panel) Sphere formation in specific cell culture conditions, without serum and with the addition of specific growth factor; (Right Panel) doulble staining for CD44 and CD117 in spheroid by immunofluorescence. Zhang S, Balch C, Chan MW, Matei D, Schilder JM, Yan PS, Huang T and Nephew KP. Identification and characterization of ovarian cancer initiating cells from primary human tumors. Cancer Research 2008; 68(11): 4311-4320.*

The same authors demonstrated that these spheroid-forming cells were characterized by the over-expression of stemness-associated genes, such as Bmi-1, Oct-4, Notch-1, Nanog, Nestin, ABCG2, and by the ability to resist canonical chemotherapy (cisplatin and paclitaxel). Moreover, the most important stemness property was the higher tumorigenic potential of $CD44^+/CD117^+$ cells when injected into immunodeficient mice, compared to the $CD44^+/CD117^-$ counterpart. More in detail, the injection of 100 $CD44^+/CD117^+$ cells was tumorigenic in nude mice with tumor latency ranging from 73 to 102 days, whereas the injection of up to 10^6 bulk tumor cells failed to form tumors also with extended latency [29].

In a recent study of my research group, we could confirm that ovarian $CD44^+/CD117^+$ cells, isolated *ex vivo* from ascitic effusions of EOC patients, are endowed with all previous mentioned stemness properties [30].

Other studies have reported different ovarian CSC markers [31]. One of the most widely described is CD133 or Prominin, a membrane glycoprotein encoded by the CD133/Prom-1 gene and firstly recognized as a hematopoietic stem cells marker. In 2009, Baba and collaborators [32] have identified $CD133^+$ cells in ovarian cancer cell lines and in primary ovarian tumors. More in detail, they reported that $CD133^+$ cells were able to generate tumors when injected into immunodeficient mice more efficiently than the $CD133^-$ population. Furthermore, the tumors derived from the injection of $CD133^+$ cells consisted of both $CD133^+$ and $CD133^-$ cells, suggesting that $CD133^+$ cell subset was able to form two phenotypically different cell populations. Unfortunately, other reports have demonstrated that also isolated $CD133^-$ cells are able to generate tumors. One possible explanation for this result is that a rare population of $CD133^+$ cells, responsible for the tumor-initiating capacity, contaminated the isolated $CD133^-$ cells. Nevertheless, the other hypothesis is that another small cancer cell subset, which does not express CD133, could give rise to tumor-initiating cells [33] (Figure 10). Thus, the specificity of CD133 as a stemness marker remains uncertain.

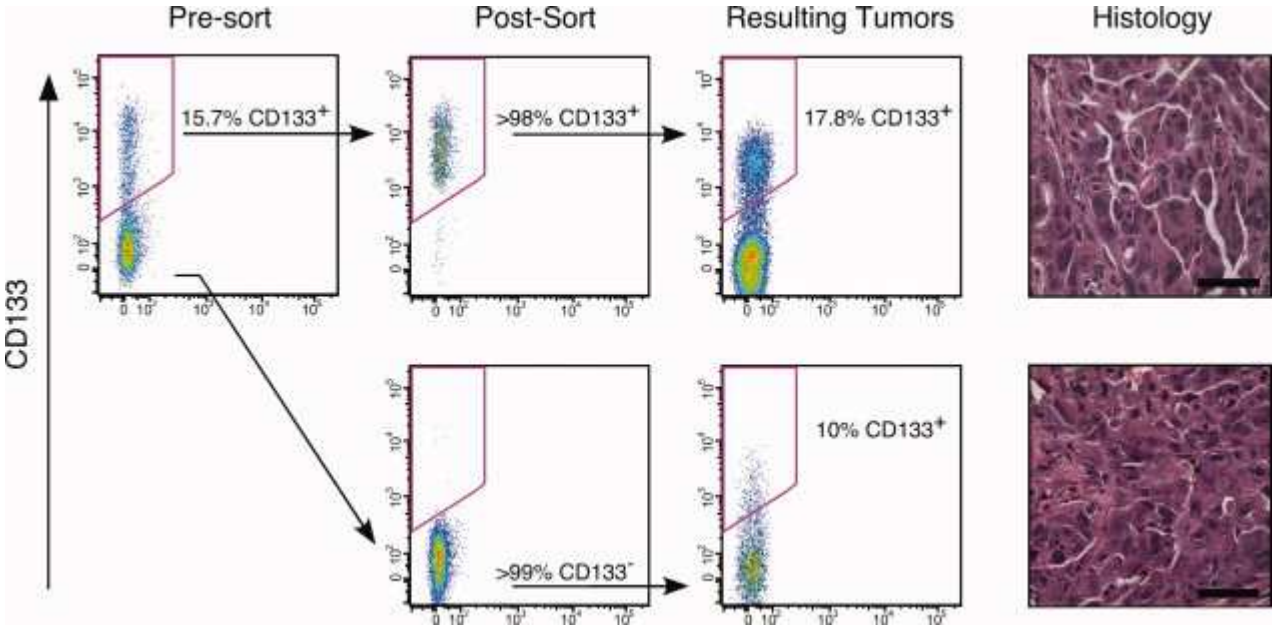

Figure 10. Sorted $CD133^+$ and $CD133^-$ cells generate histologically similar tumors that contain $CD133^+$ cells fraction. *Curley MD1, Therrien VA, Cummings CL, Sergent PA, Koulouris CR, Friel AM, Roberts DJ, Seiden MV, Scadden DT, Rueda BR, Foster R. CD133 expression defines a tumor initiating cell population in primary human ovarian cancer. Stem Cells 2009; 27:2875-2883.*

Aldehyde dehydrogenase isoform 1 (ALDH1), a detoxifying enzyme involved in chemo-resistance development, is another putative marker of ovarian cancer stem cells. Indeed, cells with high ALDH activity ($ALDH^+$), isolated from ovarian cancer cell lines, are more resistant to chemotherapy and present a higher tumorigenic potential compared to cells with low ALDH activity ($ALDH^-$) [34]. These data were supported by the research group of Landen, who

demonstrated that the down-regulation of ALDH, obtained by ALDH gene silencing, reverted chemo-resistant cells into chemo-sensitive cells both *in vitro* and in an *in vivo* xenograft model [35].

Since the combination of two or more CSC markers increases the specificity of CSC identification and isolation, Silva and collaborators evaluated the characteristics of $ALDH^+/CD133^+$ cells derived from ovarian cancer cell lines, and demonstrated that this cell population is highly proliferative. Furthermore, ovarian cancer $ALDH^+/CD133^+$ cells displayed a higher tumor potential and generated tumors with shorter latency than the $ALDH^-/CD133^+$, $ALDH^+/CD133^-$ and $ALDH^-/CD133^-$ counterpart (Table 2) [34].

	A2780 Cell Line			
Cell Type	ALDH−CD133+	ALDH+CD133+	ALDH+CD133−	ALDH−CD133−
# Cell Injected	Tumors Formed			
30	4/6	4/6	0/5	0/5
100	5/5	5/5	3/6	0/5
1000	10/10	10/10	10/10	7/10
	Primary Human Tumor Samples			
Cell Type	ALDH−CD133+	ALDH+CD133+	ALDH+CD133−	ALDH−CD133−
	Tumors Formed			
# Cell Injected				
10–500	0/9	4/9	0/9	ND
5000	ND	ND	0/5	0/9
50000	ND	ND	ND	0/9

Table 2. Tumor initiating capacity of limiting dilutions of ALDH+/- CD133+/- cells from primary human ovarian tumors. *Silva I.A., Bai S., McLean K., Yang K., Griffith K., Thomas D., Ginestier C., Johnston C., Kueck A., Reynolds R.K., Wicha M.S., Buckanovich R.J. Aldehyde dehydrogenase in combination with CD133 defines angiogenic ovarian cancer stem cells that portend poor patient survival. Cancer Res 2011; 71:3991-4001.*

Lastly, since ovarian CSC are able to extrude toxins and drugs, as chemotherapy agents, thanks to the expression of members of the ATP-binding cassette transporter family (for instance ABCG2), this stem cell subset has also been defined "Side Population" (SP). Indeed, the ability of drug extrusion has allowed the isolation of a side population of cells with an increased capacity to extrude lipophilic substrates such as the dye Hoechst 33342. Many reports have shown that SP cells present the following features: resistance to cytotoxic treatment, expression of stemness and multi-drug resistance genes, and high tumorigenic potential [36].
Nevertheless, data on the SP population show some discrepancies: firstly, in one study, despite the SP fraction has been identified in paclitaxel-resistant cell lines, it has not been identified in cisplatin-resistant cells; secondly, the correlation between SP and multidrug resistance remains still unclear [37]. Furthermore, although SP cells have been found more represented in ascites, recurrent tumors and tumors spread in the peritoneal cavity, the association of SP with disease stages was not significantly demonstrated [38].

These premises suggest that CD117 is the stemness marker which, in combination with CD44 as a tumor cell marker, best allows the identification of an ovarian cancer cell population characterized by stemness properties, such as self-renewal, chemo-resistance and tumor-initiating capacity.
Moreover, CD117-expressing cells with stemness phenotype have been detected not only in ovarian cancer cell lines, but also in primary samples of ovarian carcinoma at different stages and so with different grade of tumor dissemination and distant metastasis.

Thereby, CD117 (or c-kit), in view of its well-known function as tyrosine kinase receptor and of its high specificity as ovarian CSC markers, represent an attractive target for cancer therapy.

Some of the molecular features of ovarian CSC, which allow their survival, proliferation and ability of tumor growth maintenance, have been analyzed in the last years. Among these molecular traits, Notch, Wnt, Hedgehog and transforming growth factor-β (TGF-β) signaling are crucial for the modulation of self-renewal. Especially, TGF-β plays a key role in the regulation of epithelial to mesenchymal transition (EMT) pathways and in the promotion of CSC motility and migration [39].
Furthermore, some genes associated with ovarian tumor-initiating properties are already known. For instance, the expression of p53, a tumor suppressor gene, is involved in the pathogenesis of high-grade serous adenocarcinoma of the ovary; recently, some authors have reported that p53 expression silencing transforms ovarian tumor non-stem cells into ovarian CSC [40], supporting the idea that p53 function impairment could increase the self-renewal ability of ovarian stem-like cancer cells. Another recent report by Chau and collaborators have demonstrated the key role of c-kit in the modulation of ovarian CSC survival and chemo-resistance, through cellular processes mediated by phosphoinositide 3-kinase (PI3K)/Akt and Wnt/β-catenin-ATP-binding cassette G2 (ABCG2) pathways. C-kit was found over-expressed in ovarian CSC compared to their non-stem counterpart, and solid evidence has demonstrated that the c-kit knockdown or the c-kit activity inhibition by Imatinib treatment are able to reduce the tumorigenic potential and the chemo-resistance property of ovarian CSC [41].

Finally, also microRNA (miRNA), small non-coding RNA molecules able to modulate the expression of specific genes, has recently been reported as important regulators of tumor development and cancer stemness. In ovarian CSC, the expression profile of mir-199a and mir-200 was analyzed [42,43]. In particular, mir-199a emerged as suppressor of tumorigenicity and chemo-resistance of the ovarian $CD44^+/CD117^+$ cell subset, due to the mir-199a-mediated CD44 repression which inhibits CSC migratory and invasive properties [43].

All the above mentioned features could provide potential novel strategies to improve ovarian cancer therapy. However, a new hypothesis suggests that the self-renewal property could be supported by signals from the surrounding microenvironment. In ovarian cancer, this niche includes immune cells, stromal cells, blood vessels and extracellular matrix effectors. All these components could represent a new target for therapy; indeed, these tumor-associated cells are involved in several biological processes, such as inflammation, hypoxia and angiogenesis, which occur in CSC niche and could be crucial to determine the fate of CSC. Thus, it is mandatory to focus the attention on tumor microenvironment to discover the key mechanisms of CSC survival and growth (Figure 11) [44].

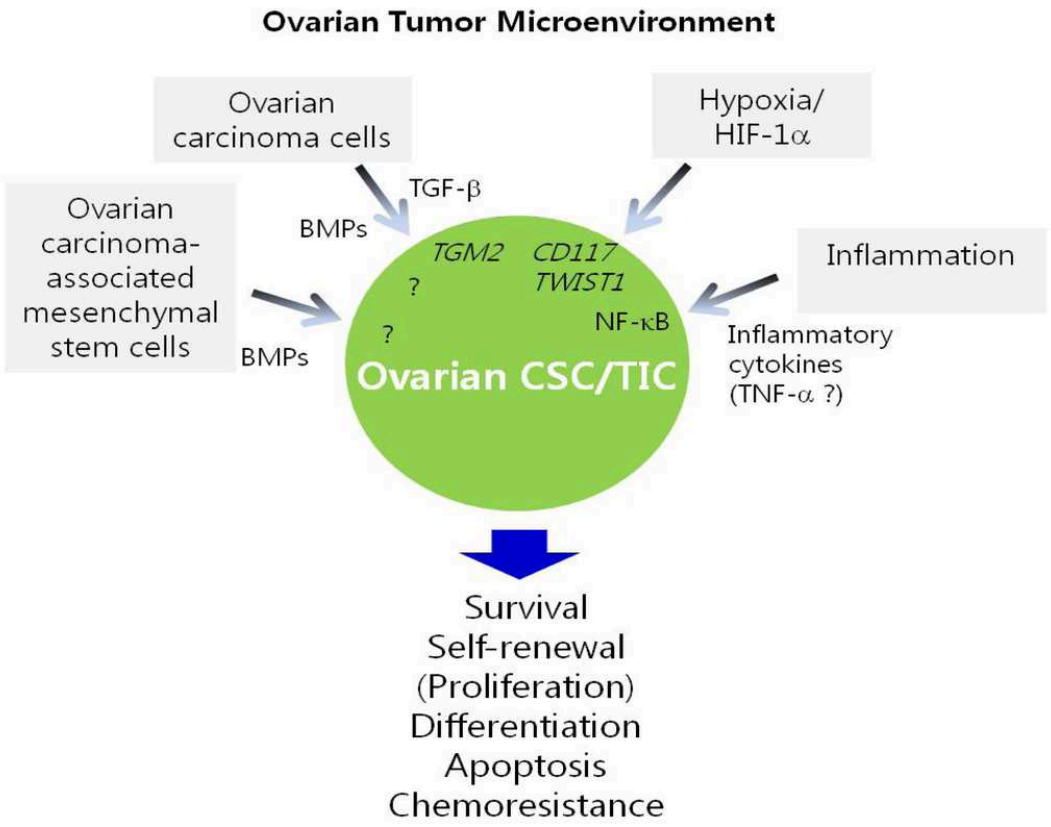

Figure 11. The tumor microenvironment involved in the maintenance and regulation of ovarian cancer stem cells. *Kwon M.J. and Shin Y.K. Regulation of ovarian cancer stem cells on tumor-initiating cells. Int. J. Mol. Sci. 2013; 14:6624-6648.*

1.4. Role of cancer stem cells in chemo-resistance and importance of targeting CSC

In view of clinical implications, chemo-resistance is one of the most interesting properties of ovarian CSC. Indeed, because of drug resistance, most chemotherapy treatments against cancer prove ineffective and fail in a short period. This failure is due to the therapeutic strategies, which do not target selectively CSC; for this reason, CSC are not eliminated by the therapies and, thus, the incidence of tumor relapse and metastasis is very high. Many different pathways are involved in drug resistance onset, including activation of anti-apoptotic effectors, inactivation of pro-apoptotic factors, and enhancement of survival signals. Among the survival signals, the most important are the following: the previously mentioned drug extrusion pumps, altered DNA repair mechanisms and cell cycle alterations. Furthermore, CSC can enter in a state of quiescence, so escaping stress conditions as chemotherapy-related cytotoxicity, oxygen and nutrient starvation and oxygen radical formation.

Therefore, it is mandatory to develop selective treatments against CSC. Unfortunately, to this aim, many obstacles have to be faced (Figure 12).

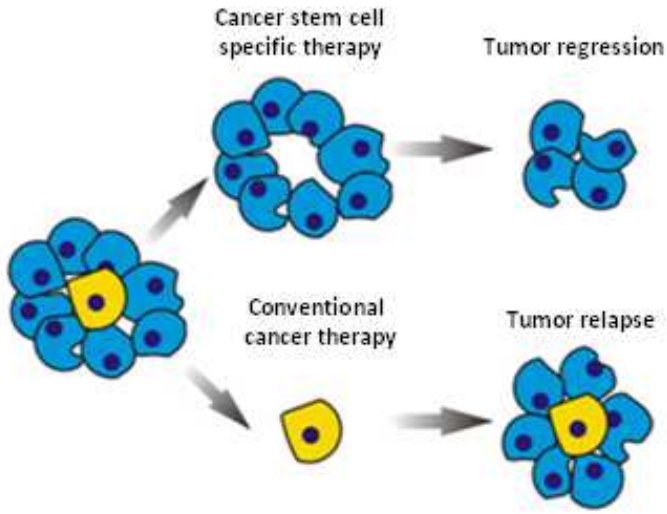

Figure 12. Cancer Stem cell specific and conventional therapy. *Pardal R, Clarke M.F., Morrison F.J. Applying the principles of stem-cell biology to cancer. Nat Rev Cancer. 2003 Dec;3(12):895-902.*

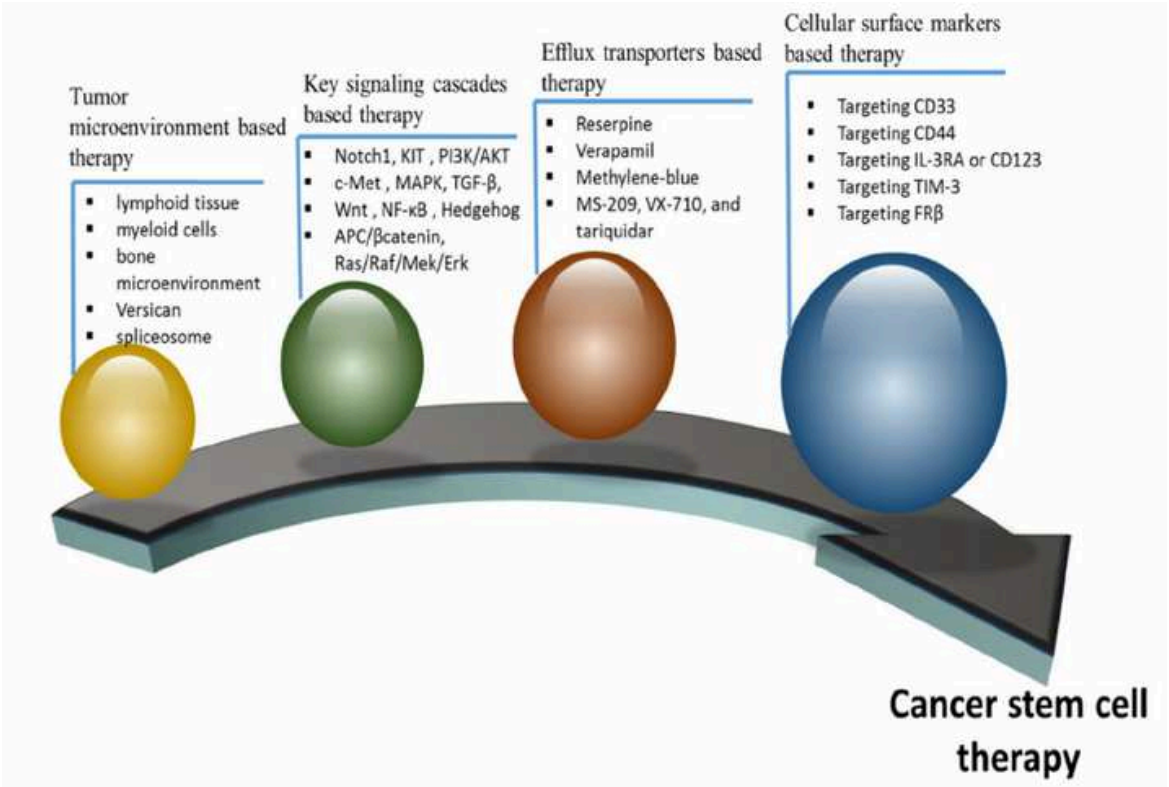

Figure 13. Therapies targeting cancer stem cells. *Targeting surface markers surface markers of CSC (blue area), specific pathways involved in the self-renewal (green area), inhibiting ABC cassette (brown area), targeting microenvironment (yellow area). Mitra AK, Agrahari V, Mandal A, Cholkar K, Natarajan C, Shah S, Joseph M, Trinh HM, Vaishya R, Yang X, Hao Y, Khurana V, Pal D. Novel delivery approaches for cancer therapeutics. J Control Release. 2015 Dec 10; 219:248-68.*

As a matter of fact, several problems in clinical applications could derive from the complexities which lie beyond anti-CSC therapy. The difficulties to face for this new approach include the necessity to find new specific CSC antigens to distinguish these cells from the non-neoplastic stem

compartment. Indeed, CSC share many characteristics and molecular epitopes with normal stem cells, thus enhancing the risk of toxic side effects related to CSC-targeting therapy.

The therapeutic strategies currently developed to specifically target CSC [45,46] (Figure 13) are the following. Firstly, a very interesting approach is the development of molecules specifically designed to target CSC. For instance, Imatinib, specifically inhibiting CD117 function, has already administered in clinical trials for the cure of many cancer types, including ovarian cancer [46]. Moreover, several drugs have already been designed to bind to ABC transporter proteins and, thus, to inhibit multi-drug resistance onset. In this setting, the first identified drug-extrusion pump inhibitor was Verapamil, often used in side population analysis by virtue of its ability to extrude the dye Hoechst. In ovarian cancer, the combination between the chemotherapy agents Docetaxel and the pump inhibitor Tariquidar is under clinical investigation [47]. Also microRNA could be regarded as target for anti-CSC therapy; indeed, as previously mentioned, the role of such molecules in CSC features has already been reported, and compounds able to inhibit or to mimic miRNA function are already under investigation for cancer treatments [48].

Another strategy to circumvent the CSC resistance to chemotherapy is the induction of differentiation and of the loss of self-renewal features of these cells. CSC differentiation can be stimulated by drugs such as retinoic acid or drugs able to generate epigenetic changes in the tumor cells. Whitworth et al. reported that the treatment with carboplatin in combination with three novel retinoid compounds could reduce the growth of ovarian CSC [49]. Furthermore, some compounds able to inhibit the pathways controlling self-renewal and cell fate decisions of stem cells and progenitor cells, such as Wnt, Hedgehog and Notch, have already been identified. In this regard, McAuliffe and collaborators have recently shown that γ-secretase inhibitor (GSI), a Notch pathway inhibitor, depletes efficiently CSC [50], thus demonstrating the importance of the Notch signaling pathway in the modulation of ovarian CSC growth and survival.

Lastly, many researchers have recently focused their attention on tumor microenvironment, since it could create a niche to protect CSC from anti-tumor drug action and from specific stress conditions, such as nutrient or oxygen starvation. In view of the crucial role of the microenvironment in supporting cancer growth, progression and migration, including metastasis formation, the interactions between CSC and tumor niche assume a prominent importance in the development of future therapeutic strategies [51].

Tumor microenvironment includes many cell types, such as immune inflammatory cells, as well as cancer-associated fibroblasts and endothelial cells; all these cells may represent a suitable therapeutic target. Moreover, tumor niche does not only mean the array of cell types surrounding cancer mass, but also means the conditions, in terms of oxygen/nutrient supply and metabolites, in which the cells grow and which constrain or permit cell survival. As Weinberg asserted in his seminal work, tumor has to be recognized as an organ, and its biology can only be understood by studying the cell types within it as well as the microenvironment that these cells create during the steps of carcinogenesis [52]. Therefore, in the last decades, cell metabolism became a key target to improve cancer therapy.

These premises highlight that cancer-associated miRNA and cancer cell metabolism represent two main fields for medical research. Therefore, further investigations to target miRNA and/or cell metabolism are needed to develop novel anti-tumor strategies.

1.5. microRNA

microRNA (miRNA) are small non-coding RNA molecules of about 22 nucleotides, which induce the post-transcriptional repression of messenger RNA (mRNA) targets, based on sequence-specific binding to the mRNA [53,54]. Indeed, the miRNA-mediated inhibition of the protein translation is due to the base-paired binding of the specific miRNA to its mRNA target [55]. As a result, the miRNA/mRNA interaction induces the silencing of the mRNA-coding gene, through different possible processes: 1) the cleavage of the mRNA strand; 2) the destabilization of the mRNA target by the shortening of its poly(A); 3) the impairment of mRNA translation into protein, since the presence of the miRNA physically prevents ribosome activity [55,56]. Thus, since miRNA act by silencing their target genes, miRNA action resembles that of small interfering RNA (siRNA) of the RNA interference (RNAi) pathway; however, while miRNA derive from sequences of RNA transcripts that fold back on themselves to form short hairpins, siRNA derive instead from longer regions of double-stranded RNA [54].

The human genome encodes over 1000 miRNA, which are estimated to be responsible for the regulation of about 60% of the genes [57,58]. The specificity of miRNA/mRNA binding is given by the full base-paired complementarity of the mRNA target to the seed region of the miRNA, which is a short sequence of the non-coding RNA (nucleotide 2-8) at the 5' end [58-60]. Once the seed region of the miRNA totally recognized its complementary sequence at the target mRNA 3'-UTR, the complete base-paired alignment of the nucleotides outside the seed region is not necessary to miRNA inhibitory function; thus, in several cases the miRNA/mRNA pairing is not sufficient to induce the cleavage of the target transcript [55]. Moreover, since the miRNA-silencing effect is mediated by a few nucleotides, a given miRNA may have hundreds of different mRNA targets, and a given target might be regulated by multiple miRNA [57,61]. Thereby, miRNA are involved in many biological processes, such as cell proliferation and apoptosis that are two of the most widely accepted tumor-associated pathways; indeed, the role of miRNA in carcinogenesis has already been reported in several cancer types [62,63].

miRNA can be transcribed either from intragenic region, within introns or exons of other genes, or from their specific coding genes. Approximately 70% of the characterized miRNA genes are intragenic, lying in the introns of coding or non-coding genes or in the exons of long non-coding gene sequences [64]. In these cases, the miRNA-coding sequence is usually found in a sense orientation, and is usually transcribed in synergy with the host gene [65-68]. In other cases, miRNA derive from independent genes, which are often oriented antisense to neighboring genes and are so probably transcribed as an independent unit [69-72].

About 40% of all miRNA are organized in clusters and are transcribed in a polycistronic unit from which mature miRNA are processed [72,73]. Generally, these clustered miRNA are members of a family, showing overlapping functions [64,72]. Each cell type has a combination of either isolated

or clustered expressing miRNA, which regulate coding genes in a tissue-specific manner and, therefore, are essential for the maintenance of cell identity and functional phenotype [74].

The biogenesis of miRNA is well-known (Figure 14).

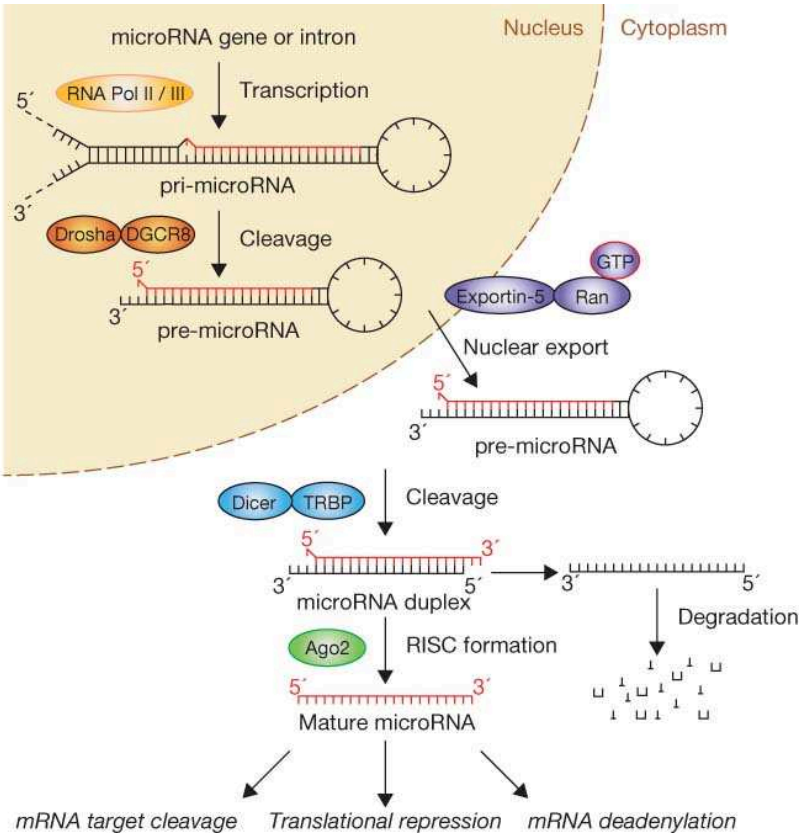

Figure 14. The canonical pathway of microRNA processing*. Winter J, Jung S, Keller S, Gregory RI, Diederichs S. Many roads to maturity: microRNA biogenesis pathways and their regulation. Nat Cell Biol. 2009 Mar;11(3):228-34.*

miRNA genes are usually transcribed into the nucleus by RNA Polymerase II (Pol II) [72,75]. The polymerase often binds to a promoter located near the DNA sequence intended to became the hairpin loop of the pre-miRNA. The resulting transcript is capped at the 5' end with a 7-methylguanosine nucleotide, then is polyadenylated through the addition of a tail of multiple adenosine molecules [poly(A) tail] at the 3' end, and finally is subjected to splicing. miRNA are initially transcribed as a part of an ~80 nucleotide-long RNA stem loop, which in turn forms part of a several hundred nucleotide-long miRNA molecule termed pri-miRNA (primary miRNA) [65,72]. A single pri-miRNA may contain from one to six miRNA precursors, and the hairpin is flanked by sequences necessary for the efficiency of the entire process. Because of its looped structure, the hairpins of the pri-miRNA are formed by double-stranded RNA (dsRNA), which is recognized by a nuclear protein known as DiGeorge Syndrome Critical Region 8 (DGCR8). DGCR8 associates with the RNasi III Drosha forming the Microprocessor Complex [76,77]. In this complex, DGCR8 orients the catalytic domain of Drosha to cleave the RNA in a definite site near the hairpin, thus liberating the pri-miRNA from the looped structure. The product resulting from this process is the pre-miRNA (precursor-miRNA) [78,79]. Otherwise, when the miRNA-coding sequence is located

within the introns of other genes, the pre-miRNA is directly spliced out of introns bypassing the Microprocessor Complex [80]; in these cases, the pre-miRNA are also termed Mirtrons.
Independently of their origin, pre-miRNA are transported from the nucleus to the cytoplasm by the shuttle protein Exportin-5 [81]. In the cytoplasm, the RNase III Dicer cleaves the pre-miRNA hairpin, yielding an imperfect miRNA duplex of about 22 nucleotides in length [82]. Based on the thermodynamic stability [83-85], only one strand is selected to be incorporated into the RNA-induced silencing complex (RISC), which also contains Dicer and other associated proteins [86]. RISC is also termed microRNA ribonucleoprotein complex (miRNP), and RISC with incorporated miRNA is also known as miRISC [87].
The strand that is not incorporated in the RISC complex is usually referred as "passenger strand" and is normally degraded. However, sometimes both strands become functional miRNA targeting different mRNA populations [88].
Other key members of the RISC complex are the proteins of the Argonaute family. These proteins are essential for the miRNA/mRNA interaction, since they bind the miRNA and orient it for the contact with the target mRNA [87,89].
Additional RISC components include TRBP (HIV trans-activating response RNA – TAR binding protein) [90], PACT (protein activator of the interferon-induced protein kinase), the SMN complex, FMRP (fragile X mental retardation protein), Tudor-SN (Tudor staphylococcal nuclease-domain-containing protein), MOV10 (the putative DNA helicase), and the RNA recognition motif containing protein TNRC6B [81,91,92].

Besides the aforementioned canonical mechanisms of miRNA gene regulation through 3'-UTR interactions, other "not canonical" miRNA-mediated mechanisms of mRNA expression modulation are emerging [93-98]. Some miRNA have been shown to bind to the open reading frame or the 5'-UTR of the target genes and, in a few cases, they have been shown to activate rather than to inhibit gene expression [93,94]. Other studies reported that miRNA can interact with ribonucleoprotein in a RISC-independent manner [95,96], or, in other cases, they can regulate gene expression by binding directly to DNA [97-99].

Overall, these data demonstrate the complexity and widespread regulation of gene expression by miRNA.

1.5.1. microRNA and cancer

Since miRNA are strictly involved in the normal functioning of human cells, it is easy to understand that miRNA deregulation could be associated to many diseases [100]. Cancer is the first human disease known to be associated to miRNA expression alteration [62,63], and the term "oncomir" is used to indicate tumor-linked miRNA [101].
The role of miRNA in cancer development has firstly been identified in hematologic malignancies. In this setting, the down-regulation of mir-15a and mir-16-1 has been reported in B-cells of patients affected by chronic lymphocytic leukemia (CLL) [102].
Further studies have established that also solid cancers exhibit a distinctive miRNA expression profile compared to normal tissues. Moreover, genome-wide profiling showed that the miRNA expression signatures allow to discriminate different cancer types with high accuracy, and to identify the primary tissue of origin of poorly differentiated tumors [74,103]. Thus, miRNA

represent reliable indicators of the cancer types [74], underlying their crucial role in tumor development.

Further studies have reported that selected groups of distinct miRNA were commonly up- or down-regulated concurrently in distinct types of human neoplasms. For instance, miR-17 and miR-21 were found to be consistently up-regulated in colon, lung, stomach, prostate and pancreatic tumors, whereas breast, lung and colon cancer exhibited a significant up-regulation of mir-155 [103]. These data have been validated over time in different cohorts of patients, and similar results were observed in other types of cancer as well (Table 3) [62]. On the other hand, miR-29 was reported to be down-regulated in CLL, acute myeloid leukemia (AML), lung and breast cancer, rhabdomyosarcoma, cholangiocarcinoma, liver cancer and mantle cell lymphoma [104-111]. Among the miRNA recurrently deregulated in cancer, it is necessary to mention also miR-15-a/miR-16-1, which was down-regulated in CLL, prostate and pituitary adenomas [102,112,113], and members of the let-7 family, which showed a reduced expression in lung, colon, breast, ovarian and stomach cancer [106,107,114-120]. Some of the miRNA classified as deregulated in cancer are reported in Table 3.

Common miRNAs altered in human cancers.

MicroRNA	Expression in cancer	Function	Targets
Let-7a-2	Down in breast, lung, colon, ovarian, and stomach cancer	Tumor suppressor	KRAS, HMGA2, MYC, DICER, BCLXL, IMP-1, CDC34, IL6
miR-15/16	Down in CLL, prostate cancer, and pituitary adenomas	Tumor suppressor	BCL2, COX2, CHECK1, CCNE1, CCND1, CCND2, BMI-1, FGF2, FGFR1, VEGF, VEGFR2, CDC25a
miR-29 family	Down in AML, CLL, lung and breast cancer, lymphoma, hepatocarcinoma, rhabdomyosarcoma	Tumor suppressor	CDK6, MCL1, TCL1, DNMT1, DNMT3a, DNMT3b
miR-34 family	Down in colon, lung, breast, kidney, and bladder cancer	Tumor suppressor	SIRT1, BCL2, NOTCH, HMGA2, MYC, MET, AXL. NANOG, SOX2, MYCN, SNAIL
miR-26a	Down in liver cancer	Tumor suppressor	CCND2, CCNE2
miR-200 family	Down in aggressive breast and ovarian cancer	Tumor suppressor	ZEB1, ZEB2, BMI-1, SUZ-12, FN1, LEPR, CTNNB1, JAG1, MALM2, MALM3, p38 alpha
miR-155	Up in high risk CLL, AML, breast, lung, colon cancer, and lymphoma	Oncogene	SOCS1, BACH1, MEIS1, ETS1, FOXO3A, hMSH2, hMSH6, hMLH1, SMAD5, WEE1, SHIP1, CEBPB
miR-21	Up in lung, breast, pancreas stomach, ovary prostate cancer, and CLL, AML, glioblastoma, myeloma	Oncogene	PTEN, TPM1, PDCD4, SPRY1, TIMP3, RECK
miR-221/-222	Up in invasive ductal carcinoma, lung cancer, hepatocellular carcinoma, papillary thyroid cancer	Oncogene	p27(Kip1), p57(Kip2), PTEN, TIMP3, FOXO3A, ERalpha, KIT, TRSP1, DICER, APAF1, PUMA, PTPμ
miR-17/92	Up in lung, breast, colon	Oncogene	PTEN, BIM, HIF1, PTPRO, p63, E2F2, E2F3, TSP-1, CTGF, p21(WAF1), JAK1, SMAD4, TGFbetaII, MnSOD, GPX2, TRXR2

Table 3. Common miRNAs altered in human cancers. *Di Leva G, Croce CM. The role of microRNAs in the Tumorigenesis of Ovarian Cancer. Front Oncol. 2013 Jun 13;3:153.*

These similarities in miRNA expression patterns of different tumor tissues led to the hypothesis that miRNA deregulation in cancer is not a random event, thus suggesting that up-regulated miRNA may act as oncogenes, whereas the down-regulated miRNA as tumor suppressors. Consequently, it was postulated that miRNA-encoding genes can be subjected to genomic alteration leading to

expression up-regulation (e.g, translocations, amplifications) or loss-of-function (e.g., deletions, insertions, mutations) [62]. Nevertheless, though several evidence has been found consistent with these postulated mutations in miRNA-coding genes, other mechanisms able to modify miRNA expression have been proposed and, in some cases, validated. Among them, the most frequently found in cancer tissues are the following: defects in the biogenesis mechanism, such as the altered expression of Drosha, Dicer, and Exportin; chromosomal rearrangements; alterations in miRNA transcriptional regulation, such as modification in transcription factor functions or epigenetic modifications at the promoter region of the miRNA-coding genes [63].

Besides the evidence indicating that miRNA expression is deregulated in several cancer types, functional investigations have provided insights into the role of miRNA in carcinogenesis [62]. As a matter of fact, the tumor suppressor miRNA let-7, mir-29 and mir-34 have been shown to target oncogenes which play critical roles in several cancer pathways, such as ras [115], mcl-1 [109,121] and myc [122,123]. Other gain- and loss-of-function experiments, in combination with target prediction analyses, have demonstrated that mir-21 [124] and mir-16 [125] are involved in apoptotic signaling, and also that mir-17-92 cluster [126] and mi-155 [127] induced lymphoproliferative disorder development. Moreover, in addition to classical tumor suppression or oncogene functions, miRNA have been recently found to be implicated in cell migration and metastasis. Indeed, some authors have reported that mir-10a [128], mir-126 and mir-135 [129] act as regulators of invasion and distant site metastasis in breast and lung cancer.

In view of the evident involvement of miRNA in tumorigenesis, targeting miRNA has recently assumed a high relevance in the attempt to improve anti-cancer therapy. Indeed, the rationale for using miRNA as anti-cancer drugs is based on two major assumptions: miRNA expression is deregulated in cancer compared to normal tissues, and the cancer phenotype can be changed by targeting miRNA expression [62]. To target miRNA expression, two main categories of strategies have been proposed: direct and indirect strategies. The direct strategies imply the use of oligonucleotides or virus-based constructs either to block the expression of an oncogenic miRNA or to enhance the expression of a tumor suppressor miRNA. More in detail, antisense oligonucleotide working as competitive inhibitors of miRNA have recently been designed to block the expression of oncogenic miRNA [130-134]. These oligonucleotides are defined “antagomirs” or “miR-Mask”, based on the technology from which derive. To the same scope of miRNA expression inhibition, some studies have reported the use of other competitive miRNA inhibitors, termed “miRNA sponges”, which consist on mammalian vector-derived transcripts containing multiple binding sites to the selected miRNA [135-137]. On the other hand, the over-expression of tumor suppressor genes can be achieved by introducing synthetic oligonucleotides that are identical to the selected miRNA and are known as miRNA mimics [108-110,112,118,121]. Recently, the use of adenovirus associated vectors (AAV) has been tested to increase the expression of a selected miRNA; this approach has obtained interesting results in view of its minimal toxicity, and some clinical trials are underway [138,139]. Finally, the indirect strategy involves the use of drugs to modulate miRNA expression by targeting their transcription and processing [140-145] (Figure 15).

Based on these premises, in order to underline the importance of miRNA investigation, it is mandatory to underline that targeting miRNA, rather than specific genes or proteins, may be more effective, as miRNA often finely tune entire pathways [48].

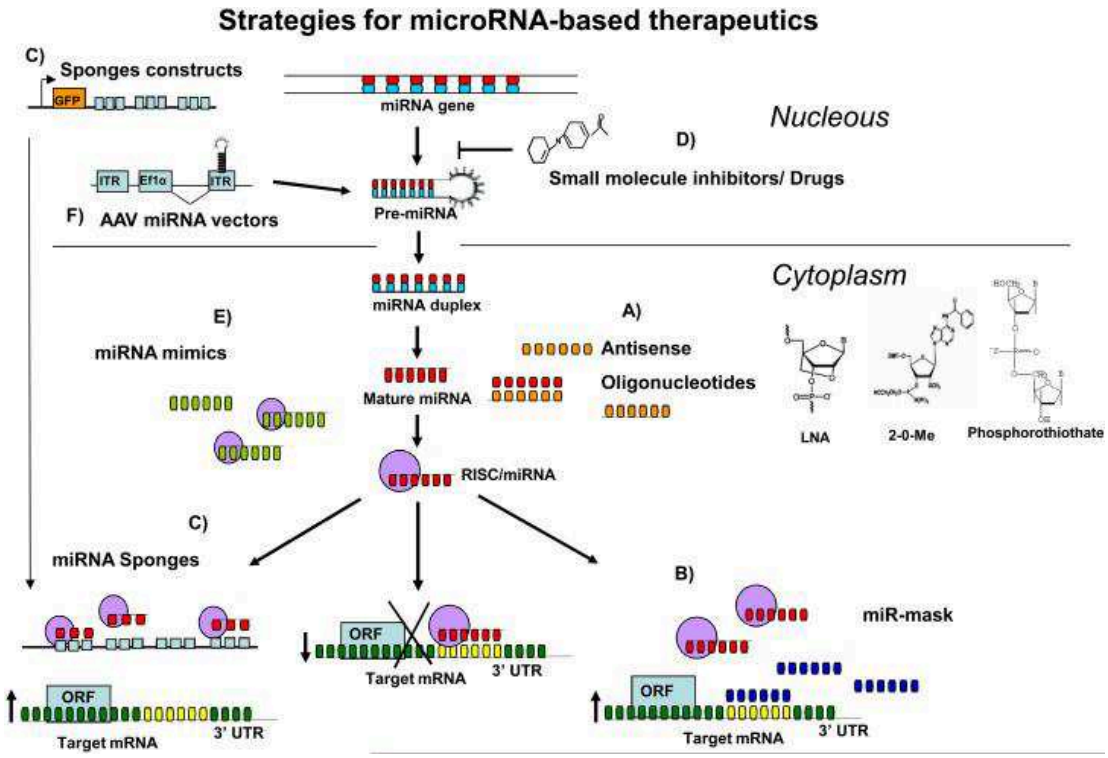

Figure 15. Strategies for miRNA-based therapies*. Garzon R, Marcucci G, Croce CM. Targeting microRNAs in cancer: rationale, strategies and challenges. Nat Rev Drug Discov. 2010 Oct;9(10):775-89.*

However, despite the recent evidence about the possibility of using miRNA as a therapeutic tool, there are no miRNA-based drugs available for clinical applications, because of the challenges related to this approach [146]. Indeed, as miRNA may affect simultaneously the expression of an array of genes, they may cause several side effects. Moreover, the delivery to the correct tissue, which is an essential aspect for an efficient miRNA function and for the prevention of unwanted off-target effects, is very hardly achievable [48]. Anyway, the impact that miRNA tools could have in the improvement of anti-cancer treatments remains very promising; therefore, novel mechanisms to employ miRNA targets in cancer therapy need to be exploited in order to face the hurdles associated to this miRNA therapeutic approach.

1.5.2. miRNA and ovarian cancer (CSC and non-CSC)

The results mentioned in the previous paragraph have underlined the importance of analyzing miRNA signatures in cancer tissues, in the attempt to identify novel miRNA to use as predictors of tumor progression or as targets for cancer therapy. This is actually true for all human malignancies, but very more important for aggressive cancers, which still represent a medical issue because of their poor responsiveness to chemotherapy. In this regard, ovarian carcinoma urgently needs the development of new therapeutic strategies, in view of its aggressiveness despite the current clinical treatments. Thus, efforts have to focus on the identification of ovarian cancer biomarkers that may aid in early diagnosis and prognosis, as well as on the characterization of therapeutic targets to circumvent chemo-resistance and to prolong survival at advanced stages [48]. In this context, miRNA have provided a significant contribution, since many studies have already demonstrated the involvement of miRNA in the occurrence and/or progression of ovarian carcinoma [48,146-150].

The first report of miRNA deregulation in ovarian cancer was published in 2007 by Iorio and colleagues, who showed that miRNA expression profiles could discriminate between ovarian cancer specimens and normal ovarian tissues [149]. For instance, in this study mir-200a/b/c and mir-141 were found highly up-regulated in cancer, whereas mir-199a, mir-145, mir-140 and mir-125b1 were most significantly down-regulated. Moreover, they observed that these miRNA signatures correlated with the different the histological subtypes of ovarian carcinoma. Indeed, some miRNA were found deregulated in all subtypes (mucinous, endometrioid, and clear cells), while the over- or under-expression of other miRNA was associated only to one or few tumor subgroups, thus allowing to discriminate among them (Figure 16).

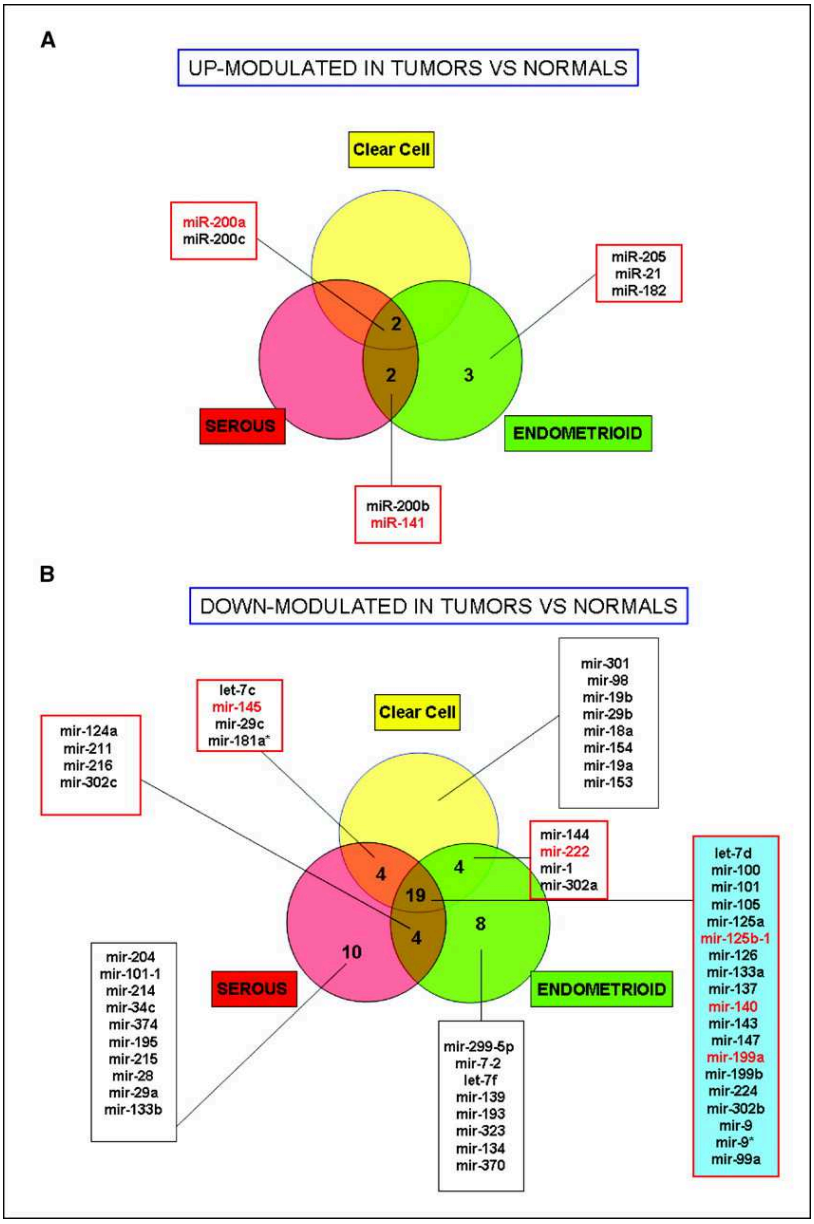

Figure 16. miRNA signatures characterizing different ovarian carcinoma histotypes compared with the normal tissue. *Iorio MV, Visone R, Di Leva G et al. MicroRNA signatures in human ovarian cancer. Cancer Res. 2007 Sep 15;67(18):8699-707.*

The results from this first study have been later confirmed by the same research group and by other authors [151-153], and are still considered among the most relevant and promising data about

miRNA and ovarian cancer [48]. Indeed, further genome-wide miRNA analyses extended the miRNA deregulation network in ovarian tumors [146], but never refuted the data provided by Iorio and colleagues.

In the later years, the relevance of the diagnostic and prognostic significance of miRNA signatures have been investigated, and many studies have shown different ovarian miRNA profiles along the course of the disease (Table 4). Indeed, some miRNA were found differentially expressed either in ovarian primary tumors versus metastatic lesions, or in the early-stage versus the late-stage diseases. For example, mir-200a was identified as a predictor of patient outcome in advanced-stage ovarian tumors [154,155], and another member of the mir-200 family, mir-200c, was found to be associated with progression-free survival and overall survival of stage I ovarian cancer [156]. Nevertheless, these data are not always consistent; for example, mir-200 family has been related either to poorer or better prognosis in ovarian cancer patients [156]. Therefore, miRNA expression analysis is not yet regarded as a reliable marker for ovarian carcinoma detection and monitoring. More recently, in the attempt to overcome the lack of reliability of one-single-miRNA-based diagnosis, Bagnoli and collaborators defined and validated a 35-miRNA-based predictor of ovarian cancer relapse or progression [157]. However, despite this study is very promising, it does not yet provide a clinically available platform; thus, further studies are needed to support the use of miRNA as diagnostic and/or prognostic tools [48].

Moreover, tissue-based markers still require an invasive procedure to obtain samples, thus determining a hurdle in the translation of above-mentioned discoveries to the clinics. The use of serum biomarkers represents one of the best tool for early diagnosis and for the correct prediction of the patient outcome and therapeutic response; indeed, circulating miRNA are more stable and easy detectable than those associated to specific tissues. Several studies have revealed the detectability of certain miRNA in the serum of ovarian cancer patients and the correlation of their expression level with ovarian carcinoma stages and grades [158-161]. Unfortunately, despite these data, ovarian carcinoma serum biomarkers are not yet used in the clinics. Indeed, the heterogeneity of the results, based on different sample types and on different technologies, highlights that additional studies are required to define predictive and reliable miRNA signatures for clinico-pathologic applications.

Despite the importance of miRNA profiles in the diagnosis and prognosis of ovarian malignancies, the possibility that miRNA could be involved in the responsiveness to therapies has even a more significant impact on disease management. In this context, ovarian miRNA signatures have already demonstrated their potential in the prediction of the clinical response to chemotherapy. Firstly, in 2008 Zhang's research group identified the involvement of let-7i in response to cisplatin treatment in ovarian cancer [119]. Another study performed by Eitan and colleagues provided a list of several miRNA differentially expressed in patients characterized by complete response to platinum-based chemotherapy, with no recurrence within six months, compared to patients who relapsed rapidly after treatment; this list included mir-27a, mir-23a and mir-378 [154]. Other researchers reported that higher expression of mir-200c was associated with lower relapse/progression rate [162]. Few years later, a panel of 23 miRNA associated with ovarian cancer chemo-resistance was described by Vecchione et al [163]; moreover, mir-182 and mir-125b resulted to be able to confer resistance to cisplatin in human ovarian carcinoma, probably because of their anti-apoptotic activity [164,165]. More recently, a cluster of miRNA emerged as down-modulated in early-relapsing ovarian cancers, also related to a reduced sensitivity to chemotherapy [157].

Table 4. *miRNA studies in human epithelial ovarian cancer.* *Di Leva G, Croce CM. The role of microRNAs in the Tumorigenesis of Ovarian Cancer. Front Oncol. 2013 Jun 13;3:153.*

miRNA profiling studies in human epithelial ovarian cancers.

Reference	Number of samples/subtypes	Method of analyses	Main findings
Iorio et al.	15 Normals/69 tumors 31 Serous/8 endometrioid/4 clear cells/9 poorly differentiated/1 mucinous	miRNA microarray	Ovarian cancer-specific miRNA signature Subtypes specific miRNA signature Epigenetic mechanism responsible for their aberrant expression
Yang et al.	10 Tumors and 10 "normal" HIOSE cell line	miRNA microarray	Ovarian cancer-specific miRNA signature miR-214 induces cell survival and cisplatin resistance through targeting PTEN
Laios et al.	3 Primary serous/3 recurrent serous tumors	qRT-PCR	miR-9 and miR-223 can be biomarkers in recurrent ovarian cancer
Nam et al.	22 Serous tumors/8 normals	miRNA microarray	Ovarian cancer-specific miRNA signature
Zhang et al.	106 Tumors 109 Tumors 76 Tumors 504 Tumors	miRNA microarray, aCGH, affymetrix cDNA microarray, tissue array,	miRNAs are downregulated in malignant transformation and tumor progression Genomic copy number loss and epigenetic silencing account for miRNA dysregulation
	96 Tumors	qPCR validation	
Dahiya et al.	34 Tumors and HOSE-B cell line	miRNA microarray	Ovarian cancer-specific miRNA signature
Sorrentino et al.	Drug-resistant vs. wild-type cancer cell lines	miRNA microarray	Paclitaxel and cisplatin resistance is associated with a specific miRNA fingerprint
Yang et al.	69 Tumors (42 sensitive/27 resistant)	miRNA microarray	Let-7i is a modulator of platinum-based chemotherapy Let-7i is a biomarker to predict chemotherapy response and survival
Boren et al.	16 Ovarian cancer cell lines	miRNA microarray	miRNA signature associates to cell line drug response
Wyman et al.	33 Tumors/HOSE-B cell line	Deep sequencing	Ovarian cancer-specific miRNA signature
			Subtypes specific miRNA signature
Eitan et al.	19 Tumors (stage I)/38 tumors (stage III)	miRNA microarray	miRNA signature during progression miRNA expression associated with response to platinum-chemotherapy
Hu et al.	55 Advanced-stage tumors	miRNA microarray	miR-200b-429 are biomarkers for ovarian cancer outcome
Lee et al.	33 High-grade serous tumors 2 Low-grade serous tumors	miRNA microarray	No abnormalities in miRNA expression correlated to BRCA1/2 status
	2 Serous borderline tumors		miR-34c and miR-422b are prognostic biomarkers
	3 Normal fallopian tubes		
Nagaraja et al.	10 Human clear-cell ovarian cancer cell lines and 1 normal ovarian surface epithelial cultures	Deep sequencing	Clear-cell ovarian cancer-specific miRNA signature miR-101 inhibits mTOR pathway and increases rapamycin sensitivity
Creighton et al.	8 Serous tumors 4 Serous cancer cell lines 4 NOSE cell lines	Deep sequencing	miR-31 is downregulated in cancer Reduced levels of miR-31 are correlated with defects in the p53 pathway
Vaksman et al.	21 Tumors (13 effusions/8 primary tumors)	miRNA microarray	miRNA signatures for the primary tumors and effusions
Kim et al.	103 Tumors	miRNA microarray	miRNA signature is correlated with clinico-pathological parameters (subtype, grade, survival)
Marchini et al.	144 Tumors (stage I)	miRNA microarray	Ovarian cancer-specific miRNA signature
			miR-200c is a predictor of survival and relapse
Cancer Genome Atlas Research Network	489 Serous tumors	miRNA microarray	Global analyses of mRNA expression, miRNA expression, promoter methylation, and DNA copy number

Moreover, based on the correlation between drug response and miRNA expression, many studies have tried to manipulate the mechanisms responsible for chemo-resistance by altering the levels of miRNA. For instance, Liu and colleagues have demonstrated that the efficient delivery of let-7i in chemo-resistant ovarian tumor cells could sensitize this malignant population to the drug-induced apoptotic effect [166]. Also the over-expression of mir-200c have been proved to increase the sensitivity to microtubule-targeting agents and to mitigate cancer invasiveness in ovarian carcinoma cells [167,168].

Although these findings are fascinating, we are probably far from a concrete application of miRNA as a therapeutic tool in human ovarian cancer; however, the potential translation to the clinics is very interesting and based on increasing preclinical evidence. The first miRNA based anti-cancer drug, which entered a phase I clinical trial in 2013 for hepatocellular carcinoma, would potentially be useful also for the treatment of ovarian malignancies; indeed, this drug is a mir-34a mimic, and mir-34a is frequently down-regulated in ovarian cancer. This evidence gives a concrete hope that a miRNA-based therapy could be developed for overcoming ovarian tumor recurrence [48].

Last, but not least, some researchers have recently focused their attention on the role of miRNA in the cancer stem cell subset. For example, a recent study have reported that the over-expression of mir-1207 promoted the cancer stem cell-like phenotype, in terms of enhanced spheroid formation capability and increased proportion of cancer stem-like cells [169]. Other results have suggested that mir-200a could induce the migration and invasion of ovarian cancer stem-like cells, by inhibiting the expression of E-cadherin [170]. In 2012, Nam and colleagues have provided the first miRNA profile of ovarian cancer stem-like cells, revealing a list of 37 miRNA differentially expressed in such cells compared to cancer non-stem counterpart. However, this miRNA signature was only a preliminary finding, since it was based on a single ovarian cancer cell line [171]. Moreover, unfortunately, the experiments leading to this miRNA pattern, as well as those about mir-1207 and mir-200a, were performed on cells recognized as cancer stem cells based on the surface expression of CD133, which is no longer considered as a faithful marker of ovarian CSC. Indeed, as previously mentioned, the co-expression of the surface markers CD44 and CD117 is widely accepted as the only way to correctly define ovarian cancer stem cells (CSC). Ovarian $CD44^+/CD117^+$ CSC have been evaluated, in terms of miRNA expression, in two reports. In the first, miRNA-199a has exhibited its ability to enhance the sensitivity to chemotherapeutic treatments and to suppress the tumorigenic potential of ovarian $CD44^+/CD117^+$ cancer cell fraction, probably by interacting with CD44 [43]. The second report has demonstrated that the over-expression of mir-200c could induce a significant decrease in the tumor- and metastasis-formation abilities of the $CD44^+/CD117^+$ cancer population, by modulating epithelial-to-mesenchymal transition [42]. Although these results seem very promising to a future application in the clinics, there is still not any reliable clinical miRNA-based strategy to target ovarian cancer stem cells, and further investigations, in clinically relevant samples rather than in established cell line, are needed to translate the increasing evidence about miRNA into CSC-target therapy.

Overall, these premises allow to conclude that miRNA represent a fascinating field in cancer research.

1.6. Metabolism of cancer – Warburg Effect

Altered metabolism is recently emerged as one of the most hallmarks of cancer; thus, targeting cancer metabolism represents a promising approach for anti-tumor therapy improvement. In this chapter, the clinical relevance of metabolic profile in human malignancies will be described.

Cancer cells, unlike normal cells, prefer to convert the pyruvate into lactic acid for ATP generation rather than exploit the mitochondrial oxidative phosphorylation, even in the presence of oxygen. This metabolic profile, termed aerobic glycolysis or the "Warburg Effect" (Figure 17), is very peculiar, since it allows the production of a much lower amount of ATP compared to that produced by oxidative phosphorylation; however, the ATP derived from the generation of lactic acid bypassing the mitochondrial chain is much more rapidly available to the cells.

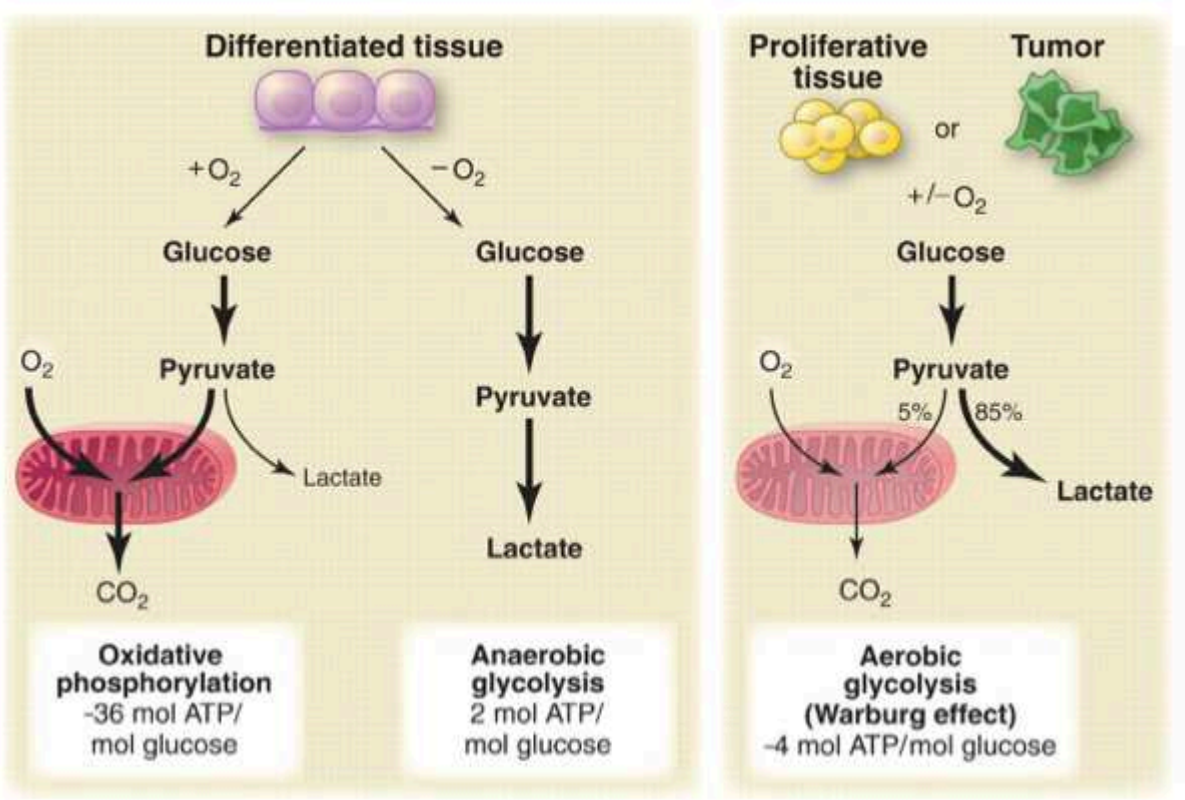

Figure 17. Warburg effect – aerobic glycolysis*. Heiden MGV et al. Understanding the Warburg effect: The Metabolic Requirments of Cell Proliferation. Science 2009; 324:1029.*

In 1930, Otto Warburg, a Nobel prize-awarded scientist for the discovery of "yellow" enzymes Flavins, noticed an abnormal metabolism in cancer cells. More in detail, through a manometer, Warburg and his collaborators observed that cancer cells did not consume more oxygen that normal tissue cells, even in normal oxygen conditions [172]. This behavior appeared unusual in view of the higher proliferation rate of cancer cells compared to normal tissue. Initially, Warburg supposed that cancer cells presented an impaired mitochondrial function and an eventual deficit in oxidative respiration [173]. Nevertheless, many subsequent studies revealed that mitochondria correctly work in most cancer cells, suggesting the existence of an alternative reason for cancer aerobic glycolysis.

To investigate the possible explanations for this phenomenon, Warburg proposed some differences between normal cells and cancer cells.
Normal cells are characterized by a specific metabolic pattern, essential to translate energy substrates (carbohydrates, proteins and lipids) into several metabolic intermediates, which are used to synthetize amino acids, glycogen, nucleic acids and other biomolecules required for normal cell function. Glucose, derived by carbohydrate processing, enters the cells through specific transporters. In the cytoplasm, many enzymes regulate glycolysis which, in the end, generates the pyruvate. This final product of glycolysis can be enzymatically converted into lactic acid (lactic fermentation), or can enter mitochondria, where it is converted to acetyl-CoA by pyruvate dehydrogenase (PDH). Acetyl-CoA condenses with oxaloacetate (OAA) to form citrate, which is completely oxidized in the Trichloroacetic (TCA) cycle to generate reducing equivalents for respiratory chain. The final energy production of this process, termed oxidative phosphorylation, is 2 molecules of ATP from glycolysis and 34 from the mitochondrial respiratory chain. Thus, in the presence of normal oxygen level, normal cells are able to produce 36 ATP molecules, which is about the 90% of the energy requirement for the cell survival.
In case of oxygen stress conditions, the cells are able to survive by switching their metabolism to anaerobic glycolysis. More in detail, the cells can convert the pyruvate to lactic acid in the cytosol, thus bypassing mitochondrial activity. The process leading to lactic acid production allows glycolys to continue by cycling NADH back to NAD^+, but induces a minimal production of ATP molecules.

According to the theory of Warburg, cancer cells exploit aerobic glycolysis, that is the conversion of most glucose molecules into lactate independently of the cell oxygen tension. Considering the fast growth of tumor masses, it seems peculiar that cancer cells privilege a metabolic profile which is inefficient in terms of energy production, even when a more efficient reaction is available. Many reasons can explain this bioenergetic alteration, and it is essential to remember that cancer cells, unlike cells of health tissues, must perform metabolic processes in very stressful conditions, such as low nutrient supply and imbalance of pH and oxygen levels. Firstly, cancer cell metabolism has to be modified to facilitate the uptake and incorporation of metabolic intermediates necessary to generate new cells: for instance, lipids for membrane synthesis, amino acids for protein synthesis, and nucleic acids for DNA duplication. Furthermore, lactic fermentation provides an acidic microenvironment, which is harmful for normal cells but not for tumor cells; thus, glycolysis and lactic acid production could represent a cellular defense mechanism for cancer cell growth. Another hypothesis is that glycolysis produces less reactive oxygen species (ROS) than oxidative respiration; so, bypassing the mitochondrial chain might allow cancer cells to elude the genomic damage induced by high ROS concentrations. In conclusion, while it is evident that lactic fermentation generates less ATP than oxidative phosphorylation, escaping mitochondrial respiration could provide more metabolic intermediates, which are probably much more important than simple ATP to maintain cancer cell growth and survival also in stress conditions.

The Warburg effect implies that cancer cells, to produce a reasonable amount of ATP, need to perform glycolysis at higher rates and require much more glucose uptake than normal cells. Despite many unanswered questions about the efficiency of aerobic glycolysis, metabolism reprogramming in cancer cells is universally accepted as one of the hallmarks of cancer [52]. As a matter of fact, the high cell glucose requirement related to the Warburg effect is clinically exploited for tumor

diagnosis and prognosis, by ^{18}F-deoxyglucose (^{18}F-FDG) positron emission tomography scanning (PET). ^{18}F-FDG is a radiolabelled glucose analogue which normally enters the cells and is phosphorylated like normal glucose: thus, the ^{18}F-FDG detection by PET represents a good reflection of the amounts of glucose taken up from body cells [174]. The technical capacity to measure the glucose levels within the tumor masses and to monitor the glucose uptake by cancer cells validate the Warburg's hypothesis, so underlining the importance of glucose consumption in cancer metabolism and in cancer clinical detection.

One of the major unsolved questions about tumor cell metabolic profile (Figure 18) is whether the alteration of cell metabolism could be due to gene mutations, or rather the great energy requirement of cancer cells could induce a peculiar metabolic profile which could translate into genetic abnormalities.

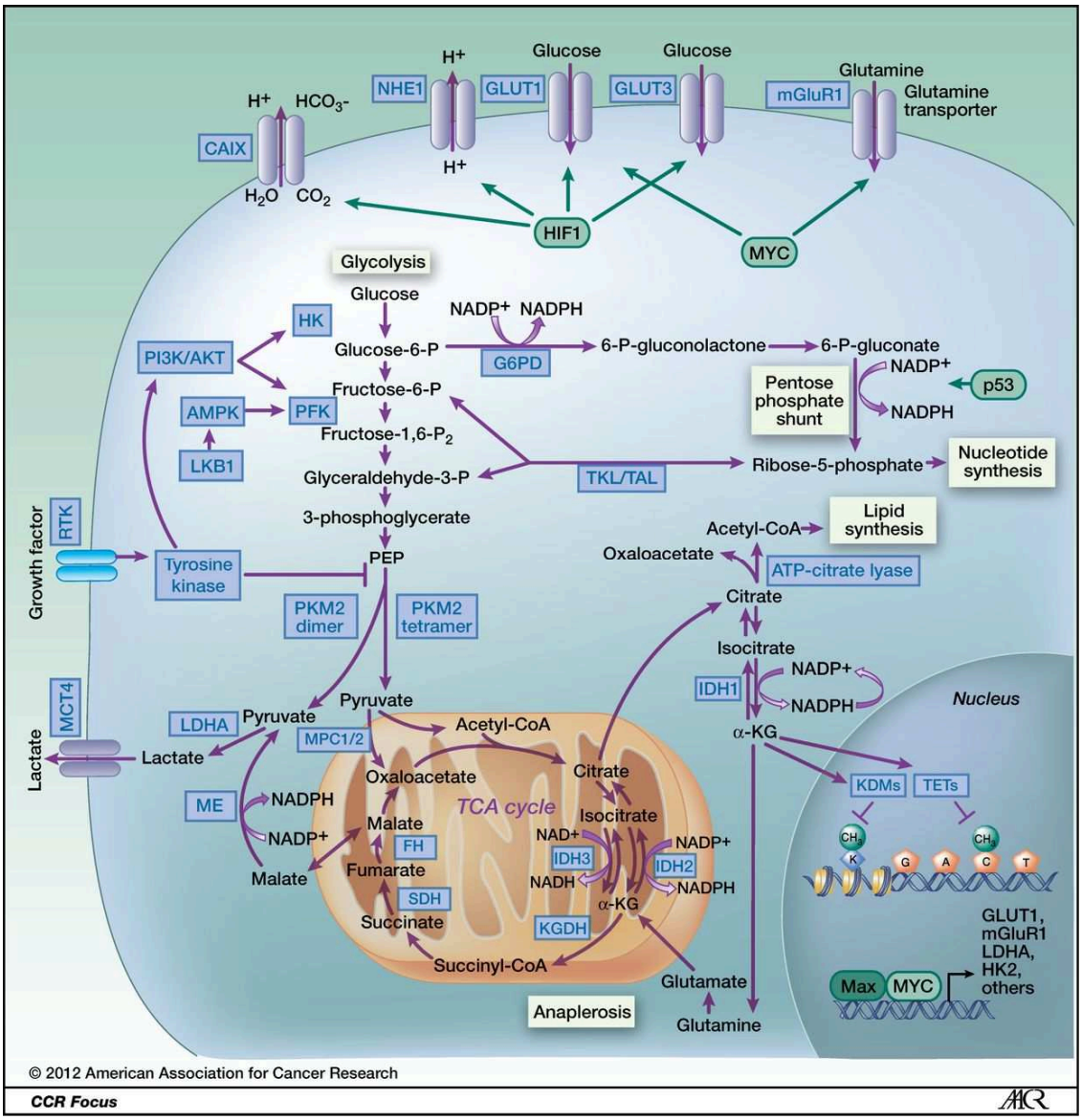

Figure 18. Schematic illustration of metabolic pathways prominent in malignant cells. *Teicher BA, Linehan WM and Helman LJ. Targeting Cancer Metabolism. Clin Cancer Res. 2012 Oct 15;18(20):5537-45*

Indeed, mutations of tumor-suppressor genes or oncogenes are a widely accepted cause of cancer; otherwise, metabolic stress conditions of the tumor-surrounding microenvironment, such as oxygen radical formation, may induce genetic alterations which increase the possibility of cancer development. In this regard, certain types of tumors are strictly related to mutations affecting the genes involved in metabolic processes, especially genes encoding for mitochondrial enzymes, thus supporting the Warburg's theory that cancer cells have basal defects in mitochondrial function.
Some of these enzymes, encoded by genes frequently mutated in cancer, are known: succinate dehydrogenase (SDH) [175], which converts succinate to fumarate in the TCA cycle; isocitrate dehydrogenase 1 (IDH1) [176], which is the key enzyme in the conversion of isocitrate to α-ketoglutarate (α-KG); and pyruvate kinase isoform 2 (PKM2) [177], which catalyzes the conversion of phosphoenol-pyruvate into pyruvate at the last steps of glycolysis.

Finally, one of the most important metabolic modulators is hypoxia-inducible factor (HIF), which is a regulatory factor involved in hypoxia, a common phenomenon occurring in cancer [178,179]. HIF regulates the expression of many metabolism-associated genes, including genes encoding for glucose transporters (GLUT), and for enzymes involved in glycolysis, such as pyruvate dehydrogenase kinase (PDHK) and lactate dehydrogenase A (LDH-A). GLUT up-regulation may allow malignant cells to compete with healthy cells in the glucose uptake process, thus increasing the glucose amount within the cells. On the other hand, the over-expression of PDK induces a strong inhibition of pyruvate dehydrogenase (PDH) activity, and the amplification of LDH-A levels can accelerate the pyruvate-to-lactate conversion. Thus, HIF target genes are able to promote the Warburg effect.

Concerning Cancer Stem Cells (CSC), the investigation of their metabolism, in comparison to that of the non-stem counterpart, is an exciting challenge. In this regard, several authors have reported that CSC could present different metabolic profiles depending on their primary site of origin and on their degree of differentiation. For instance, highly undifferentiated liver cancer cells display a more glycolytic rate than differentiated cells [180,181]. Similarly, glioma stem cells show lower glucose consumption and lower lactate production, compared to their non-stem counterpart [182]. In this context, our research group has recently demonstrated that ovarian CSC privilege oxidative phosphorylation rather than aerobic glycolysis (Figure 19) [30]. In this study, we demonstrated that ovarian CSC, compared to the non-stem counterpart, over-express the major glycolysis-associated enzymes, as well as the key enzymes of pentose phosphate pathway (PPP), essential for the synthesis of nucleic acid and for the maintenance of the cell redox potential. Although this metabolic profile could seem to correlate with a high glycolytic rate, our experiments also showed that ovarian CSC up-regulated the key enzymes involved in TCA cycle, mitochondrial respiratory chain and fatty acid β-oxidation, another cellular process promoting pyruvate entrance into the TCA cycle. This evidence suggests that ovarian cancer stem cells could display an oxidative metabolism, unlike cancer non-stem population, which relies on the Warburg effect. This hypothesis is supported by the finding that ovarian CSC, compared to non-CSC, exhibit a higher mitochondrial activity and, consequently, a higher ROS production, and are able to resist *in vitro* and *in vivo* glucose starvation.

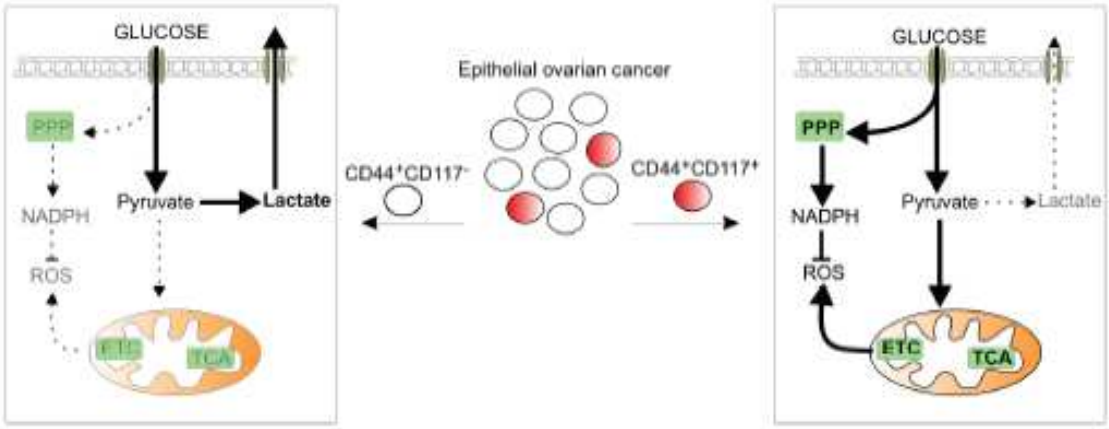

Figure 19. Schematic model of the metabolic profile of ovarian CSC and non-CSC counterpart*. Pilotto G, Pastò A, Bellio C, Ciminale V, Silic-Benussi M, Guzzo G, Rasola A, Frasson C, Nardo G, Zulato E, Nicoletto MO, Manicone M, Indraccolo S and Amadori A. Cancer stem cells from epithelial ovarian cancer patients privilege oxidative phosphorilation, and resist glucose deprivation. Oncotarget. 2014 Jun 30;5(12):4305-19.*

Overall, despite the study of cancer metabolism showed its key role in cancer development and progression, many questions are still unsolved, such as how malignant cell metabolism is modulated and whether targeting metabolic enzymes might improve the efficacy of cancer therapy overcoming therapeutic resistance.

1.7. Therapies targeting the metabolic profile in cancer – possible targeting of CSC

The reasons for targeting CSC to improve cancer therapy are related to two fundamental features, both involved in chemotherapy failure: their chemo-resistance and their metabolic alterations. Probably, these two properties are associated, but the mechanisms through which targeting metabolism could affect chemo-resistance are still unknown and need further investigation. In view of the enhanced interest in cancer metabolism, it is reasonable to foresee that new specific therapies will be developed in the next decades; currently, some drugs have already been approved for clinical use in certain types of cancer, and others are under clinical trials.

The fact that cancer cells mainly rely on aerobic glycolysis for ATP production has been investigated as a target to develop anti-cancer treatments, and many possibilities to clinically exploit cancer glucose addiction have been proposed [183] (Table 5).

- The inhibition of glucose uptake, although the toxic side effects related to the glucose starvation could represent a major challenge for the use of this approach. Some drugs have already been found to reduce the glucose entry into the cells. For instance, the flavonoid Phloretin has been shown to inhibit glucose trans-membrane uptake through GLUT1 transporter, and to present preclinical anti-cancer activity in breast and hepatocellular carcinoma [184]. Moreover, the glucose analog 2-deoxyglucose (2-DG) competes with glucose for transport into the cells and, inside the cell, it is phosphorylated into 2-DG-6-phosphate blocking glycolysis progression [185].

- The inhibition of the activity of some crucial enzymes of glycolysis. For example, the compound TT-232 is under phase II clinical trial in melanoma patient in view of its ability to bind and inhibit the glycolysis-associated enzyme pyruvate kinase isoform M2 (PKM2) [186]. PKM2, which is predominantly expressed in cancer cells, catalyzes the last step of glycolysis by transferring a phosphate group from phosphoenolpyruvate to ADP to produce ATP and pyruvate. Its involvement in carcinogenesis processes is due to its capacity to reprogram cell metabolism by shifting from an active to an inactive form; in the end, the inactive form promotes the Warburg effect by redirecting glycolysis intermediates to biogenesis pathway. Therefore, the TT-232-mediated PKM2 inhibition resulted in the reduction of proliferation rate and in the enhancement of apoptotic signaling in several tumor models [186]. Adding to this, also some miRNA was found to exhibit a suppressive activity on glycolysis enzymes; for example, mir-326 targets PKM2, while mir-200 family targets phospho-glucose isomerase (PGI) [183].

- The inhibition of pentose phosphate pathway (PPP), that is a crucial pathway because of its role in the NADH production and in the synthesis of nucleic acids, essential for oxidative stress reduction and for rapid cell proliferation, respectively. For example, tumor growth could be reduced by using an analog of NADP, termed 6-AN (6-aminonicotinamide), which inhibits glucose-6 phosphate dehydrogenase (G6PD), the first enzyme of the PPP pathway, thus increasing oxidative stress [187]. Nevertheless, Herter and collaborators demonstrated that high doses of 6-AN are neurotoxic and, at the same time, lower well-tolerated doses lack anticancer effect [188].

- The inhibition of HIF-1, that is one of the most important modulators of glucose metabolism. HIF-1 is a transcription factor that displays a key role in the cell response to oxygen deprivation. Indeed, in hypoxic conditions, HIF-1 triggers the activation of adaptive pathways, including a metabolic switch from mitochondrial respiratory chain to lactic fermentation to face the decrease of oxygen level. This transcription factor has been found up-regulated in many tumor types. Thus, HIF-1 inhibition could reduce the glycolytic rate and induce oxidative phosphorylation in cancer cells. Topotecan, a compound able to inhibit HIF-1, is already used in clinics for lung cancer patients, and the possibility to extend its clinical application had to be elucidated [189].

- The induction of oxidative phosphorylation in tumor cells, redirecting the pyruvate from lactic fermentation to Krebs cycle. Several mechanisms have been proposed to stimulate the mitochondrial respiratory chain. One of them is the down-regulation of lactate dehydrogenase (LDH) by treatment with small interfering ribonucleic acids (siRNA), which finally prevent the pyruvate-to-lactate conversion [190]. Another mechanism is the use of antisense oligonucleotides inhibiting pyruvate dehydrogenase kinase 1 (PDHK1) to activate its target pyruvate dehydrogenase (PDH), a key enzyme of TCA cycle, which in the end promotes the progression of glucose derivatives into oxidative respiration [191].

Target	Compound	Mode of action	Current clinical status
GLUTs	2-DG	Competitor with glucose	Phase I for prostate cancer terminated. Entering Phase II. Brain toxicity reported
	Phloretin	Competitive inhibitor	Preclinical
	Silybin/silibinin	Small molecule inhibitor	Phase I and Phase II, prostate cancer
HK2	2-DG	Competitor with glucose	Phase I for prostate cancer terminated. Entering Phase II. Brain toxicity reported
	Lonidamine	Small molecule inhibitor	Failed to show therapeutic benefit in Phase II for GBM. Phase II/III for benign hyperplasia suspended due to hepatotoxicity
	3-bromopyruvate	Alkylating agent, inhibitor of HK2 mitochondrial anchorage	Preclinical
PFK2/PFKFB3	3PO	Specific inhibitor	Preclinical
PKM2	TLN-232/CAP-232	Peptidic inhibitor	Phase II trials for metastatic RCC and melanoma have been halted for legal reasons
	Shikonin and alkannin	Selective inhibitors	Preclinical
LDH5	Gossypol/AT-101	Malarial LDH inhibitor	Phase I and II, many types of cancer
	FX11	Selective competitive inhibitor	Preclinical
PDK	Dichloroacetate	Small molecule inhibitor (pyruvate mimetic)	Phase I, brain cancer. Phase II, head-and-neck and non-small cell lung cancers
CA9	Indisulam	Small molecule inhibitor	Phase II, melanoma, lung, pancreatic, and metastatic breast cancers
	Girentuximab	Blocking antibody	Phase III, clear-cell RCC
Membrane-bound V-ATPase	Esomeprazole	Proton pump inhibitor	Phase II, metastatic breast cancer
NHE1	Paclitaxel	Antimitotic, reported to induce apoptosis through NHE1 inhibition (Reshkin et al., 2003)	In clinical use
	EIPA	Small molecule inhibitor, amiloride (diuretic) derivative	Preclinical
MCT1	AZD3965	Small molecule inhibitor	Entering Phase I/II, advanced solid tumors
HIF-1	BAY87-2243	Inhibitor of HIF-1 activity	Entering Phase I, advanced cancers
	EZN-2968	Antisense oligonucleotide	Entering Phase I, several types of cancers
c-Myc	Quarfloxin/CX-3453	Inhibitor of *MYC* transcription	Phase II, neuro-endocrine carcinomas
AMPK	Metformin	AMPK activator, antidiabetic drug	In clinical use for diabetes. Several clinical trials ongoing for cancer

2-DG, 2-deoxyglucose; 3PO, 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one; AMPK, AMP kinase; CA9, carbonic anhydrase-9; EIPA, 5-(N-ethyl-N-isopropyl)amiloride; FX11, 3-dihydroxy-6-methyl-7-(phenylmethyl)-4-propylnaphthalene-1-carboxylic acid; GBM, glioblastoma multiform; GLUT, glucose transporter; HIF-1, hypoxia-inducible factor-1; HK2, hexokinase 2; LDH5, lactate dehydrogenase 5; MCT1, monocarboxylate transporter 1; NHE1, sodium–proton exchanger 1; PDK, pyruvate dehydrogenase kinase; PFK2, phosphofructokinase 2; PFKFB3, phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3; PKM2, pyruvate kinase M2; RCC, renal cell carcinoma.

Table 5. Leading therapeutic compounds targeting the glycolytic metabolism of tumors. *Porporato PE, Dhup S, Dadhich RK, Coppetti T and Sonveaux P. Anticancer target in the glycolytic metabolism of tumors: a comprehensive review. Front Pharmacol. 2011 August 15.*

Furthermore, several studies identified mitochondria as attractive targets to develop novel anti-cancer strategies, and the term “mitocans” has been coined to classify mitochondria-targeting anti-tumoral drugs [192]. This enhanced interest on mitochondria is due to the fact that the Warburg’s theory about the existence of some defects in these organelles is not far-fetched, since cancer cells can exhibit several degrees of mitochondrial function impairment, such as increased trans-membrane potential and ROS production. In particular, the increase in ROS production is related to multiple changes in all the tumor progression-associated cellular processes, such as proliferation, migration, differentiation and apoptosis.

Indeed, the two most innovative drugs used to target mitochondria have to be mentioned: Metformin and CPI-613. Metformin (Figure 20), which is administered to diabetic patients, has recently received much attention in view of its potential as anti-tumoral agent. The anti-cancer effects of Metformin are probably independent on its hypoglycemic activity, and have been investigated in several *in vitro* and *in vivo* models; to date, a number of clinical trials are testing this drug as an adjuvant to classic chemotherapeutic regimens [193,194]. More in detail, this anti-diabetic compound exerts its pharmacological activity by targeting one of the complexes of the mitochondrial chain, thus inhibiting oxidative phosphorylation and activating the AMPK pathway. Since the Metformin-associated impairment of mitochondrial ATP production induces metabolic stress, it is reasonable to think that Metformin could prevent the growth of tumor mass by affecting the cellular energy status.

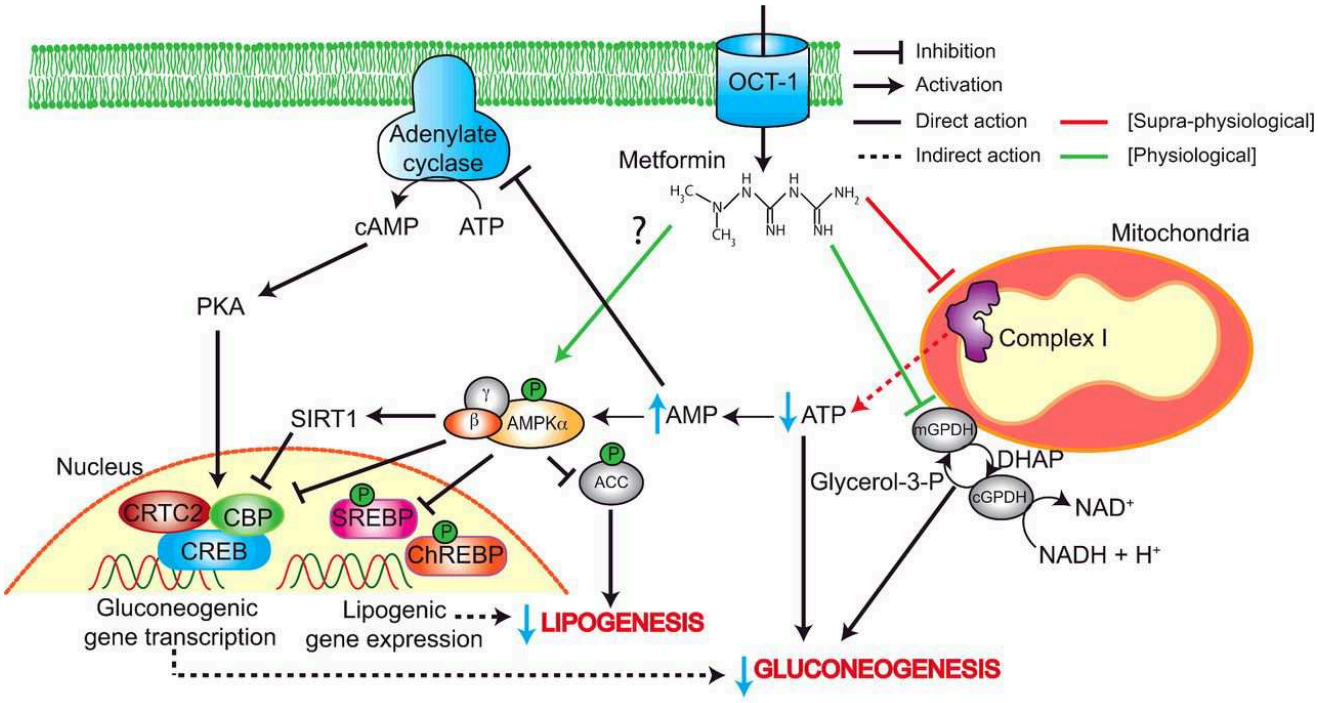

Figure 20. Mitochondrial targets of Metformin. *Pryor R, Cabreiro F. Repurposing metformin: an old drug with new tricks in its binding pockets. Biochemical J. 2015 Oct 16;471(3):307-322.*

CPI-613 is an analog of lipoic acid able to impair mitochondrial metabolism, and its selective effect against malignant cells has already been observed both *in vitro* and *in vivo* [195]. This drug is still under clinical trial, and its emerging importance as anti-cancer agent is due to the involvement of its targets in the TCA cycle: these targets are pyruvate dehydrogenase kinase (PDK) and alpha-ketoglutarate dehydrogenase (α-KGDH, or KDH) (Figure 21). More in detail, CPI-613 activates PDK and eventually promotes the phosphorylation-mediated inhibition of PDH, finally stimulating several tumor cell death pathways. Moreover, this lipoic acid-analog induces the activation of α-KGDH through an endogenous redox mechanism. Therefore, this drug simultaneously and independently affects two key processes of the mitochondrial metabolic chain.

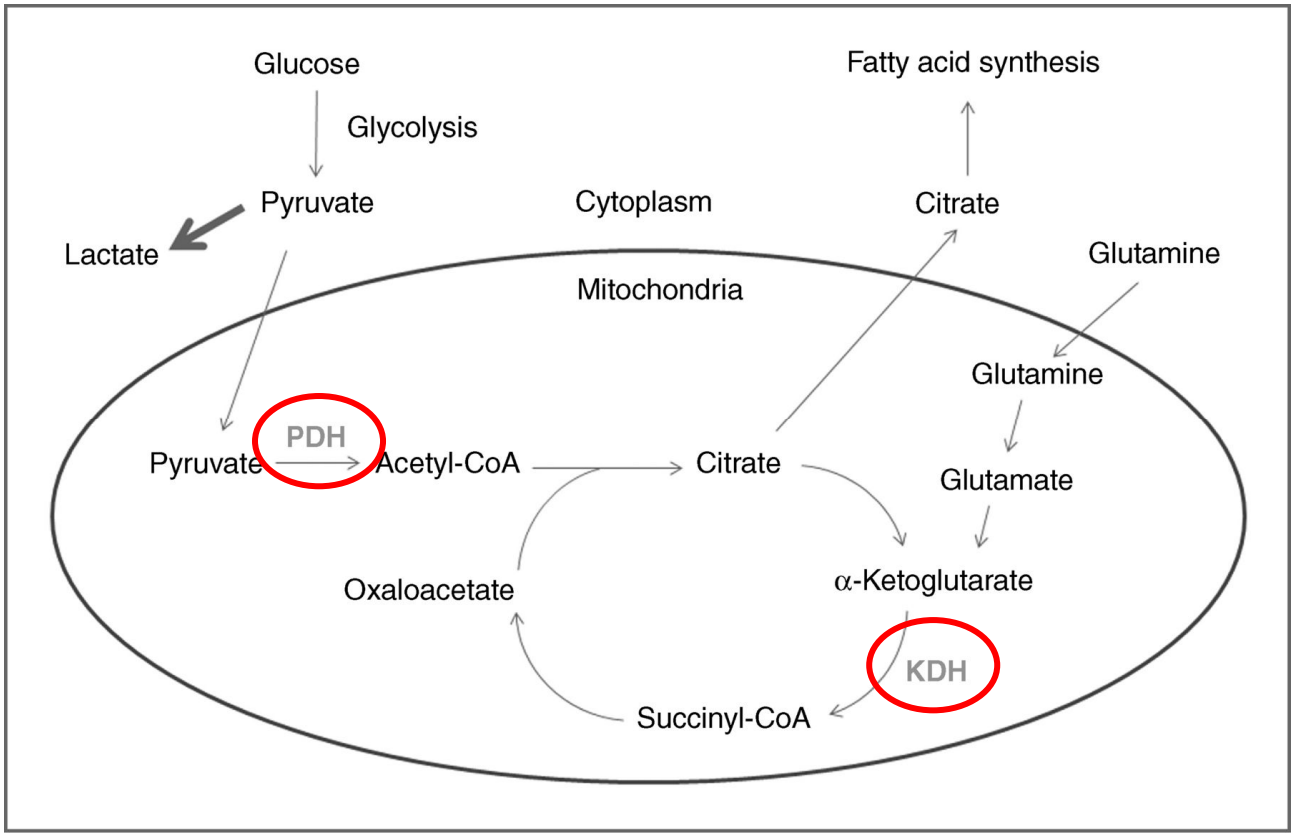

Figure 21. Schematic of carbon metabolism with the CPI-613 targets PDK and α-KGDH (KDH). *Pardee TS, Lee K, Luddy J, Maturo C, Rodriguez R, Isom S, Miller LD, Stadelman KM, Levitan D, Hurd D, Ellis LR, Harrelson R, Manuel M, Dralle S, Lyerly S and Powell BL. A Phase I Study of the First-in-Class Antimitochondrial Metabolism Agent, CPI-613, in Patients with Advanced Hematologic Malignancies. Clin Cancer Res. 2014 Oct 15;20(20):5255-64.*

Some studies have suggested the possibility that Metformin could efficiently target CSC [193] [194]. On the contrary, there is no significant evidence about the ability of CPI-613 to induce the death of CSC, probably because of the novelty of this compound.
Nevertheless, the recent evidence that CSC are characterized by a different metabolism than the cancer non-stem cells [30,182], could open exciting avenues to novel target therapy.

All these premises underline the importance to further investigate the role of cell metabolism in cancer development and progression, and provide the basis to use anti-metabolic reprogramming strategies as a roadmap for the improvement of anti-cancer therapy, circumventing chemotherapy resistance, tumor aggressiveness and recurrence.

1.8. Aims of the project

Recently, our research group demonstrated that ovarian cancer stem cells (CSC) are characterized by a different glucose metabolism compared to non-CSC; more in detail, CSC privilege oxidative phosphorylation rather than aerobic glycolysis (or Warburg effect), on which the non-stem tumor mass relies. In these experiments, we noticed that not all the analyzed epithelial ovarian cancer (EOC) patients presented the same *in vitro* glucose dependence.

Thus, in view of the importance of cancer metabolism and microRNA (miRNA) signatures for new therapeutic strategies, the aims of this project are the following:

- Investigating the possibility to distinguish different glucose metabolic profiles in EOC patient-derived samples, and to associate these distinctive metabolic traits with the different patient responses to chemotherapy.

- Analyzing the miRNA signatures of EOC patient-derived samples, in terms of miRNA profile of both CSC and cancer non-stem cells.

In the first part of the project, we intend to explore whether the metabolic differences between EOC samples could be exploited as a predictor of clinical chemotherapy response.

In the second part, we intend to investigate a miRNA signature involved in the regulation of the metabolic traits of EOC samples. We plan also to address whether this miRNA signature is related to the entire tumor mass or rather to the specific cancer stem compartment. Our future perspectives include the exploitation of these possible miRNA-mediated regulatory mechanisms as a new tool for improving ovarian cancer therapy.

2. MATERIALS AND METHODS

2.1. EOC primary samples and *in vitro* culture

This study was approved by the Institutional Ethics Committee for patient studies. After obtaining written informed consent, 70 patients diagnosed with EOC entered this study: 47 had received chemotherapy prior to sampling, whereas 23 were sampled at diagnosis before any surgery/chemotherapy. Patient's clinical and pathologic features are summarized in Table 7. Ovarian tumor cells were isolated from ascitic effusion fluids derived from EOC-bearing patients or from immunocompromised mice intraperitoneally injected with EOC cells (patient derived xenograft–PDX) by centrifugation at 500g and red blood cell lysis, as reported elsewhere [196]. Then the cells were maintained in RPMI-1640 medium (Euroclone, Milan, Italy), supplemented with 10% fetal bovine serum (FBS; GIBCO Invitrogen, Monza, Italy), 1%sodium pyruvate (Lonza, Basel, Switzerland), and 1% L-glutamine (Gibco). Cells were cultured at 37°C, 5% CO_2, and harvested at confluence using trypsin-EDTA (Invitrogen). All the experiments were performed on gated or FACS-sorted CD45 (lympho-myeloid cell marker)-negative and CD44 (tumor marker)-positive ($CD45^-/CD44^+$) cells to exclude myeloid and lymphoid contamination and to select only cancer cells.

2.2. Glucose deprivation assay and *in vitro* cell treatments

EOC ascitic effusion cells were cultured at $6x10^4$ cells/ml in complete or glucose-deprived RPMI medium (Sigma-Aldrich, St. Louis, MO). After 14 days, cell viability of $CD45^-/CD44^+$ tumor cells was evaluated by flow cytometry with Live/Dead (Life Technologies, Monza, Italy) staining, which allow the determination of cell viability on the basis of cell membrane integrity and esterase activity, as described elsewhere [197].

Carboplatin was added to the complete medium, and cell viability evaluated 72 h later by AnnexinV/PI staining, a two-color assay to discriminate live cells, early/late apoptotic cells and necrotic cells, as described elsewhere [197].

2.3. Animal studies and patient derived xenografts (PDX)

Severe combined immunodeficiency (SCID) and RAG-$2\gamma^{-/-}$ mice were obtained from internal breeding. Procedures involving animals and their care were performed according to institutional guidelines that comply with national and international laws and policies (EEC Council Directive 86/609, OJ L358, 12 December 1987). Patient derived xenografts (PDX) were generated by injecting intraperitoneally (i.p.) into SCID mice 10^6 tumor cells derived from ascitic effusions of EOC-bearing patients, after red blood cell lysis and lymphocyte removal, as reported elsewhere [198]. About two months later, animals developed tumors, which contained a predominant ascitic component processed as ascitic effusions of EOC-bearing patients.

For *in vivo* carboplatin treatment, after red blood lysis and lymphocyte removal, $5x10^5$ $CD45^-/CD44^+$ tumor cells isolated from high-grade serous ovarian cancer xenografts (classified as GA or GNA at baseline) were injected subcutaneously (s.c.) in 200 µl of Matrigel in both dorsolateral flanks of RAG-$2\gamma^{-/-}$ mice. When tumors reached 100 mm^3 volume, mice were randomized in two groups, and either treated with 4 doses of carboplatin (50 mg/Kg weekly) or with equal saline

amounts as a control. Tumor growth was evaluated by caliper measurements. Mice were sacrificed when the tumors of the control group reached 600 mm^3 volume.

To investigate the possible correlation between PLT response and glucose metabolism, we selected two EOC xenografts which were obtained by subcutaneous injection into nude mice (Envigo RMS, Corezzana, Italy), and which were sensitive to cisplatin treatment (#S). Mice were treated with multiple cycles of cisplatin (5 mg/Kg once every 3 weeks/cycle) in order to obtain the cisplatin-resistant counterpart (#R); meanwhile the sensitive counterpart was maintained by serial passage in mice receiving phosphate saline buffer (PBS). Experiments were performed after a total of 5-8 cisplatin cycles.

For *in vivo* 2-deoxiglucose (2-DG; Sigma Aldrich) treatment, RAG-2$\gamma^{-/-}$ mice were injected in both dorsolateral flanks with cancer cells from GA/GNA PDX, as above described for carboplatin treatment. When tumors reached 100 mm^3 volume, mice were divided in two groups, one receiving intraperitoneally three times/week 2 g/kg of 2-DG (2-DG group), the other a same volume of saline solution (control group). As for carboplatin treatment, tumor growth was evaluated by caliper measurements, and mice were sacrificed when the tumors of control group reached 600 mm^3 volume.

2.4. Flow cytometry analysis and FACS-sorting

To flow cytometry analysis or FACS-sorting cell separation, the cells were stained with the following anti-human antibodies: anti-CD45 (1:10; Miltenyi Biotec, Bergish Gladbach, Germany) to exclude lympho-myeloid cell fraction; anti-CD44 (1:1000; Abcam, Cambridge, U.K.) to identify tumor cells; anti-CD117 (non-activating AC126 clone, 1:10; Miltenyi Biotec) to isolate cancer stem cells; and anti-GLUT1 (1:100; Abcam) to evaluate the surface expression of such transporter protein. Live/Dead (1:600; Invitrogen) labelling was used to discriminate living cells. After adding the antibodies, the cells were incubated for 30 minutes at 4°C; then the antibodies were eliminated by centrifugation and cell suspension in PBS. The unconjugated antibodies required an additional incubation for 30 minutes at 4°C with the appropriate secondary antibody (Alexa 1:500, Invitrogen).
All the flow cytometry analyses were performed using a FACS LSRII (BD Bioscience, Franklin Lakes, NJ); data were collected from at least $1x10^5$ cells/sample and elaborated with FlowJo software (TreeStar, Ashland, OR).

FACS-sorting was performed with a MoFlo Astrios cell sorter (Coulter Electronics, Brea, CA), after gating $CD45^-/CD44^+$ tumor cells or $CD45^-/CD44^+/CD117^+$ and $CD45^-/CD44^+/CD117^-$ cells. The purity of the sorted population always exceeded 90%.

To distinguish living cell population from early/late apoptotic or necrotic subset, the cells were labelled with AnnexinV/PI staining kit (Roche, Basel, Switzerland) for 15 minutes at 37°C, according to kit protocol instructions. Unless otherwise specified, results were expressed as the ratio between the percentage of $AnnexinV^-/PI^-$ cells at the experimental time points and the percentage at time 0.

Aldehyde dehydrogenase (ALDH) activity was determined by a fluorogenic dye (ALDEFLUOR® assay; Stem Cell Technologies, Vancouver, Canada), according to assay protocol instructions and

as described elsewhere [199]. The specific ALDH inhibitor diethylaminobenzaldehyde (DEAB) was used as a control.

To evaluate the proliferation rate, EOC effusion cells were stained with carboxyfluorescein-diacetate-succinimidylester (CFSE; Life Technologies) as described elsewhere [200,201], and seeded at $6x10^4$ cells/well in RPMI medium. Flow cytometry analysis was performed 72 h later.

2.5. Seahorse analysis

The oxygen consumption rate (OCR) and Extracellular acidification rate (ECAR) were determining using the SeaHorse XF Extracellular Flux Analyzer (Seahorse Biosciences, San Jose, CA) [30]. FACS-sorted $CD45^-/CD44^+$ tumor cells were seeded at $2x10^4$/well in 24-well plates (XF24 V7, Seahorse Biosciences, Copenhagen, Denmark) in complete RPMI medium, and incubated for 4 h at 37°C, 5% CO_2. In the case of not-adherent cells, the XF24 V6 microplates were coated with 20 µl of Matrigel (Cultrex, Trevigen, Gaithersburg, MD) to allow cell adhesion to the bottom of the plates. The assay was initiated by medium replacing with DME base supplemented with 10% FBS, 1% sodium pyruvate, 1% penicillin-streptomycin, 1% glucose (Sigma Aldrich) and 1% L-glutamine. The cartridge of the instrument was loaded to dispense over 2 hours-analysis four different metabolic inhibitors at 20 minute intervals: oligomycin (1 µM), followed by FCCP (0,6 µM), antimycin (1 µM) and rotenone (1 µM; all from Sigma Aldrich). Three sequential measurements of OCR and ECAR were done at time 0 and after each reagent addition.

2.6. Western blotting (WB)

FACS-sorted $CD45^-/CD44^+$ tumor cells were lysed and subjected to SDS-polyacrylamide gel electrophoresis and WB. Immunoreactivity was evaluated hybridizing the membranes with anti-human antibodies against PARP (1:1000, Cell Signaling, Danvers, MA), MCT4 (1:1000, Santa Cruz, Dallas, TX), actin (1:500, Sigma-Aldrich), Cyclin D1 (1:1000, Cell Signaling), Casein Kinase 1 Delta (1:5000, Cell Signalling), NUMB (1:1000, Cell Signaling) and α-tubulin (1:4000, Sigma-Aldrich). Then the blots were hybridized with a 1:5000 dilution of the appropriate secondary HRP-conjugated antibody (Amersham-Pharmacia, Amersham, U.K.). Finally, the signal was detected by chemiluminescence with SuperSignal kit (Life Technologies), and lane densitometry was analyzed by standard procedures.

2.7. RNA extraction, reverse transcription and qRT-PCR

Total RNA was extracted following the TRIzol method according to manufacturer's instruction. cDNA was synthesized from 0.5-1 µg of total RNA using the Superscript II reverse transcriptase (Life Technologies), then it was added to the mix containing the gene-specific primers and the appropriate reagent for fluorescent detection (Platinum® SYBR® Green qPCR SuperMix-UDG with ROX; Invitrogen). Each sample was run in triplicate on ABI PRISM® 7900HT Sequence Detection System (PE Biosystems, Foster City, CA). Results were analyzed using the comparative ΔΔCt method; ΔΔCt values were utilized to calculate the $RQ=2^{-\Delta\Delta Ct}$. Data were expressed as the fold difference in gene expression (normalized to the housekeeping β2-microglobulin gene) relative to a reference sample, as indicated in the individual figure legends. qRT-PCR efficiency ranged from 95% to 105%. Primer sequences are listed in Table 6.

Gene Name	*Forward*	*Reverse*
β2micro	5'-TCTCTCTTTCTGGCCTGGAG-3'	5'-TCTCTGCTGGATGACGTGAG-3'
ALDH1A	5'-CCCGTTGGTTATGCTCATTT-3'	5'-TGCTCTGCTGGTTTGACAAC-3'
GLUT-1	5'-GATGATGCGGGAGAAGAAGG-3'	5'-AAGACAGCGTTGATGCCAGAC-3'
HKII	5'-GAAGATGCTGCCCACCTTTG-3'	5'-CACCCAAAGCACACGGAAGT-3'
PFK	5'-ACTGACGCCTGTCGCTTATG-3'	5'-GAGCGGGTTAGGTCCCTTCT-3'
GAPDH	5'-GAAGGTGAAGGTCGGAGT-3'	5'-CATGGGTGGAATCATATTGGAA-3'
LDH-A	5'-GATTCAGCCCGATTCCGTTAC-3'	5' ACTCCATACAGGCACACTGG-3'
LDH-B	5'- TGGGTGTTGGACAAGTTGGT-3'	5'- AGCAAGTTCATCAGCCAGAGA-3'
CYCLIN A	5'-GGACCTTCACCAGACCTACC-3'	5'-AGTGTCTCTGGTGGGTTGAG-3'
CYCLIN B	5'-ACTGCCTGCTCTCACATCTT-3'	5'-TGTTGCAGGCAGGACAGATA-3'
CYCLIN D	5'- GGCGGAGGAGAACAAACAGA-3'	5'- GGAGGGCGGATTGGAAATGA-3'
CYCLIN E	5'-GTGGATGGCATCAAACAGGG-3'	5'-GCACCTTCCATAGCAGCATC-3'
MRP1	5'-CAAGGTGGATGCGAATGAGG-3'	5'-TGAGGAAGTAGGGCCCAAAG-3'
MRP2	5'-GGACACATCTGCCATTCGAC-3'	5'-CCAGGTTCACATCTCGGACT-3'
ABCG2	5-CTCTTCGGCTTGCAACAACT-3'	5'-TTCTCCTCCAGACACACCAC-3'
CSNK1D	5′-GCCCTAGTTATCGTAACAG-3′	5′-CGCCAATAAAGAGTCTGTCA-3′
MCT4	5'- CCAGGCCCACGGCAGGTTTC-3'	5'- GCCACCGTAGTCACTGGC C-3'
NANOG	5'-CCAAAGGATGAAGTGCAAGC-3'	5'-CAAGTTGGGTTGGTCCAAGT-3'
SOX2	5'-CCGCGTCAAGAGGCCCATGAA-3'	5'-CCCGCTTCTCGGTCTCGGACAA-3'
OCT4	5'-TGGCGTGGAGACTTTGCA-3'	5'-GGTTCCCTCTGAGTTGCTTC-3'

Table 6. Primers for qRT-PCR. *The figure shows the sequences of the primers used to evaluate the mRNA level of the mentioned genes by qRT-PCR*

2.8. microRNA arrays and microRNA-specific qRT-PCR

For each microRNA (miRNA) array, $CD45^-/CD44^+/CD117^+$ cancer stem cells (CSC) or $CD45^-/CD44^+/CD117^-$ non-CSC were isolated by FACS-sorting from five primary EOC-samples. For each single chip hybridization, the five FACS-sorted EOC primary samples were pooled after patient classification as GA or GNA, to distinguish the samples both for CSC/non-CSC features and for GA/GNA phenotype. Totally, we performed 4 miRNA arrays for CSC and 4 for non-CSC, to a total number of 20 patients (10 classified as GA and 10 as GNA) analyzed both for cancer stem fraction and cancer non-stem counterpart. miRNA were purified from total RNA and hybridized on microRNA microarray chip (Affymetrix microRNA 4.0; Santa Clara, California, United States). Limma statistical analysis was performed to find out significant expression differences.

The differential expression level (logFC) and its statistical significance (p-value<0.05) were evaluated to select some miRNA among the list emerged from the arrays; the relative expression of these selected miRNA was further validated by qRT-PCR.

For qRT-PCR, the miRNA-specific cDNA was synthetized from total RNA through miRNA-specific probes (TaqMan® MicroRNA Assays, RT probes; Applied Biosystems) added to the appropriate reverse transcription kit (TaqMan™ MicroRNA Reverse Transcription Kit, Applied Biosystems), according to kit protocol instructions. To finally perform the qRT-PCR, the cDNA was mixed with miRNA-specific TaqMan probes (TaqMan® MicroRNA Assays, TM probes; Applied Biosystems) and with the TaqMan fluorescent detection kit (LightCycler® 480 Probes Master; Roche). As for gene expression evaluation, each sample was run in triplicate on ABI PRISM® 7900HT Sequence Detection System, and results were analyzed using the comparative ΔΔCt method. Data were expressed as the fold difference in miRNA expression relative to a

reference sample, as indicated in the individual figure legends; qRT-PCR efficiency ranged from 95% to 105%. Has-mir-16 was used as housekeeping miRNA to normalize the data, since it was equally expressed in all our samples, as revealed by miRNA arrays and by qRT-PCR performed to choose the best housekeeping for our cell model.

Spearman's Rank Correlation was performed to represent the heat map of microRNA array expression data.

2.9. Spheroid formation

To enrich samples from EOC PDX in cancer stem cell fraction, we promoted *in vitro* spheroid formation, since spheroid-forming cells represent a stem-like subset, as reported elsewhere [30]. To spheroid formation promotion, ascitic effusion cells from EOC xenografts were seeded in poly-2-hydroxyethyl methacrylate (PhEMA)-coated plates (BD Bioscience, Franklin Lakes, NJ), at the density of $2x10^4$ cells/well. The cells were cultured in serum-free DMEMF12 medium supplemented with bFGF (10ng/ml; Peprotech, Rocky Hill, NJ), EGF (20 ng/ml; Peprotech) and B27 (Gibco – Life technologies). Medium was replaced every 7 days, and after 2 weeks the cells were harvested and cancer stem cell enrichment was evaluated by qRT-PCR for CD117 gene expression. To a reliable cancer stem-like cell enrichment, CD117 expression had always to exceed a 10-fold increase in spheroid-forming cell culture conditions compared to cells cultured in normal adhesion conditions. Cancer stem cell enriched cultures (indicated as spheroid-forming cells in all figures) were used to microRNA silencing treatments.

2.10. *In vitro* miRNA silencing

EOC xenograft cells were plated at the concentration of 0,1 x 10^6 cells/well on normal adhesion p6 plates with RPMI complete medium for 24h. Then, the RPMI medium was replaced with OPTIMEM and anti-mir-602 antagomiR (has-mir-602 mirVana ™ miRNA inhibitor; Life Technologies) was added to a total of 150 pmol/well; to allow cell transfection, lipofectamine (Lipofectamine ® RNAiMAX; Invitrogen) was mixed with the miRNA inhibitor, as protocol instructions. Similarly, an equal number of cells were treated with a negative control miRNA inhibitor (mirVana™ miRNA inhibitor Negative Control #1; Life technologies), to use as a control for data normalization on mir-602 independent transfection effects. Moreover, to monitor antagomiR uptake efficiency by flow cytometry analyses, some cells were transfected with a carboxyfluorescein-labeled RNA oligonucleotide (FAM™-labeled Anti-miR™ Negative Control; Applied Biosystems). After an overnight incubation, the OPTIMEM medium supplemented with miRNA inhibitors was replaced with normal complete RPMI medium, and the mir-602 silencing was evaluated by qRT-PCT at 24h, 48h, 72h and 6 days from medium replacement. At each time point the cells were also harvested to perform all the experiments for mir-602 function investigation.

In all reported silencing experiments, transfection efficiency was highest than 50%, and mir-602 expression showed at least 5-fold decrease in mir-602 antagomiR treated cells compared to control subset.

2.11. miRNA/target interactions and related pathways

The targets of the analyzed miRNA were identified using miRTarBase database, which reports all the experimentally validated miRNA/target interactions.
The pathways involving the miRNA targets are reported on Reactome database.

2.12. Statistical analysis

Continuous variables were summarized with mean, standard deviation and median; categorical variables were reported as counts and percentages. Comparisons between mean values were performed by two-tail Student's *t* test. The χ^2 test was used for the association between *in vitro* glucose addiction and clinical platinum response. Correlations between variables were evaluated by the Rho of Pearson. Progression Free Survival (PFS) was calculated from the completion of first-line chemotherapy to the time of last follow-up visit or event. The survival probability was estimated by means of the Kaplan-Meier method and heterogeneity in survival among strata was assessed through the log-rank test. The median PFS was reported along with 95% confidence intervals (CI). A *P*-value <0.05 was considered as statistically significant. All data analyses were performed using the SAS statistical package (SAS, release 9.2; SAS Institute).

As mentioned in the proper paragraph, Limma analysis and Spearman's Rank Correlation were performed to the statistical evaluation of miRNA array data.

3. RESULTS

3.1. GLUCOSE ADDICTION AND PLATINUM-SENSITIVITY OF EOC SAMPLES

3.1.1. *In vitro* glucose starvation discloses two distinct metabolic phenotypes of EOC cells

In a recent study, focused on metabolic features of ovarian CSC and their cancer non-stem counterpart, we noticed that not all EOC patient-derived cells showed the same metabolic profile. Thus, we wondered whether this different metabolic behavior could correlate with the patient response to chemotherapy.

To investigate a possible association between response to chemotherapy and metabolic features of tumor cells, we collected EOC ascitic effusions from 47 carboplatin-treated patients (Table 7) which were categorized as clinically platinum(PLT)-sensitive or -resistant according to FIGO classification.

	Platinum-sensitive[a] N=19(%)	**Platinum-resistant N=28(%)**	**Total N=47 (%)**	***P* value[b]**
Histotype				**NS**
Endometrioid	1 (5.2)	5 (17.8)	6 (12.71)	
Serous	16 (84.2)	21(75)	37 (78.7)	
Other (mucinous/clear cells)	2 (10.5)	2 (7.1)	4 (8.5)	
Stage				**NS**
3A	4 (21.0)	3 (10.7)	7 (13.85)	
3B	0 (0.0)	1(3.6)	1 (4.62)	
3C	12 (63.1)	19 (67.8)	31(67.69)	
4	3 (15.78)	5 (17.8)	8(17.02)	
Grading				**NS**
G1	3 (15.7)	2 (7.1)	5 (10.6)	
G2	2 (10.5)	6 (21.4)	8 (17.02)	
G3	14 (73.7)	20 (71.4)	34 (72.3)	
Age (years)				
Mean ± SD	64.1±11	64.2±9.40	64.15±10.02	

Table 7. Clinical characteristics of EOC-bearing patients. *a) EOC patients were categorized as PLT-sensitive or PLT-resistant according to time to disease recurrence after platinum therapy completion (FIGO classification): patients who relapsed within 6 months were classified as PLT-resistant, whereas patients relapsing after 6 months were classified as PLT-sensitive. b) According to χ^2 test, NS not significant.*

Tumor cells isolated from patients' ascitic fluids were cultured *in vitro* either in the presence or in the absence of glucose for two weeks. Interestingly, while under normal culture conditions the viability of tumor cells from PLT-sensitive and resistant patients was comparable (Figure 22 A), in PLT-sensitive samples cell viability dropped dramatically upon glucose deprivation, and it was in

all cases below the median value calculated for all samples (13%). Instead, PLT-resistant samples collectively displayed lower sensitivity to glucose starvation, although heterogeneous responses were recorded (Figure 22 A). Thus, we arbitrarily chose median viability under glucose starvation (13.0%) as a cut-off value to discern two groups, which possibly reflected different states of glucose addiction: glucose deprivation-sensitive (Glucose Addicted, GA) patients (14-d viability <13.0%), and glucose deprivation-resistant (Glucose non-Addicted, GNA) patients (14-d viability ≥13.0%) (Figure 22 B and Table 8).

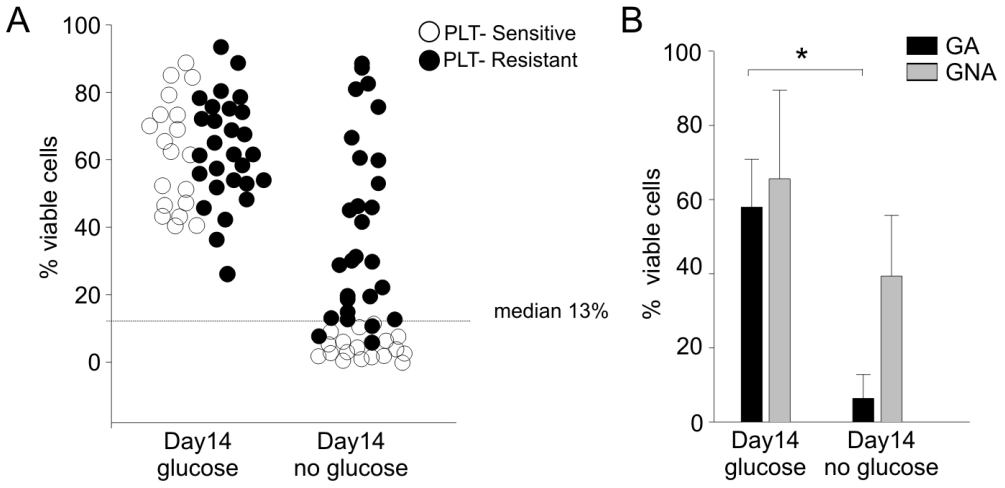

Figure 22. Glucose addiction discriminates two EOC patient categories. ***(A)*** *Flow cytometry analysis of cell viability in ascitic effusion cells isolated from 47 EOC patients categorized as PLT-sensitive/ resistant on the basis of their clinical response to PLT treatment. Cell viability of CD45$^-$/CD44$^+$ tumor cells was recorded after 14-day in vitro culture in the presence (glucose) and in the absence (no glucose) of glucose by Live/Dead staining. Each circle represents an individual patient, and the dotted line indicates the median value (13.0%), chosen as cut-off value.* ***(B)*** *The bar graph summarizes mean values (± SD) of tumor cells from EOC patients categorized as glucose addicted (GA) or glucose non-addicted (GNA) according to their cell viability after 14 d under glucose starvation. *P < 0.05.*

	Platinum-sensitive N=19(%)	**Platinum-resistant N=28(%)**	**Total N=47 (%)**	***P value***
GA/GNA status[c]				*P*<0.001
GA < 13.0%	19	3	22	
GNA ≥ 13.0%	0	25	25	

Table 8. Association between in vitro glucose addiction of tumor cells and clinical PLT response. *c) EOC patients were categorized as GA or GNA at diagnosis, according to the percent viability of their tumor cells under 14-day in vitro glucose starvation.*

To experimentally validate the apparent correlation between *in vitro* glucose addiction of EOC cells and clinical response to platinum salts, we compared *in vitro* cell viability after 72 h of carboplatin treatment of tumor cells isolated from EOC samples defined as GA (n=6) or GNA (n=6) according to their cell viability under glucose starvation. As shown in Figure 23 A, mean lethal dose 50 (LD50) was 5.77 ± 1.03 µg/ml for GA patients and 8.9 ± 1.4 µg/ml for GNA samples. These results correlated with AnnexinV/PI staining of GA and GNA samples after treatment with three different concentrations of carboplatin. (Figure 23 B).

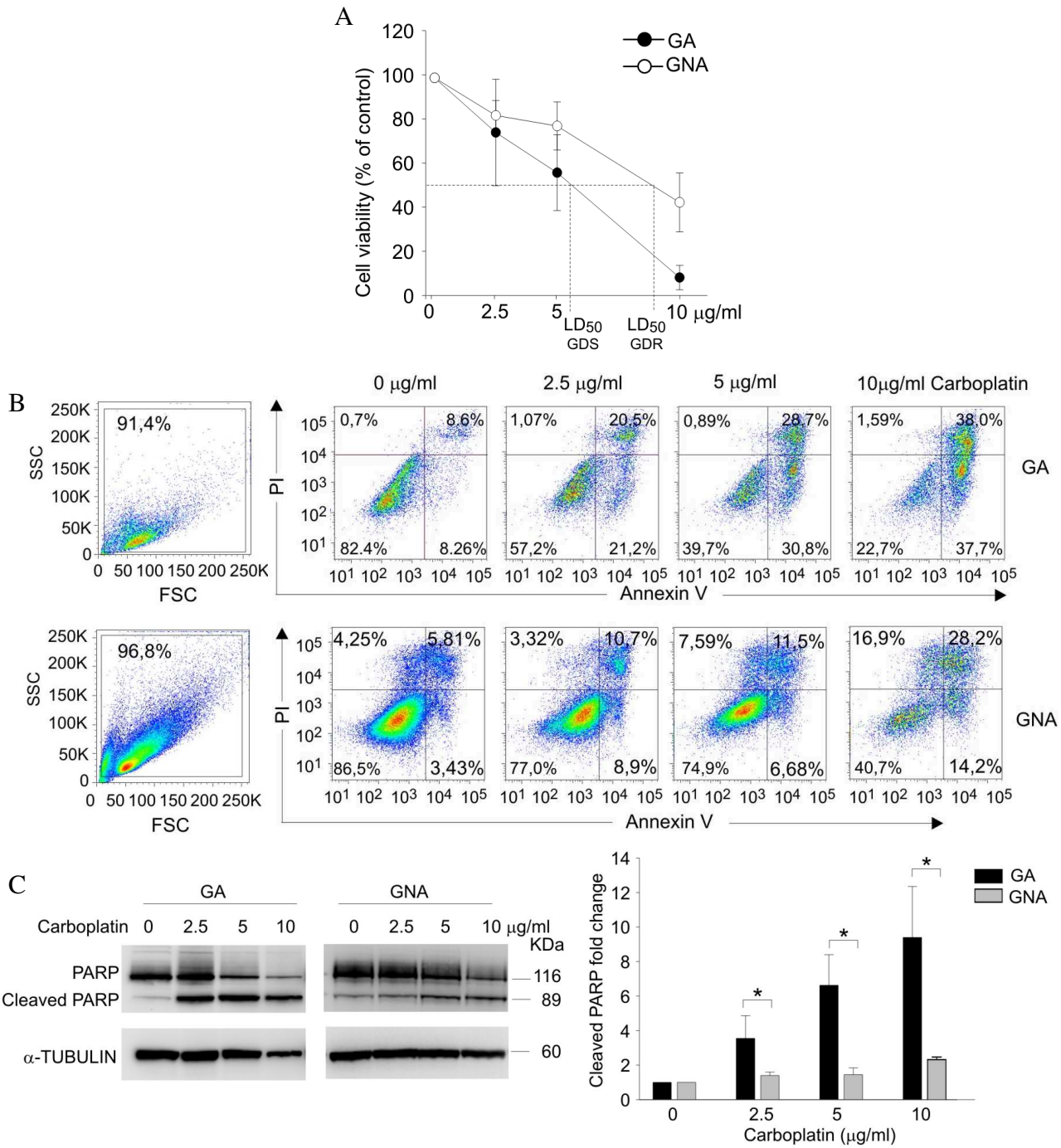

Figure 23. GA/GNA phenotype correlates with sensitivity to in vitro PLT-treatment. ***(A)*** *Cell viability analysis of AnnexinV/PI staining in CD45$^-$/CD44$^+$ tumor cells from GA (n=6) and GNA (n=6) patients treated in vitro for 72 h with different doses of carboplatin (2.5, 5 and 10 µg/ml). Data were expressed as mean percent viability (± SD) compared to control. The LD_{50} value for GA and GNA samples is indicated on the abscissa axis.* ***(B)*** *Flow cytometry analysis of cell viability by AnnexinV/PI staining in CD45$^-$/CD44$^+$ tumor cells from two representative GA and GNA patients, treated in vitro for 72 h with different carboplatin concentrations.* ***(C)*** *WB analysis of total and cleaved PARP in FACS-sorted CD45$^-$/CD44$^+$ tumor cells of GA and GNA patients, treated in vitro for 72 h with different doses of carboplatin. One representative blot is shown in the left panel; the right histogram depicts mean values (± SD) of 6 GA vs 6 GNA patients. Signal intensities of the PARP bands were normalized against the α-tubulin signal. Expression ratios were calculated by dividing normalized signal intensity values obtained for untreated GA or GNA cells. *P < 0.05.*

In fact, apoptosis was detected at the lowest carboplatin concentration used (2.5 µg/ml) in tumor cells from GA patients, whereas in GNA samples as much as 10 µg/ml were needed to induce a sizable increase in apoptosis (Figure 23 B). To confirm these data, we evaluated by WB the effects of carboplatin treatment on PARP cleavage, a well-known marker of apoptosis [202]. As shown in Figure 23 C, while in GA samples the fraction of cleaved PARP increased in a dose-dependent manner, in cells from GNA patients an increase in cleaved PARP was evident only when the maximal dose of carboplatin was used.

Finally, we addressed the apparent *in vitro* correlation between PLT sensitivity and glucose addiction also by *in vivo* experiments in a xenotransplantation model. We have previously shown that EOC ascitic effusion cells can be successfully transplanted into immunodeficient hosts [30]. Interestingly, EOC xenografts generated from ascitic effusions of GA patients maintained a high *in vitro* sensitivity to glucose deprivation with a high mortality in the absence of this nutrient, whereas viability of xenografts from GNA samples was not affected by glucose starvation (Table 9).

	Primary sample		***Xenografts***	
Patient ID	***% cell viability***	***GA/GNA phenotype***	***% cell viability***	***GA/GNA phenotype***
#14	1.8	GA	5.37	GA
#24	7.6	GA	12.3	GA
#29	11.2	GA	8.9	GA
#36	10.6	GA	4.31	GA
#39	5.7	GA	3.5	GA
#49	2.39	GA	2.92	GA
#49bis	6.35	GA	2.79	GA
#52	1.41	GA	9.21	GA
#57	0.37	GA	0.15	GA
#60	8.1	GA	11.7	GA
#62	4.9	GA	9.47	GA
#70	2.29	GA	5.4	GA
#74	6.76	GA	7.9	GA
#79	10.9	GA	6.01	GA

	Primary sample		***Xenografts***	
Patient ID	***% cell viability***	***GA/GNA phenotype***	***% cell viability***	***GA/GNA phenotype***
#15	45	GNA	53.4	GNA
#17	59.8	GNA	20.4	GNA
#32	66.5	GNA	82.0	GNA
#37	19.6	GNA	24.6	GNA
#41	43.1	GNA	36.4	GNA
#44	21.8	GNA	35.2	GNA
#46	18.6	GNA	13.6	GNA
#50	28.7	GNA	32.4	GNA
#53	46.2	GNA	21.1	GNA
#54	13.2	GNA	22.7	GNA
#58	75.6	GNA	80.1	GNA
#69	41.5	GNA	24.6	GNA
#82	21.2	GNA	47.2	GNA

Table 9. The GA/GNA phenotype is maintained after xenotransplantation[a]. *a) EOC patients were categorized as GA or GNA according to the viability of their tumor cells after 14-day in vitro glucose starvation as detailed in Figure 22 A (primary sample). The xenografts were generated by orthotopic injection of EOC tumor cells into SCID mice; the resulting tumor cells isolated from ascitic effusion were then assayed for their GA/GNA phenotype as above.*

Furthermore, when RAG-2γ-/- mice subcutaneously injected with xenografts from GA patients were treated with a glucose analogue (2DG) which prevents glucose utilization, we observed a significant delay in tumor growth after a short treatment of only four doses of 2-DG (Figure 24 A). On the contrary, in mice injected with xenografts from a GNA patient, up to eight doses of 2-DG

were needed to spot a significant difference in tumor volume between treated and control animals (Figure 24 B). Similarly, we observed a significant control of *in vivo* tumor growth by carboplatin administration in RAG-2γ-/- mice subcutaneously injected with tumor cells isolated from GA donors, whereas up to 3 weeks were needed to significantly reduce tumor development in mice injected with tumor cells from GNA patients (Figure 24 C, D).

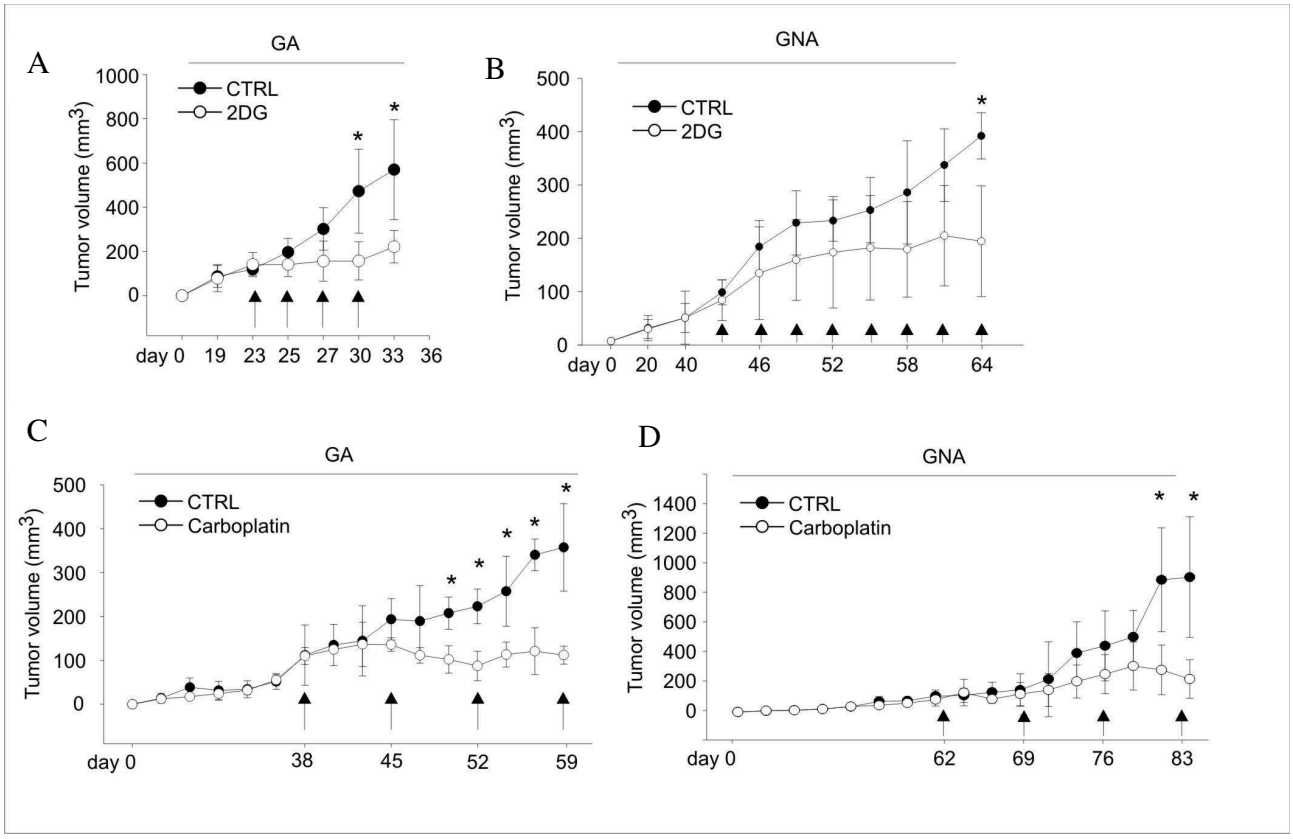

Figure 24. Tumor cells from GA and GNA patients present different in vivo PLT-sensitivity. (A-B)*Tumor growth curves in RAG-2γ$^{-/-}$ mice untreated (CTRL) and treated with the glucose analogue (2DG) after subcutaneous (s.c.) injection of xenografts cells from GA (A) and GNA (B) patients. When tumor volume reached 100 mm^3, the mice were randomized to receive the different treatments. The arrows indicate the days of 2DG administration; data show mean values (± SD) of six tumors/group. *P< 0.05.* ***(C-D)*** *Effect of carboplatin administration on the growth of tumors generated by s.c. injection of tumor xenotrasplants from GA (C) and GNA (D) patients into RAG-2γ$^{-/-}$ mice. When tumor volume reached 100 mm^3, the mice were randomized to receive carboplatin or saline solution as a control (CTRL). The arrows indicate the days of drug administration; data show mean values (± SD) of six tumors/group. *P< 0.05.*

On the whole, these data indicate that *in vitro* and *in vivo* PLT sensitivity is closely associated in most patients with a metabolic profile oriented towards strict glucose addiction.

3.1.2. GNA samples are characterized by low proliferation rate and high MDR pump expression

These observations prompted us to explore the mechanisms that could explain why platinum-based therapy was more efficient in GA compared to GNA patients. PLT sensitivity relies on two critical parameters: the proliferation rate (which renders tumor cells a more suitable target for the DNA-damaging effects of the drug) and the ability of malignant cells to extrude toxic compounds through multidrug-resistance (MDR) pumps and detoxifying enzymes [11,205]. We first monitored cell

proliferation in EOC primary samples after staining with CFSE, a membrane dye that enables the evaluation of cell division rate by flow cytometry [201]. Three gates were identified at the beginning of the experiment (T0) according to the dye intensity (Figure 25 A, left panel); after 72 h *in vitro* culture, the cell percentage in each gate was recorded. As shown in Figure 25 A (right panel), cells from GNA patients persisted longer in gate #1 compared to GA cells, indicating a slower proliferation rate. We also addressed expression of Cyclins. As shown in Figure 25 B, mRNA levels of *CYCLIN A*, *B*, *D* and *E* were significantly higher in tumor cells from GA patients compared to GNA samples. For Cyclin D1, WB analysis fully confirmed these results (Figure 25 C).

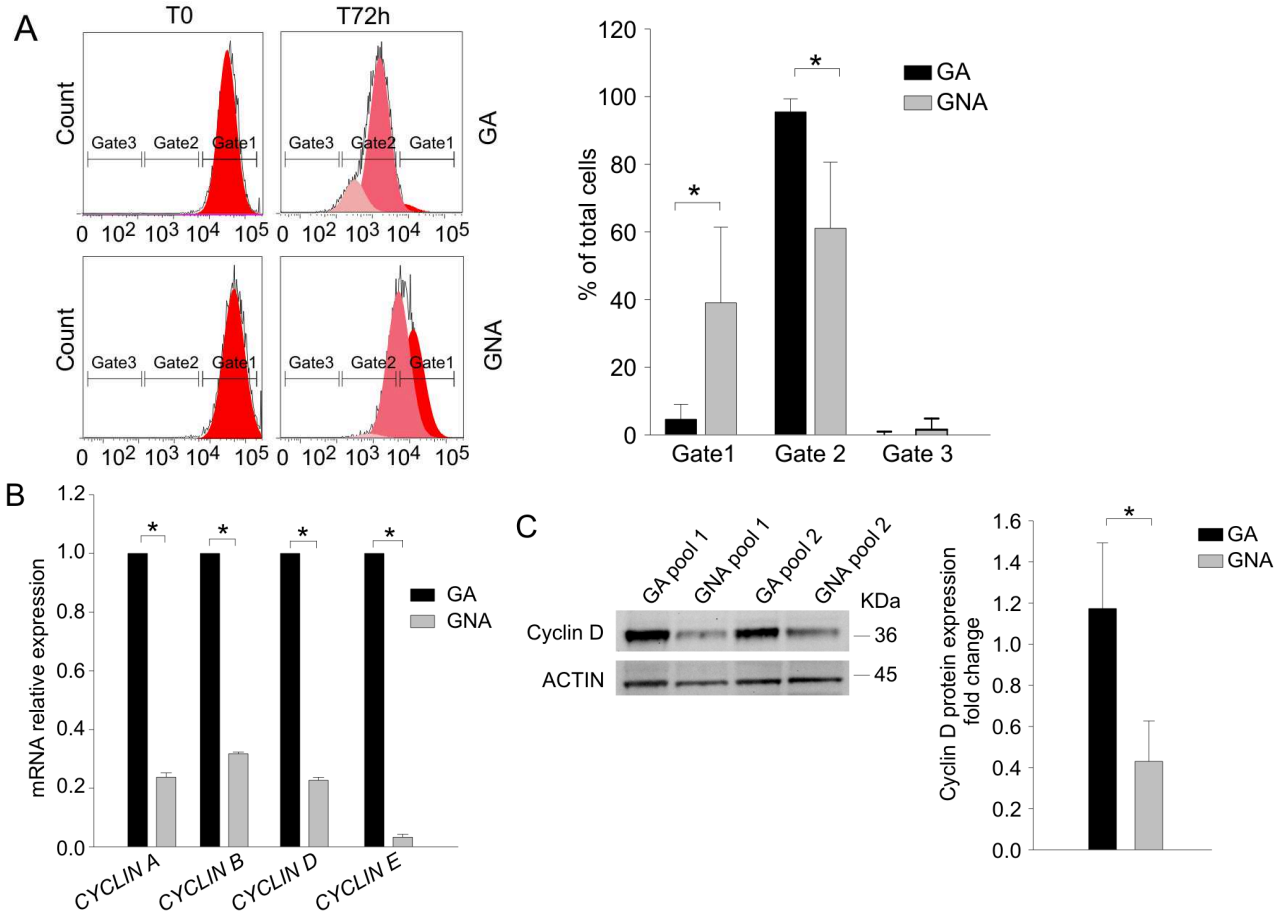

Figure 25. GNA patients present lower proliferation rate ***(A)*** *The proliferation rate of GA vs. GNA ascitic effusion cells was evaluated by CFSE staining in CD45⁻/CD44⁺ tumor cells. Three gates were set according to dye intensity (time 0; left panel). For each gate, the mean percentage of CFSE⁺ cells was quantified 72 h after staining. One representative experiment is shown on the left; mean values (± SD) in 5 GA and 4 GNA patients are plotted on the right. *P < 0.05.* ***(B)*** *qRT-PCR analysis of cyclin expression in FACS sorted CD45⁻/CD44⁺ tumor cells from EOC ascitic effusions. Data were expressed as mean relative expression values in tumor cells from GA (n=6) compared to GNA (n=6) patients (± SD). *P < 0.001.* ***(C)*** *Left panel: Representative WB analysis of Cyclin D expression in pools of CD45⁻/CD44⁺ tumor cells from GA and GNA patients (pools #1) and patient-derived xenografts (pools #2). Each pool consisted of 3 GA or GNA samples. Right panel: The signal intensity of the Cyclin D bands was normalized against the actin signal; expression ratios were calculated by dividing normalized signal intensity values obtained for GNA cells by those obtained for GA cells. The graph shows mean expression ratios (± SD) in 8 different pools. *P < 0.05.*

Moreover, we quantified *ex vivo* mRNA expression levels of several *MDR* pump genes and detoxifying enzyme in tumor cells from GA and GNA patients. As shown in Figure 26 A, GNA samples displayed significantly higher expression of three out of four of the genes analyzed. *ALDH1A* mRNA levels did not significantly differ between the two patient categories, mainly due to its heterogeneous expression in GNA samples (Figure 26 A); however, when enzymatic activity of ALDH was measured by the ALDEFLUOR® assay, GNA samples showed a 3-fold higher expression of this detoxifying enzyme (Figure 26 B).

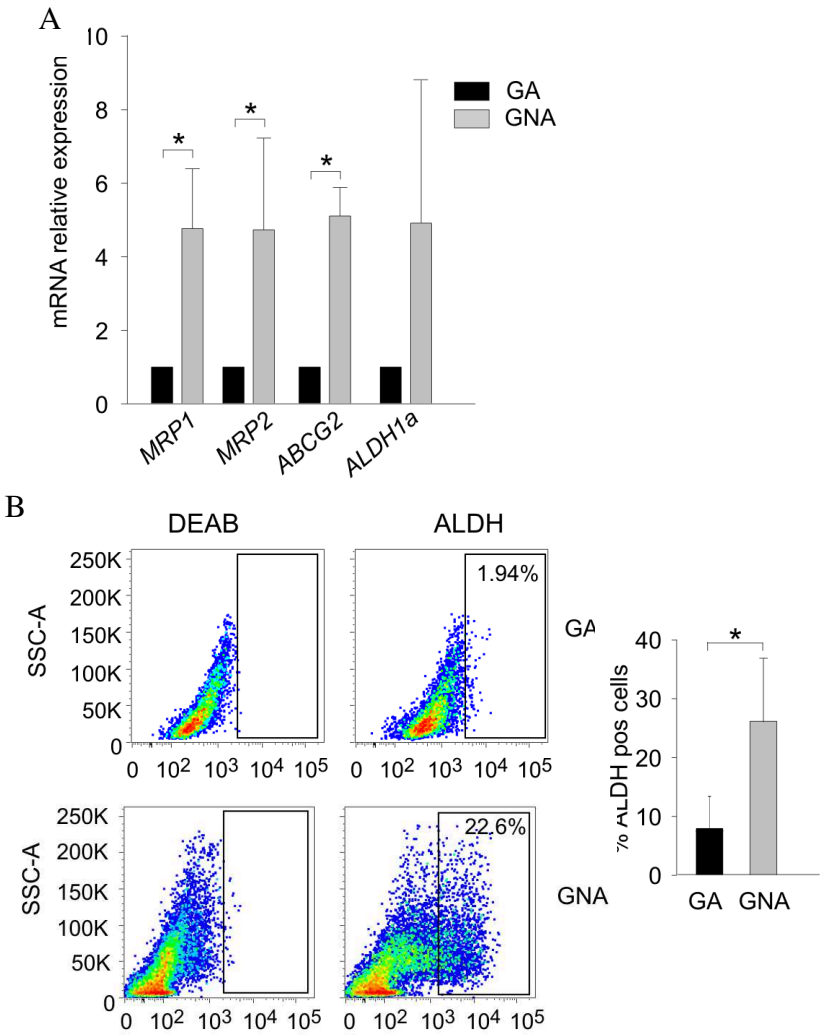

Figure 26. GNA patients present higher MDR pump expression. ***(A)*** *qRT-PCR analysis of multidrug resistance (MDR) pump expression in FACS sorted CD45⁻/CD44⁺ascitic effusion cells from GA (n=6) and GNA (n=6) patients. Data were expressed as mean relative expression values (± SD). *P < 0.05.* ***(B)*** *Ex vivo flow cytometry analysis of ALDH activity in CD45⁻/CD44⁺ EOC ascitic effusion cells from GA (n=20) and GNA (n=15) patients. Gates were set on the DEAB control, and values indicate the percentage of ALDH⁺ cells. One representative experiment is shown on the left; the right histogram shows mean percent values (± SD). *P < 0.05.*

It has been demonstrated [11,205,206] that higher MDR expression and resistance to platinum treatment are key hallmarks of cancer stem cells (CSC). We and others have demonstrated that in ovarian cancer [30] and glioblastoma [182,207] CSC are characterized by OXPHOS metabolism and resistance to glucose starvation. Hence, we evaluated in GA and GNA samples the percentage of cells co-expressing CD44 and CD117, a recognized marker of EOC CSC [29,30]. We did not found any difference between GA and GNA patients (data not shown), thus implying that the different phenotype of GA and GNA samples was likely not attributable to their CSC content. However, although the number of CSC is similar within GA and GNA samples, it is possible that the CSC molecular profile differs between the EOC phenotypes; therefore, CSC microRNA signature will be described in the results of the second part of the project.

3.1.3. Tumor cells from GA patients show a higher glycolytic activity compared to GNA subjects

Since these results could underlie differences in the machinery controlling glycolytic activity between GA and GNA patients, we addressed the glucose metabolic profiling by firstly evaluating the expression of GLUT1, one of the major glucose transporters [203,204]. By flow cytometry, we found significantly higher surface expression of GLUT1 in tumor cells from GA (21.13 ± 4.07%) compared to GNA samples (8.44 ± 5.60%, Figure 27 A).

We next compared mRNA expression levels of several genes encoding for key components involved in glycolysis, such as hexokinase II (*HKII*), phosphofructokinase (*PFK*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and lactate dehydrogenase (*LDH*). As depicted in Figure 27 B, tumor cells isolated from GA patients showed significantly higher expression of all transcripts analyzed, compared to GNA samples.

These results were supported by Seahorse analysis of extracellular acidification rate (ECAR, a surrogate marker of lactic acid production) in short-term *ex vivo* cultures. Tumor cells from GA patients presented significantly higher ECAR values compared to GNA samples (Figure 27 C), suggesting higher rates of lactate production. Accordingly, WB analysis showed a significantly higher expression of MCT4, the major lactate transporter, in tumor cells from GA patients (Figure 27 D).

On the other hand, oxygen consumption rate (OCR) did not show any significant difference between the two groups (Figure 28).

Overall, these observations indicate that tumor cells from glucose-addicted EOC patients show a higher glycolytic activity, compared to GNA subjects.

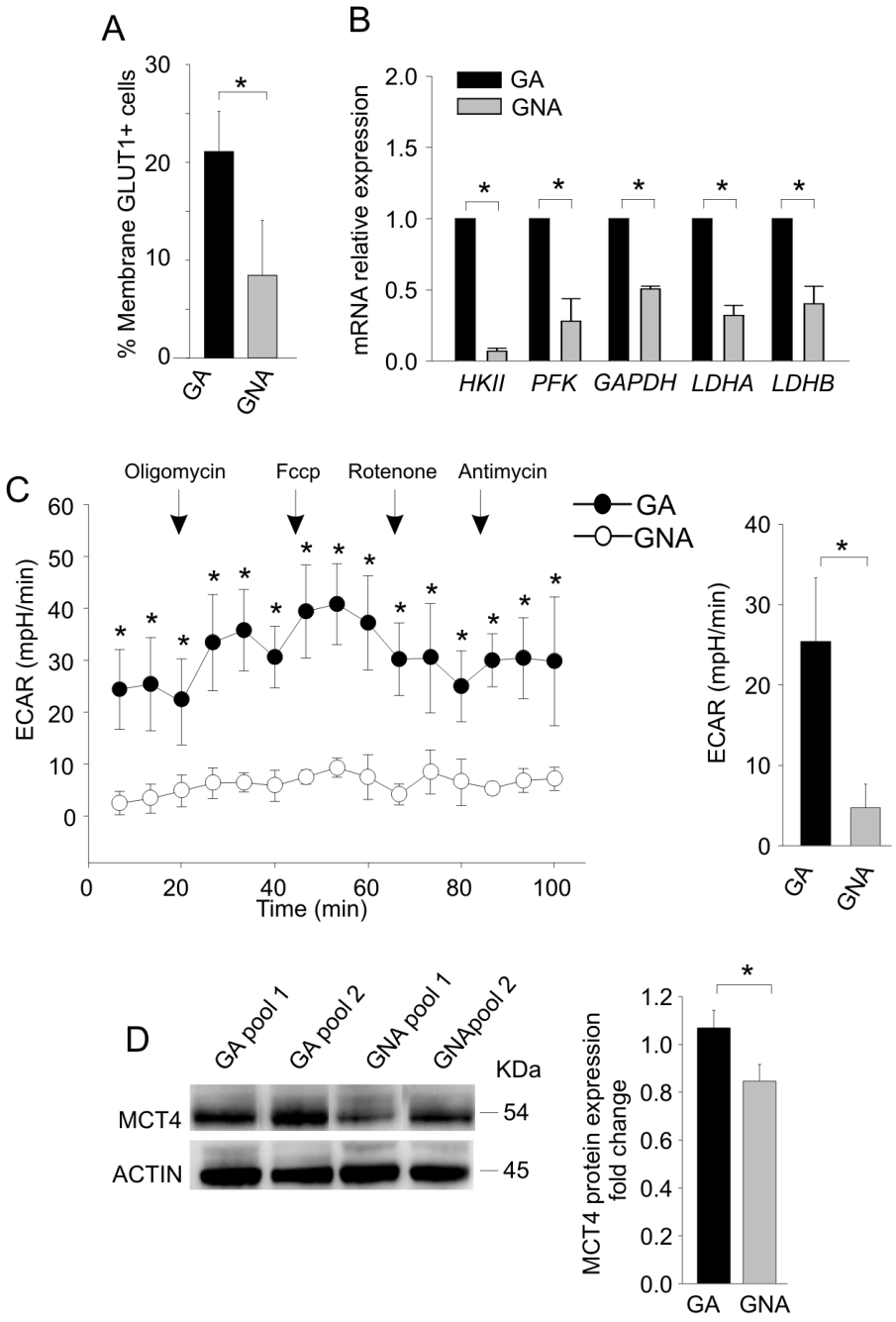

Figure 27. GA and GNA patients present different metabolic profiles. ***(A)*** *Flow cytometry evaluation of membrane GLUT1. Data expressed as mean percent values in tumor cells from GA and GNA patients (±SD). *P < 0.05.* ***(B)*** *qRT-PCR analysis of key enzymes of the glucose metabolic chain in FACS sorted CD45$^-$/CD44$^+$ tumor cells from GA (n=6) and GNA (n=6) patients. *P < 0.01.* ***(C)*** *ECAR analysis in CD45$^-$/CD44$^+$ tumor cells from GA and GNA patients. One representative experiment is shown on the left; the first three points of the graph indicate the basal ECAR ratio. At different time points, the indicated mitochondrial inhibitors were added. The histogram on the right shows the mean values of the basal ECAR in 6 GA vs 6 GNA patients (±SD). *P <0.05.* ***(D)*** *WB analysis of MCT4 lactate transporter in pooled CD45$^-$/CD44$^+$ tumor cells from GA or GNA patients (pools #1) and xenografts (pools #2). Each pool consisted of 3 GA or 3 GNA samples. After normalization against actin, expression ratios were calculated by dividing GNA signal intensity values by those of GA cells. On the left a representative blot, on the right the graph depicts mean expression ratios in 8 different pools (±SD). *P < 0.05.*

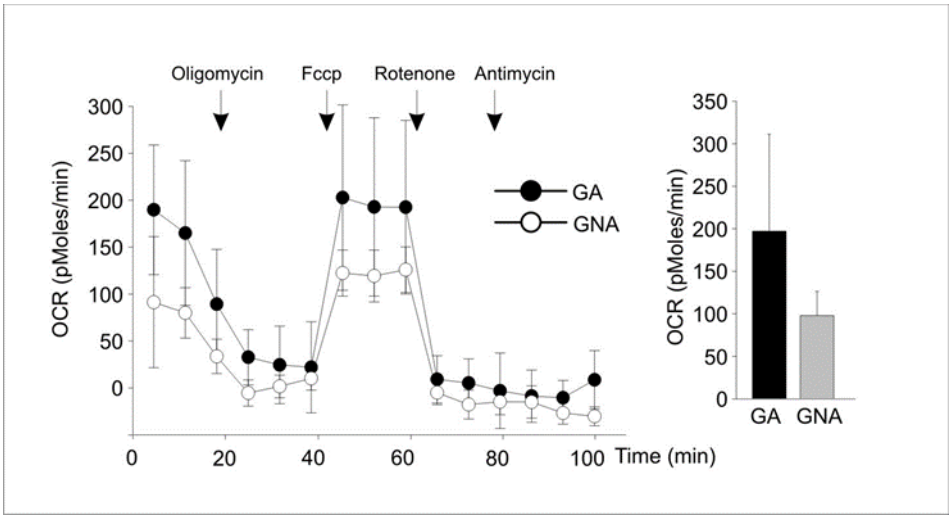

Figure 28. Tumor cells from GA and GNA patients do not differ in terms of oxygen consumption. *Analysis of oxygen consumption rate (OCR) in CD45$^-$/CD44$^+$ ascitic effusion cells from GA and GNA patients. Mitochondrial inhibitors were added as indicated. One representative experiment is shown on the left; the right histogram shows mean values (±SD) of the basal OCR in 6 GA and 6 GNA patients.*

3.1.4. PLT may favor the GNA phenotype in patients and in an EOC-derived xenograft model

The close association between glucose metabolism and PLT sensitivity was further strengthened in a mouse model, consisting on xenografts obtained by the subcutaneous injection of a PLT-sensitive sample of EOC cells into nude mice. The first-generation xenografts were all platinum-sensitive; some of them were maintained PLT-naïve by serial passages in mice, meanwhile other were made PLT-resistant by prolonged *in vivo* platinum treatment, as detailed in Materials and Methods. Thus, to investigate the association between metabolism and chemoresistance, we analyzed both parental PLT-sensitive (#S) and their PLT-resistant (#R) derivatives. Tumor cells isolated from #S and #R xenografts were preliminary treated *in vitro* for 72 h with 20 μg/ml of carboplatin; as expected, a strong reduction of cell viability was measured in #S samples, whereas almost 90% of the cells survived treatment in #R samples (Figure 29 A). In addition, when we evaluated ALDH activity at tumor harvest, a three-fold higher expression of this detoxifying enzyme was found in #R samples (mean 65% ± 6.6%), compared to the PLT-sensitive counterpart (#S, mean 21.2 ± 4.8%, Figure 29 B).

Moreover, when these cells were maintained *in vitro* under glucose deprivation, cell viability was significantly higher in #R compared to #S samples (Figure 30 A). To better evaluate the metabolic alterations induced by prolonged PLT treatment, we also analyzed mRNA expression levels of several genes involved in glucose metabolism, including *GLUT1*, *HKII*, *PFK, GAPDH*, *LDHA, LDHB* and *MCT4.* As shown in Figure 30 B, genes encoding for enzymes that transform pyruvate into lactate and regulate its extrusion outside the cells were down-regulated in #R samples.

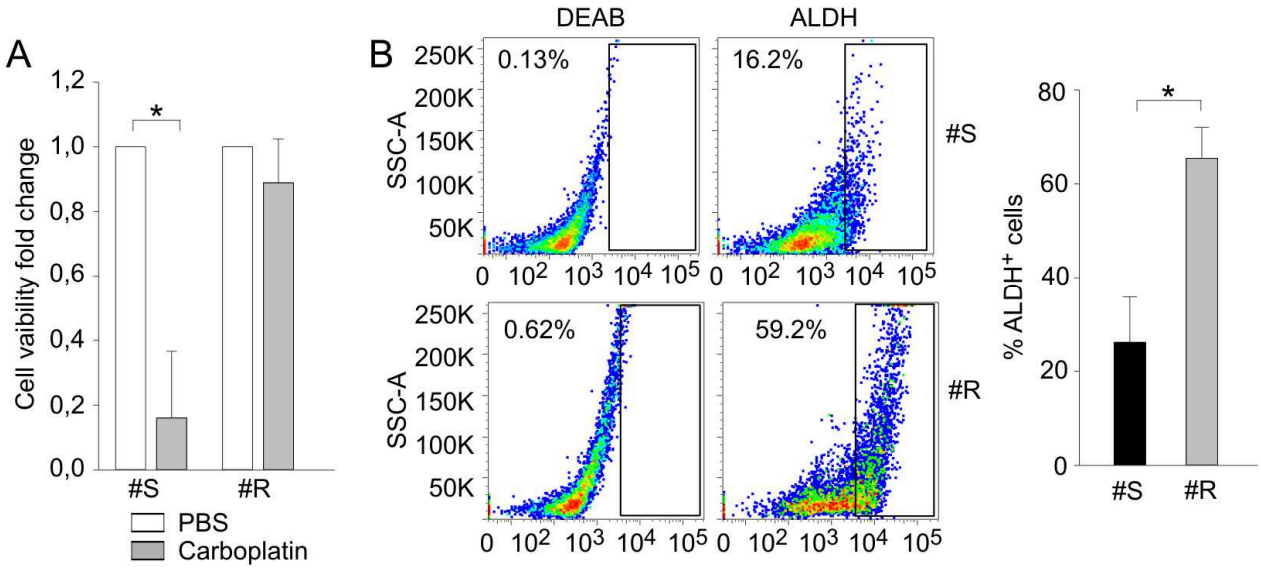

Figure 29. Prolonged PLT-treatment revert GA into GNA phenotype in a xenotransplantation model. (A) *Cell viability analysis performed with AnnexinV/PI staining in PLT-sensitive EOC xenografts (#S) and their resistant counterpart (#R) after 72 h of carboplatin treatment. Data are expressed as ratio between treated and untreated counterpart.* ***(B)*** *ALDH activity analysis in PLT-sensitive EOC xenografts (#S) and their resistant counterpart (#R) obtained as described in Materials and Methods. On the left, one representative sample is shown; the right histogram shows mean values (±SD) of three biological replicates for two different samples.*

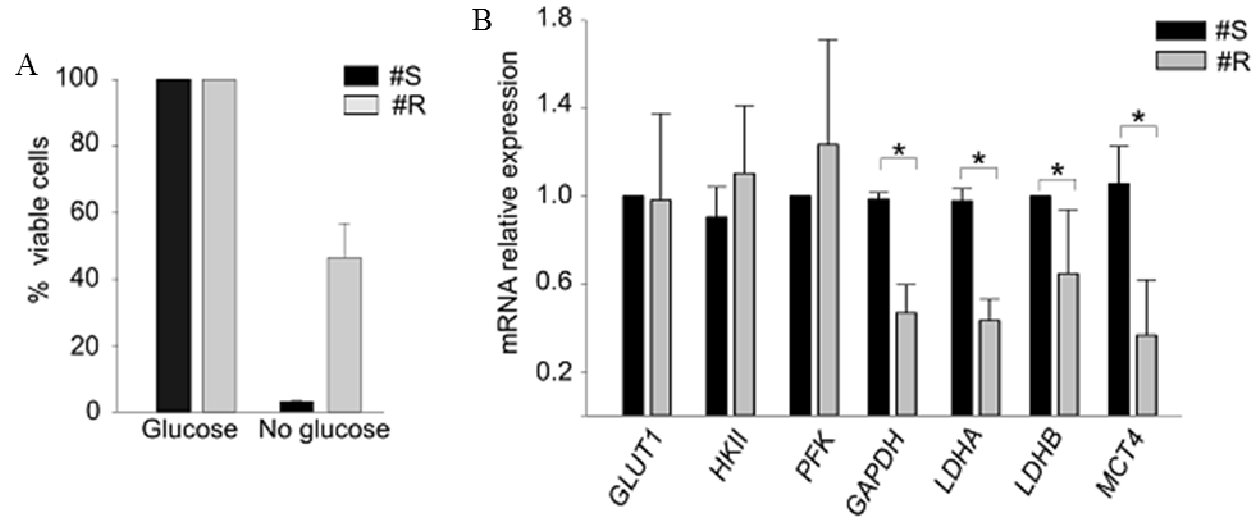

Figure 30. Prolonged PLT-treatment influences the response to glucose starvation in a xenotransplantation model. (A) *Cell viability analysis of PLT-sensitive EOC xenografts (#S) and their resistant counterpart (#R) recorded after 7-day in vitro culture in the presence (glucose) and in the absence (no glucose) of glucose by Live/Dead staining.* ***(B)*** *qRT-PCR analysis of key enzymes involved in the glucose metabolic chain in PLT-sensitive EOC xenografts (#S) and their resistant counterpart (#R). Data are expressed as mean relative expression values (± SD). *P < 0.05.*

On the whole, these data confirm the strict parallelism between glucose addiction and PLT sensitivity, and evidence that prolonged PLT treatment is associated with metabolic alterations.

3.1.5. The GA/GNA phenotype may predict clinical responsiveness in untreated patients

Nonetheless, it is possible that the GA/GNA phenotype and glucose addiction could be an intrinsic metabolic feature that some EOC patients display before chemotherapy. Indeed, when we determined the GA/GNA phenotype in serial tumor samples obtained from the same patients at various time points after administration of carboplatin, we found in 4 out of 14 cases analyzed a conversion from GA to GNA phenotype, whereas in no case the opposite transition was found (not shown). To further clarify this issue, we evaluated in a prospective cohort of 23 patients, which did not received any treatment before sampling, the 14-d viability of their tumor cells in the absence of glucose (Figure 31 A).

At diagnosis, 13 patients (56.5%) were categorized as GA, and 10 (43.6%) as GNA; all underwent first-line PLT-based chemotherapy, and the time to tumor relapse was recorded over 18 months of follow-up. As shown in Table 10, in this prospective cohort no correlation emerged between PLT-sensitive/resistant phenotypes and tumor stage, grading or histotype. On the other hand, a significant association between the GA/GNA phenotype and clinical sensitivity/resistance to first-line PLT chemotherapy was evidenced (Table 10).

	PLT-sensitive[a] N=11 (%)	**PLT-resistant N=12 (%)**	**Total N=23 (%)**	***p* value[b]**
Histotype				NS
Endometrioid	2 (18.2)	0 (0)	2 (8)	
Serous	9 (81.8)	7 (58.3)	16 (69)	
Other (mucinous/clear cells)	-	5 (41.6)	5 (21)	
Stage				NS
3A-3B	2 (18.2)	2 (16.6)	4 (17.3)	
3C-4	9 (81.8)	10 (83.4)	19 (82.6)	
Grading				NS
G1-G2	2 (18.2)	3 (25)	5 (21)	
G3	9 (81.8)	9 (75)	18 (78)	
Age (years)				
Mean ± SD	63.3±9.53	74.7±7.1		
Surgery				NS
Yes	7 (63.6)	5 (41.6)	10 (43.5)	
No	4 (36.4)	7 (58.3)	13 (56.5)	
GA/GNA status[c]				*P*<0.001
GA < 13.0%	11 (100)	2 (16.6)	13 (56.5)	
GNA ≥ 13.0%	0 (0)	10 (83.4)	10 (43.5)	

Table 10. Clinical characteristics of untreated EOC-bearing patients and association between in vitro glucose addiction of tumor cells and clinical PLT response. *a) EOC patients were categorized as PLT-sensitive or PLT-resistant according to time to disease recurrence after first-line platinum therapy; patients who relapsed within 6 months were classified as PLT-resistant, whereas patients relapsing after 6 months were classified as PLT-sensitive (according to FIGO classification). b) According to χ^2 test, NS not significant. c) EOC patients were categorized as GA or GNA at diagnosis, according to the percent viability of their tumor cells under 14-day in vitro glucose starvation (<13.0 % or ≥13.0 %, respectively).*

In addition, as shown by the Kaplan-Meier curves (Figure 31 B), GA patients showed better median progression-free survival (PFS) (9.1 months, 95% CI: 7.0-12.0; left panel) and better overall survival (right panel), compared to GNA patients (2.7 months, 95% CI: 1.0-3.0; *P <0.001*) over the follow-up period.

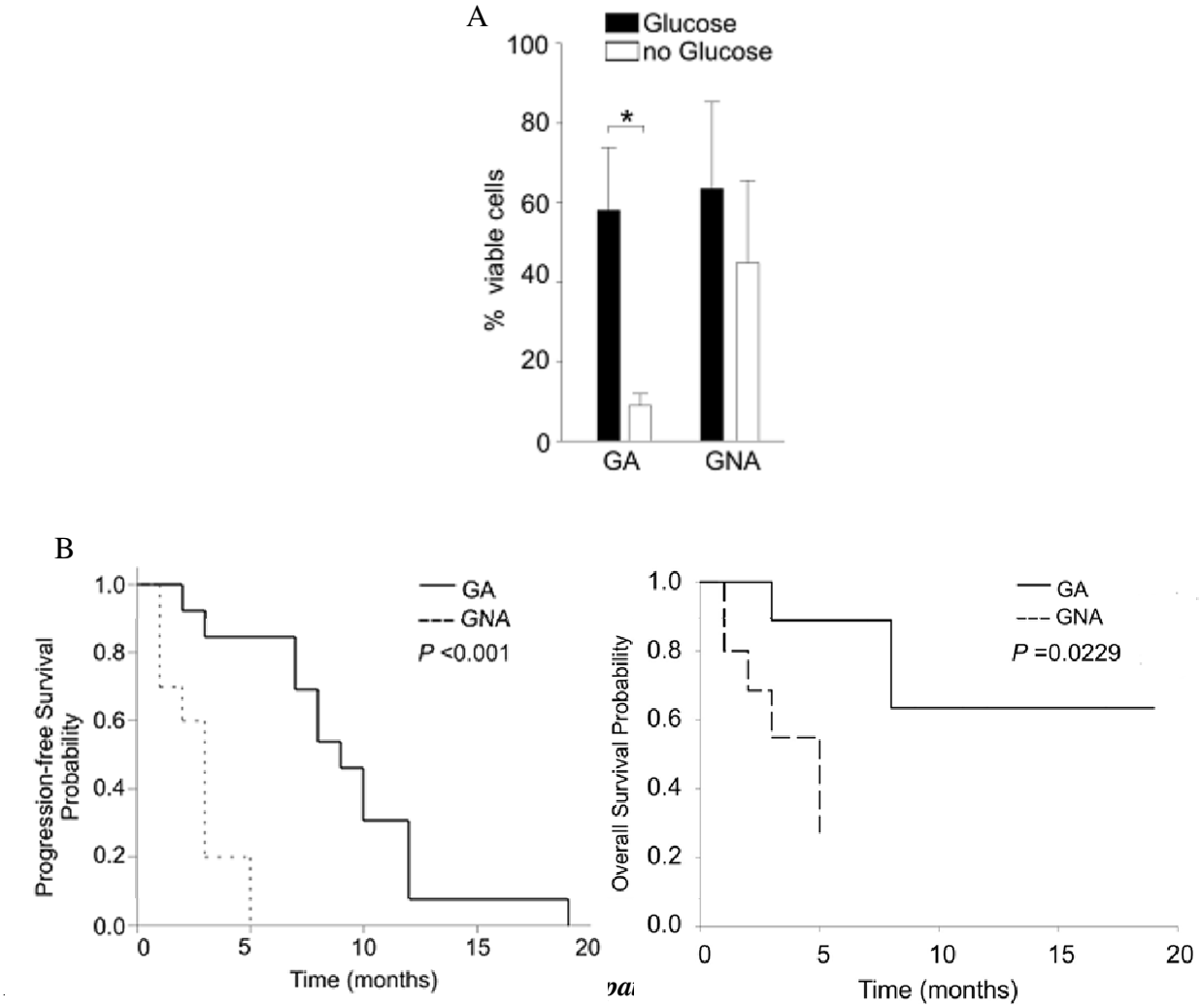

Figure 31. The GA/GNA phenotype is associated with patient progression-free survival and overall survival. (A) *Flow cytometry analysis of cell viability after 14 d in the presence or absence of glucose. The graph summarizes mean values (± SD) of tumor cells isolated from 23 EOC samples of untreated patients categorized as GA or GNA according to their cell viability under glucose starvation. *P < 0.05.* ***(B)*** *Kaplan-Meier curve showing the association between the GA/GNA phenotype and progression-free survival (left panel) or overall survival (right panel) in a cohort of 23 untreated EOC patients (GA=13, GNA=10).*

Although limited by the low number of patients analyzed, our results seem to indicate that GNA status could predict refractoriness to PLT treatment in naïve EOC patients. Nonetheless, the possibility exists that PLT treatment schedules could further exacerbate this phenotype and modulate the metabolic profile detected in patient-derived cancer cells.

3.2. miRNA PROFILE ANALYSIS

3.2.1. miRNA arrays

Deregulated glucose metabolism is often observed in cancer [208], but whether this metabolic trait influences the response to cytotoxic drugs is still unknown. At the molecular level, microRNA (miRNA) involvement into cell metabolism has already been demonstrated in several normal and tumoral tissues [209,210], and it has also been reported that several miRNAs are deregulated in ovarian carcinoma compared to ovarian normal tissue [146-149].
Thus, we decided to perform microRNA arrays on ovarian tumor cells isolated from ascitic effusions of both GA and GNA patients. To further investigate the possible involvement of CSC into metabolic alterations, the cells were isolated by FACS-sorting to separate CSC from non-CSC based on the co-expression of the surface markers CD44 and CD117, and to exclude the lymphoid/myeloid cells.

For each miRNA array hybridization, we pooled tumor cells from five patients. Totally, we performed 8 miRNA arrays, 4 CSC isolated from GA (2 arrays) and GNA (2 arrays) patients, and 4 non-stem cells (2 from GA and 2 from GNA patients), from a total of 20 patients.

Preliminary results did not reveal any significant difference in miRNA profile between non-CSC from GA and GNA patients, as well as between CSC of GA patients versus CSC isolated from GNA patients (data not shown). However, this analysis demonstrated a clear and statistically significant different miRNA profile between CSC and tumor non-stem cells, independently of the patient status (GA/GNA).

As shown on the heat map in Figure 32, the statistical analysis provided a list of several miRNA significantly deregulated in ovarian CSC compared to their non-stem counterpart: 79 miRNA were down-regulated and 201 miRNA were up-regulated in the CSC subset, considering a p-value < 0.05 (data not shown), while 9 miRNA were down-regulated in CSC and 32 miRNA were up-regulated, considering a p-value < 0.01 (Table 11).

Among them, we decided to focus our attention on 4 miRNA, 2 chosen within the list with p-value<0.05 (not shown) and two within the list with p-value<0.01 (in bold font in table 11), since they were characterized by both a low p-value and a high relative differential expression in CSC compared to non-stem cells (logFC). These miRNA are the following: hsa-mir-324-5p (p-value<0.003, logFC=-1.7) and hsa-mir-31-3p (p-value<0.02, logFC=-2.4) which are down-regulated in CSC, and hsa-mir-602 (p-value<0.005, logFC=2.24) and hsa-mir-150-3p (p-value<0.03, logFC=1,8) which are up-regulated in CSC.

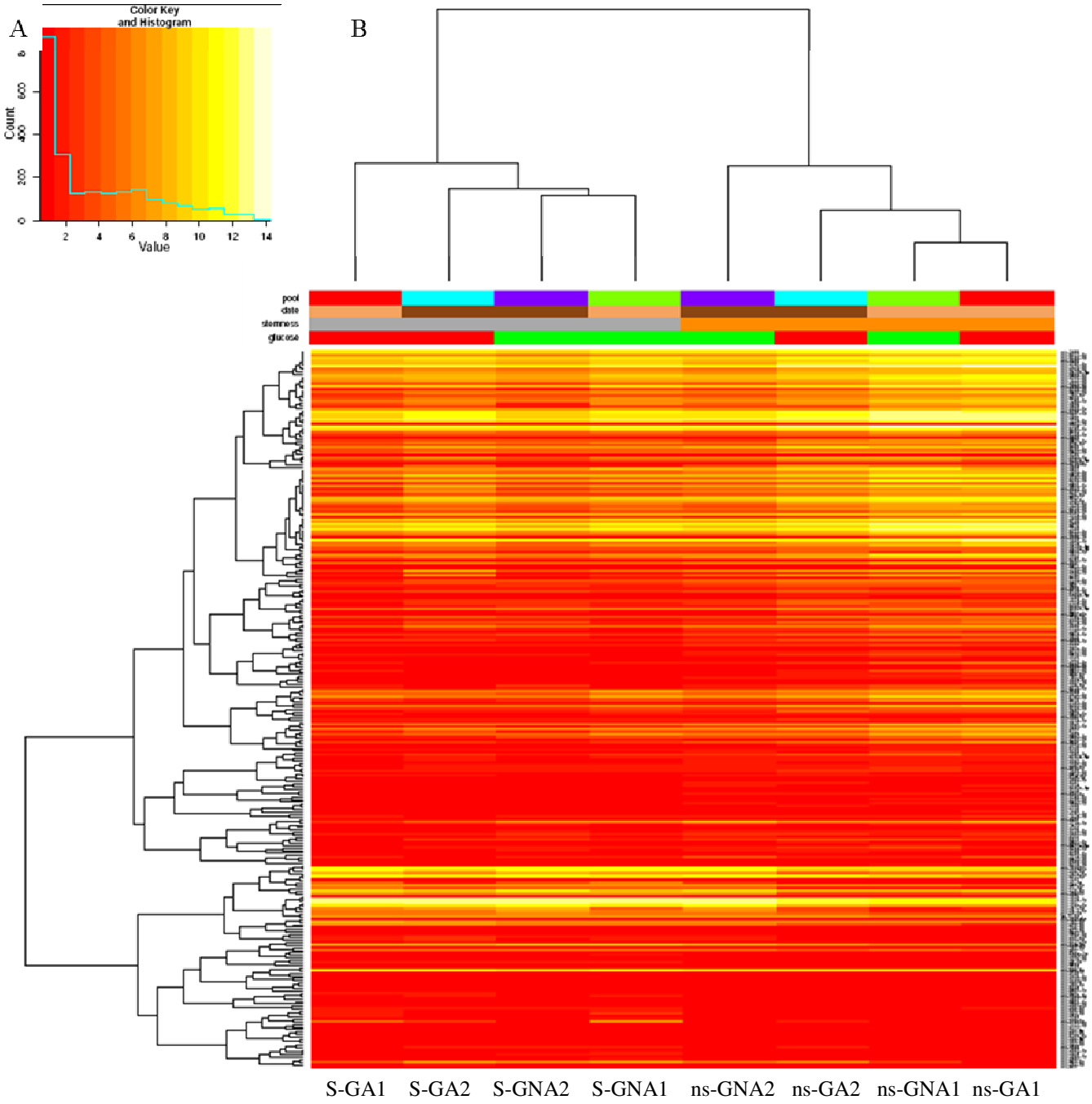

Figure 32. microRNA arrays of ovarian cancer stem cells (CSC) and non-CSC of GA and GNA samples. ***(A)*** *Color key to evaluate the up- or down- regulation.* ***(B)*** *Heatmap of microRNA expression measured on the 8 following samples: 2 samples of ovarian FACS-sorted CD45$^-$/CD44$^+$/CD117$^+$ CSC from GA patients (S-GA); 2 samples of ovarian FACS-sorted CD45$^-$/CD44$^+$/CD117$^-$ non-CSC from GA patients (nS-GA); 2 samples of ovarian FACS-sorted CD45$^-$/CD44$^+$/CD117$^+$ CSC from GNA patients (S-GNA); 2 samples of ovarian FACS-sorted CD45$^-$/CD44$^+$/CD117$^-$ non-CSC from GNA patients (nS-GNA). Each samples is a pool derived from 5 different patients, and Spearman's Rank Correlation was performed on data to statistical analyses.*

	miRNA	logFC	p-value
Down-regulated miRNA	**hsa-mir-324-5p**	**-1,7153**	**0,00259**
	hsa-mir-5694	-0,393	0,00087
	hsa-mir-98-5p	-0,3827	0,00265
	hsa-mir-449a	-0,3506	0,00458
	hsa-mir-561-5p	-0,3119	0,00595
	hsa-mir-3914	-0,2996	0,00696
	hsa-mir-873-3p	-0,2867	0,00499
	hsa-mir-644a	-0,2622	0,00532
	hsa-mir-3127-3p	-0,2346	0,00968
Up-regulated miRNA	hsa-mir-6780b-3p	0,33918	0,00848
	hsa-mir-1470	0,36857	0,00942
	hsa-mir-3131	0,48903	0,0079
	hsa-mir-6815-5p	0,59697	0,00793
	hsa-mir-4726-5p	0,63259	0,00849
	hsa-mir-3714	0,63563	0,00448
	hsa-mir-6508-5p	0,65962	0,00853
	hsa-mir-187-5p	0,67443	0,00765
	hsa-mir-6890-5p	0,67899	0,00859
	hsa-mir-4697-5p	0,69543	0,00361
	hsa-mir-6782-5p	0,8662	0,00844
	hsa-mir-3181	0,95563	0,00358
	hsa-mir-6721-5p	1,08324	0,00594
	hsa-mir-939-5p	1,20513	0,00704
	hsa-mir-4299	1,23076	0,00097
	hsa-mir-665	1,23961	0,00124
	hsa-mir-7109-5p	1,5326	0,00816
	hsa-mir-4449	1,54318	0,00571
	hsa-mir-4688	1,56239	0,00693
	hsa-mir-6861-5p	1,59545	0,00359
	hsa-mir-6787-5p	1,59791	0,00943
	hsa-mir-4467	1,6406	0,00501
	hsa-mir-6126	1,66975	0,00424
	hsa-mir-6722-3p	1,87817	0,00957
	hsa-mir-6824-5p	1,94519	0,00351
	hsa-mir-4656	2,01942	0,00967
	hsa-mir-4640-5p	2,03648	0,0026
	hsa-mir-6805-5p	2,11404	0,0079
	hsa-mir-3195	2,1904	0,00369
	hsa-mir-602	**2,2438**	**0,0043**
	hsa-mir-1909-3p	2,44495	0,00658
	hsa-mir-4721	2,55452	0,00916

Table 11. miRNA significantly deregulated in ovarian CSC compared to non-CSC. *LogFC is the relative differential expression index, whereas the p-value is the index for the statistical significance of the data. The table shows the list of the 9 miRNA down-regulated and the 32 miRNA up-regulated in CSC with a p-value lower than 0.01.*

3.2.2. mir-602 is overexpressed in ovarian CSC

To validate the different expression levels of the 4 selected miRNA, we evaluated by qRT-PCR their mRNA expression levels in CSC versus non-CSC isolated from 14 primary EOC samples. The results confirmed that mir-602 was significantly up-regulated in CSC while mir-31-3p was significantly down-regulated compared to the non-CSC counterpart. However, qRT-PCR did not confirm the miRNA array data obtained for mir-150-3p and mir-324-5p (Figure 33 A). To confirm the data obtained for mir-602 and mir-31-3p, we decided to evaluate their expression in cancer stem-like cells from patient derived xenografts (PDX) maintained in spheroid-forming conditions, or in PDX non-stem cells cultured in normal medium (5 samples for both conditions). Before the analysis of miRNA expression, we verified that cells maintained in spheroid-forming conditions were enriched in CSC by evaluating the percentage of $CD44^+/CD117^+$ cells (data not shown). Even in this case, a significant over-expression of mir-602 and down-regulation of mir-31-3p in ovarian cancer stem-like cells was evident (Figure 33 B).

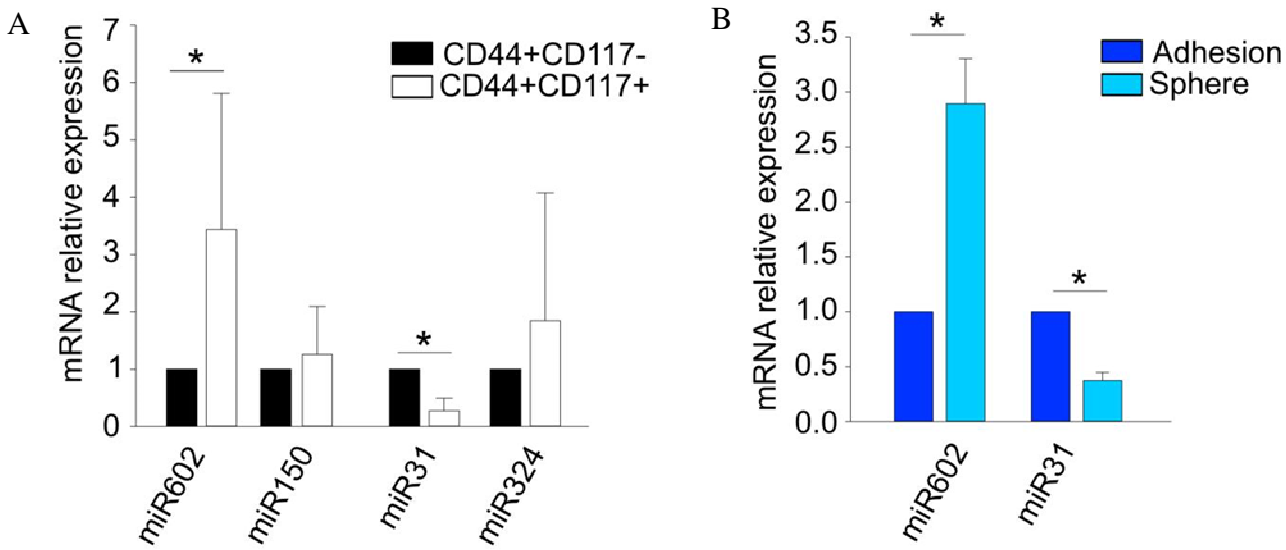

Figure 33. Relative expression level of the four miRNA selected from the miRNA arrays*. (**A**) mRNA relative expression level of the 4 miRNA chosen among the list emerging from miRNA arrays. The expression was measured on seven primary pooled samples of FACS-sorted $CD44^+/CD117^+$ cells and seven FACS-sorted $CD44^+/CD117^-$ cells, both derived from ascitic effusions of EOC-bearing women. Each pool includes five patients. (**B**) mRNA relative expression level of mir-31-3p and mir-602. The expression was measured on ovarian patients derived xenografts (PDX): five samples of spheroid-forming cells and five samples of adherent cells.*

3.2.3. Casein Kinase 1 Delta, target of mir-602, is down-regulated in ovarian CSC

Once validated by qRT-PCR the differential expression of mir-602 and mir-31-3p between CSC and non-CSC, we *in silico* analyzed the validated targets of these two miRNA. The analysis reported a long list of targets for mir-31-3p (data not shown), whereas only a few genes were identified for mir-602. In particular, only 5 genes were experimentally validated for mir-602: PPFIA3; CSNK1D; MAPKAPK5; SOX4; NDUFS3. Among these, we chose to focus our attention on Casein Kinase 1 Delta (CSNK1D), since its involvement in cell proliferation and in breast cancer cell growth has already been reported [211]. The predicted mir-602/CSNK1D binding sites are depicted on Figure 34.

ID	Duplex structure	Position
1	miRNA 3' cccGGCGUCG-----ACA-GCGGGCACAg 5' Target 5' agtCCGTGGTGTGTGTGTGTGCCCGTGTg 3'	704 - 732
2	miRNA 3' cccggCGUCGACA-GCGGGCACAg 5' Target 5' gcgagGCAGCTCTGCGCCCGGGTt 3'	1960 - 1983
3	miRNA 3' ccCGG--CGUCGA-------CAGC-GGGCACag 5' Target 5' ttGCCCAGTATCTAGTCCCCGTCGCCCCGTGca 3'	2033 - 2065

Figure 34. mir-602/CSNK1D binding sites*. The figure shows the mir-602/CSNK1D interactions predicted by miRanda and reported by miRTarBase, that is the experimentally validated microRNA/target interaction database.*

Firstly, we tested by qRT-PCR the mRNA expression levels of CSNK1D in CSC and non-CSC isolated from 6 primary pooled EOC samples (Figure 35; left bars), as well as in spheroid-forming cells samples compared to adherent cells obtained from 3 PDX samples (Figure 35; right bars).

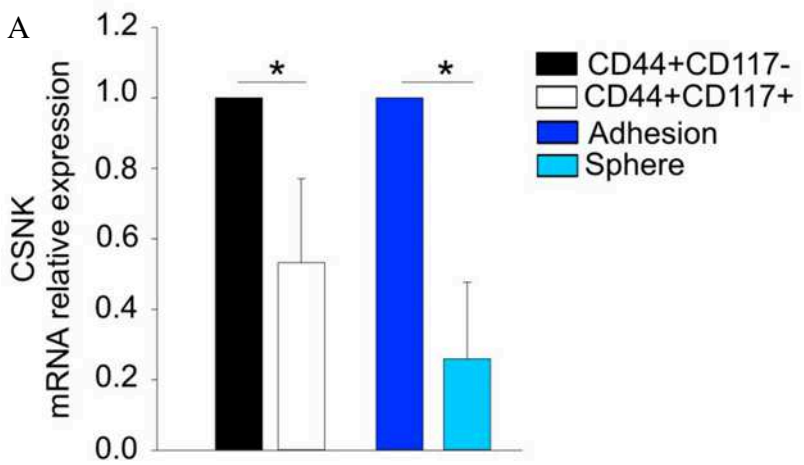

Figure 35. mRNA expression levels of CSNK1D in ovarian CSC versus non-stem cells. *(Right bars) The mRNA expression level of CSNK1D was measured on six pooled primary samples of FACS-sorted CD44$^+$/CD117$^+$ cells and CD44$^+$/CD117$^-$ cells derived from EOC-bearing women. Each pool includes five patients. (Left bars) The kinase mRNA expression level was measured on five samples of spheroid-forming cells and five of adherent cells derived from ovarian PDX.*

The results showed that the mRNA level of CSNK1D was significantly down-regulated in CSC, as well as in spheroid-forming cells of all samples, according to up-regulation of mir-602. To validate these data at protein level, we performed Western blot analysis for CSNK1D in three PDX samples maintained in spheroid-forming conditions with their controls maintained in normal culture conditions; results showed that CSNK1D was significantly down-regulated in CSC compared to the non-stem counterpart also at protein level (Figure 36). In the same PDX samples, we also evaluated

the protein expression level of NUMB, a RNA-binding protein which acts as a stemness-associated gene repressor. As expected, this protein was expressed at lower levels in spheroid-forming cells, compared to the adherent counterpart, thus showing the same trend of CSNK1D (Figure 36).

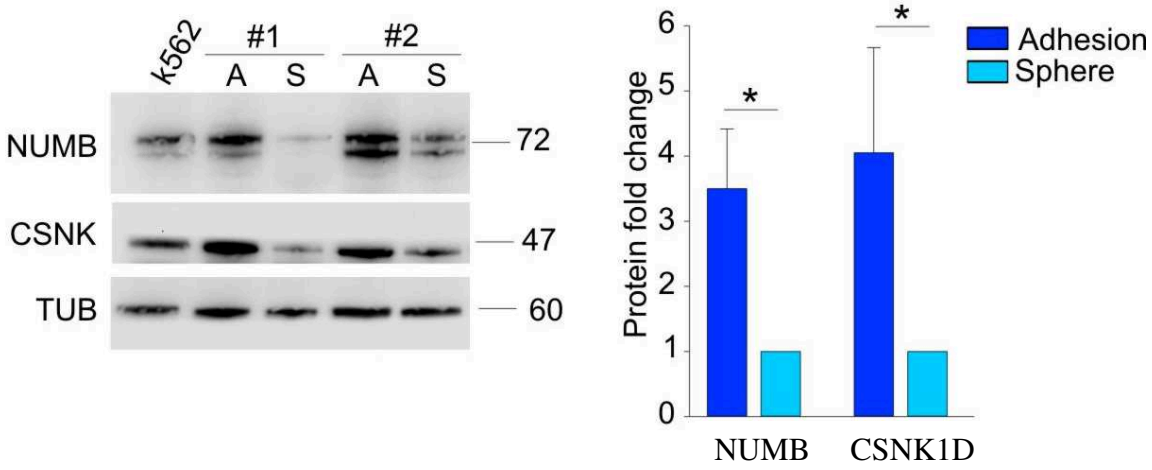

Figure 36. Expression levels of CSNK1D and NUMB in ovarian CSC compared to non-stem cells. *The protein expression level of CSNK1D and NUMB were evaluated by Western blot in five samples of spheroid-forming cells and their adherent counterpart derived from ovarian PDX. On the right, the mean of three experiments is reported. On the left, a representative blot is shown.*

3.2.4. The *in vitro* inhibition of mir-602 is associated with down-regulation of stemness genes

To further investigate the role of mir-602 in ovarian CSC biology, we decided to inhibit this microRNA in ovarian cancer cells. To this end, we treated *in vitro* five samples of PDX cells with small single-stranded RNA inhibitors (antagomiR) designed to specifically bind to and transiently inhibit mir-602, used at a concentration of 50 nM. As a control, we treated the same cells with a non-specific inhibitor, as specified in the Materials and Methods. After 24, 48 and 72 hours from the treatment, we verified the antagomiR-induced down-regulation of mir-602. Only at the time points of 48h and 72h a significant down-regulation of the mir-602 was evident (Figure 37). At these time points, we evaluated the effect of the mir-602 inhibition on the expression of the major stemness-associated genes, such as ALDH, NANOG, SOX, OCT4, by qRT-PCR (Figure 38). Data obtained in five PDX samples showed that the inhibition of mir-602 was associated with the down-regulation of all the analyzed stemness genes. This preliminary result suggests that mir-602 could be involved in stemness maintenance. However, since miRNA exhibit their function through the down-modulation of their target genes, the up-regulation of mir-602 in CSC compartment, where the stemness genes are more expressed than in the non-stem counterpart, implies that mir-602 influences stemness pathways through an indirect regulatory mechanism. This observation is supported by the lack of sequence complementarity between the 3'-UTR mRNA of the evaluated genes and the seed region of mir-602 (miRWalk database of predicted targets). Thus, we speculate that mir-602 could modulate stemness pathways through the effect of its target CSNK1D. To elucidate this issue, we firstly intend to evaluate the modulatory effect of mir-602 inhibition on CSNK1D, by measuring the expression level of this kinase after the antagomiR treatment. Then, we plan to stably down- or over-express both mir-602 and CSNK1D, in order to investigate the modulation of cell stemness features, such as spheroid formation, multidrug resistance and high

tumorigenic potential, mediated by either miRNA or kinase. All the experiments will be performed on PDX samples, where cell transfection and transduction are possible.

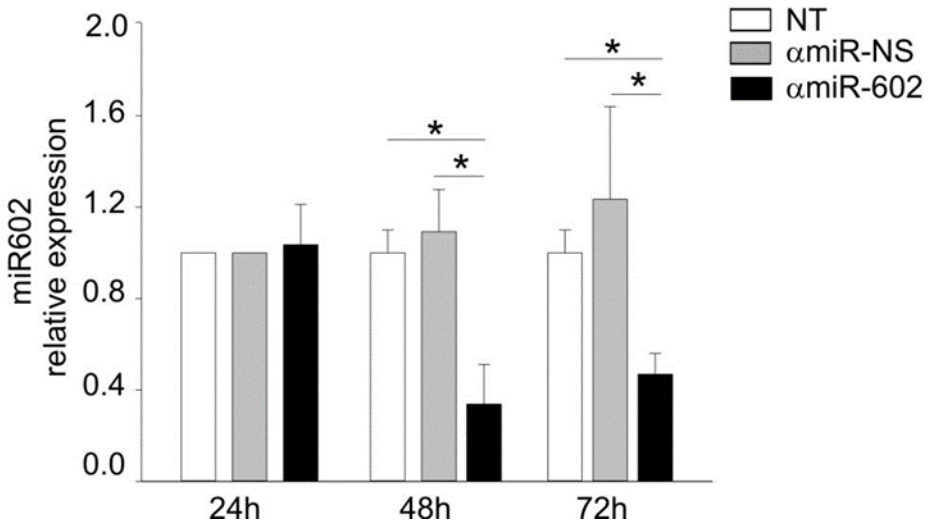

Figure 37. mir-602 antagomiR reduces the miRNA expression level. *Five samples of PDX cells was treated with 50 nM of non-specific miRNA inhibitor (αmiR-NS), or with the same concentration of specific mir-602 antagomiR (αmiR-602). As a further control, the cells were also plated in the medium used for the cell transfection, but without any treatments (NT). mir-602 relative expression level in PDX was evaluated by qRT-PCR after 24, 48 and 72 hours from the treatments. P-value<0.05.*

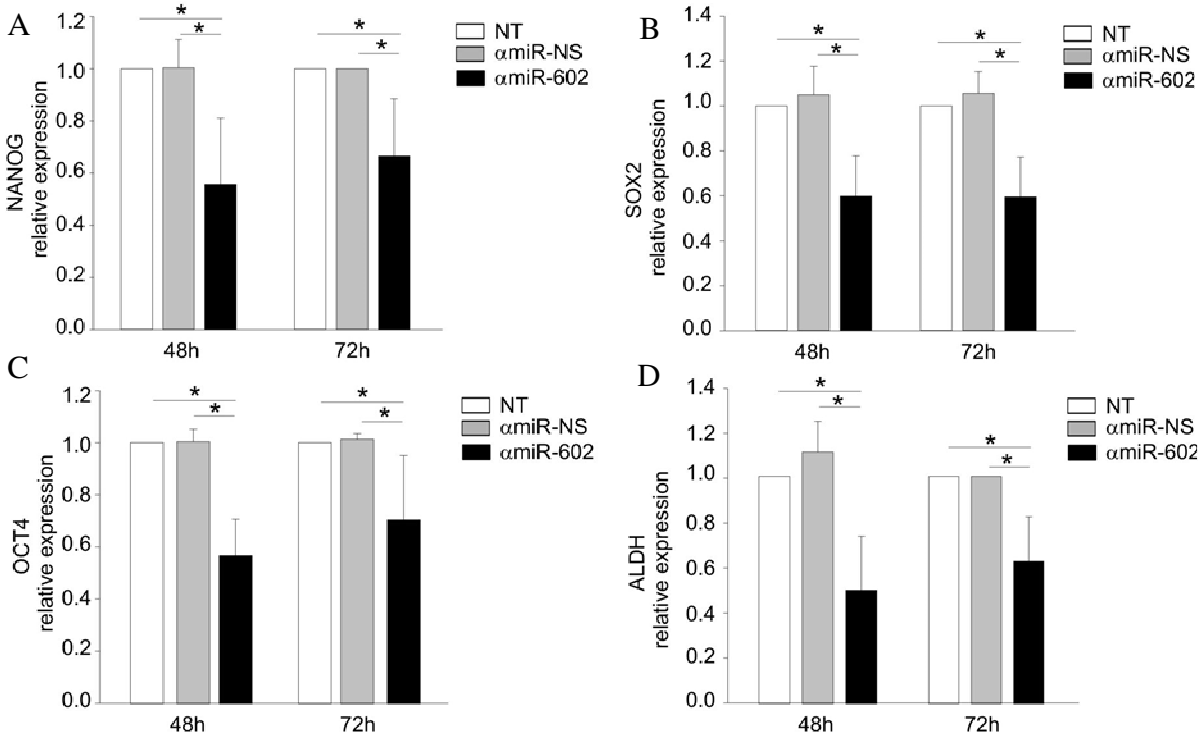

Figure 38. mir-602 antagomiR treatment affects the expression of stemness-associated genes*. mRNA expression levels of stemness genes was tested on five samples of ovarian xenograft cells treated for 48h with a non-specific miRNA inhibitor (anti-mir negative ctrl) and an inhibitor designed to specifically bind mir-602 (anti-mir602). As a further control, the cells were also plated in the medium used for the cell transfection, but without any treatments (NT).*

4. DISCUSSION

Epithelial Ovarian Cancer (EOC) is the most lethal gynecological malignancy, and it represent the fifth cause of death for cancer in women [2]. Although progresses have been made in EOC treatments by improved cyto-reductive surgery and platinum-based therapeutic regimens, the 5-years survival rate of advanced-stages EOC remain below 30% [3,212]. This poor prognosis relies on the one hand on the late diagnosis and on the other on the chemotherapy resistance occurring only a few months after the completion of first-line treatment.

While the late diagnosis is clearly due to the lack of specific symptoms that leads patients to neglect the early-phase disease, the reasons for drug resistance and tumor recurrence remain unclear. In this context, recent evidence suggests that EOC, akin most tumors [14], contains a tiny population of cells, termed Cancer Stem Cells (CSC), which are regarded as responsible for chemotherapy resistance. Indeed, as demonstrated by Zhang and colleagues, this small cell fraction (1-2%) is endowed with the entire spectrum of stemness properties, such as spheroid-formation ability, over-expression of the most common stemness-associated genes, ability to generate tumors at high efficiency when injected into immunodeficient mice, and capability to differentiate into many cell tissue types. The CSC isolated by Zhang were characterized by the co-expression on their surface of two markers: CD44 (hyaluronic acid receptor), and CD117 (c-kit), that is the stem cell factor (SCF) receptor [29] . More recently, our research group confirmed the data about the stemness properties of $CD44^+/CD117^+$ cells. Moreover, in this study we demonstrated that ovarian $CD44^+/CD117^+$ CSC present a different metabolic profile compared to non-stem counterpart. More in detail, ovarian CSC were found to privilege oxidative phosphorylation rather than aerobic glycolysis (Warburg effect), unlike the non-stem counterpart, which relies on aerobic glycolysis for their energy and metabolites supply. This peculiar CSC behavior could explain the lower glucose addiction of CSC compared to non-stem cells, which was observed in our experiments [30]. In this setting, independently of the presence of the CSC fraction, we also noticed that not all the analyzed samples behaved in the same way in terms of cell dependence on glucose; thus, we wondered whether glucose addiction could define different EOC patient populations, and especially whether this feature could correlate to different patient responses to chemotherapy. Indeed, EOC patients are divided into two main groups based on response to platinum (PLT) regimens: patients presenting a complete response, without recurrence within six months after the completion of treatment, are regarded as sensitive to chemotherapy; patients who progress very rapidly despite the platinum administration, with tumor relapse within the six-months following treatment period, are defined as resistant to chemotherapy. Since the drug resistance onset is the main hurdle to improve EOC patient outcome, and cancer cell metabolism is regarded as a promising field for developing new anti-tumor strategies, the first aim of our project was to investigate the possibility to distinguish different glucose metabolic profiles in samples from EOC patients, and eventually to exploit these metabolic differences as a predictor of clinical chemotherapy response.

The second aim of the project was to identify the microRNA (miRNA) possibly involved in the different glucose metabolic profile of individual EOC samples. Moreover, in view of the distinctive metabolic profile of CSC compared to their non-stem counterpart, we wondered whether the molecular trait leading to the metabolic differences among EOC samples could be related to the entire tumor mass or rather to a specific stem compartment. In this context, the study of miRNA, that are small non-coding RNA molecules able to repress the expression of specific genes, may be

very promising. The emerging importance of these oligonucleotides in the molecular characterization of cancer cells is related to their potential in regulating several genes and related pathways, unlike genes that usually control only one molecular signaling [62,63]. Moreover, several miRNA have been reported as deregulated in EOC compared to ovarian normal tissues [146-150,213], as well as in ovarian CSC compared to the non-stem counterpart [42,43]. For these reasons, we explored miRNA signatures in order to explain the above previous results.

Thus, this project is divided into two major parts:

- The study of the glucose metabolic profile of EOC patient-derived samples and its correlation with the clinical response to chemotherapy
- The analysis of the miRNA signatures of EOC patient-derived samples, in terms of miRNA profile of both CSC and non-stem cells

Concerning the first part, although platinum resistance is a well-established phenomenon and several mechanisms of resistance have been advanced [11,211], whether the metabolic properties of tumor cells influence response to or are modulated by cytotoxic drugs is unknown. We investigated these issues in primary samples from ovarian cancer patients and in a xenotransplant model generated by the injection of EOC patient-derived cells into immunodeficient mice. To this aim, we cultured the cells in the absence of glucose for 14 days and, based on the median value of cell viability (13%), we identified two categories of EOC patients. In particular, according to *in vitro* sensitivity of tumor cells to glucose starvation, samples could be discriminated into glucose addicted (GA) and glucose non-addicted (GNA). Interestingly, we found that GA and GNA sample groups matched with patient clinical classification as PLT-sensitive and PLT-resistant, respectively. Moreover, our data showed a more pronounced Warburg-like profile in GA samples, in terms of higher levels of the glucose (GLUT1) and the lactate (MCT4) transporter, higher medium acidification rate and higher expression of glycolysis-associated genes, compared to GNA samples. Therefore, our results clearly identify two distinct metabolic profiles within EOC patients. According to the correlation of GA/GNA phenotype with patient response to platinum treatment, GA cells presented higher *in vitro* and *in vivo* sensitivity to PLT, which could be in part explained by a more active glycolytic machinery as well as a higher proliferation rate, given the well-established connections between proliferation and Warburg effect [214]. Nonetheless, in this context the contribution to PLT sensitivity of lower MDR pump expression in GA patients (Figure 26 A) must not to be overlooked.

These results are partially at odds with previous findings obtained by metabolic characterization of tumor cells selected for platinum-resistance *in vitro*. Indeed, while Jin *et al*. reported that platinum-resistance was associated with down-modulation of glycolysis in ovarian cancer cell lines [215], Catanzaro *et al*. showed that cisplatin-resistant ovarian cancer cells were characterized by reduced mitochondrial activity and higher glucose-dependency, when compared to the cisplatin-sensitive counterpart. These features were associated with increased glucose-uptake and consumption as well as increased expression and enzymatic activity of the pentose phosphate pathway enzyme glucose-6-phosphate dehydrogenase [216]. These contrasting results may depend on the marked differences in the experimental models used in these studies. Our findings have been obtained by analyzing metabolic traits of tumors treated *in vivo* - either in the patients or in xenotranplants - with platinum, whereas Catanzaro *et al*. investigated the adaptive metabolic changes of tumor cells exposed *in*

vitro to this drug. This difference could be important, in light of recent publications remarking the impact of the environment on the metabolic phenotype of tumor cells [217]. Along this line, Matassa *et al*. recently observed that oxidative metabolism drives inflammation-induced platinum resistance in ovarian cancer [218]. These results, together with ours, challenge the current assumption that increased glycolysis correlates with poor prognosis, at least in ovarian cancer.

A possible explanation for the different metabolic profiles of EOC samples is that GNA samples could exploit autophagy pathways to support their energy needs, since their glucose utilization is apparently more dispensable. This hypothesis is based on the evidence of aberrant autophagic activity as an emerging hallmark of cancer [219]; indeed, this catabolic pathway enables cells to degrade intra-cellular organelles in order to recycle their metabolites, thus representing a mechanism for tumor cells to survive the energetic stress of tumor microenvironment [220]. Moreover, it has been recently reported that autophagy is activated in response to cytotoxic drugs, and could mediate the acquisition of a chemo-resistant phenotype in some tumor cells [221,222]. Thus, based on these premises, autophagy will be the focus of our further investigations. In this regard, we firstly intend to evaluate the basal autophagic activity and the response to autophagy inhibitor of both GA and GNA samples [223]. According to our speculations, we would expect to find a more active basal autophagy, as well as a higher sensitivity to autophagy inhibition, in GNA samples compared to GA. The figure 39 depicts a schematic view of our hypothesis about the autophagy exploitation by the different categories of EOC samples.

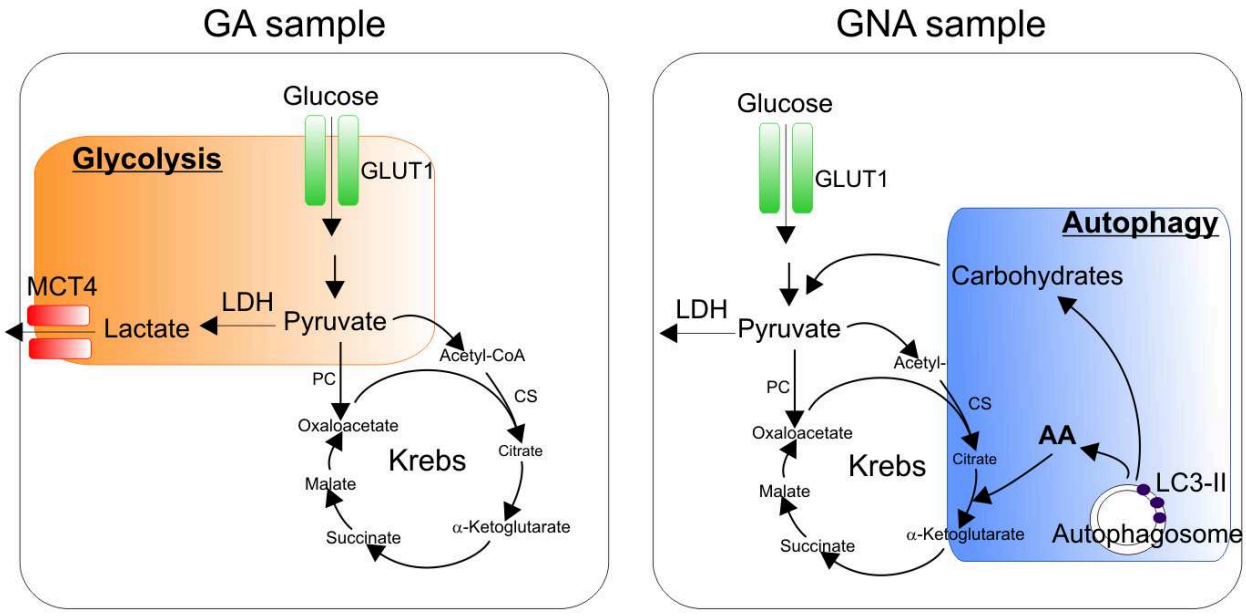

Figure 39. Schematic representation of metabolic pathways in EOC patients*. GA patients mostly rely on the Warburg effect with high levels of glucose utilization through glycolysis (orange box). GNA samples, instead, are less dependent on glycolysis, thus implying the utilization of alternative energetic/catabolic pathway such as autophagy (blue box), which degrades damaged cellular organelles in order to recycle their components and produce metabolic intermediates such as carbohydrates or amino acids (AA) that fuel the Krebs cycle.*

An intriguing finding of our work is that certain metabolic features of tumor cells could be modulated by carboplatin. Although we could not perform metabolic characterization of EOC samples at diagnosis in a large number of patients, repeated sampling from a same patient after

PLT-treatment revealed a switch from GA to GNA status after platinum treatment in about 1/4 of the cases analyzed (data not shown); in contrast, we never observed a conversion from the GNA to the GA phenotype. The surprising parallelism between PLT resistance and resistance to glucose starvation was also confirmed in a xenotransplantation model, where we demonstrated that acquisition of a PLT-resistant phenotype was accompanied by a dramatic change in the expression of the glucose metabolism-associated machinery (Figure 30). In support of this finding, PLT resistance onset was always associated with the achievement of *in vitro* glucose starvation resistance. Currently, it is not clear whether PLT-treatment could in some instances modulate the metabolic profile of tumor cells or rather select a naïve population of less glycolytic cells (GNA phenotype). In the latter scenario, GNA samples would contain a more prominent population of PLT-resistant and less glycolytic cells compared to GA samples, in which the GNA population could still be present but as a minority. Further studies are warranted to explore more in depth the mechanistic aspects of this issue.

Finally, we observed that the *in vitro* glucose addiction of primary tumor cells could anticipate the response to first-line PLT-based schedules of the patients from which they were isolated. In fact, the different PLT sensitivity of GA and GNA patients was confirmed *in vivo* in a limited cohort of 23 EOC bearing patients whose samples were collected before any chemotherapic treatment. As shown by Kaplan-Meier analysis (Figure 31 B), patients categorized at diagnosis as GA according to their glucose addiction and prospectively followed for over 18 months, were defined as PLT-sensitive according to the FIGO definition [224], and showed significantly higher median PFS compared to GNA patients.

In conclusion, the first part of our study demonstrates that EOC samples can be stratified according to their glucose addiction. In particular, by analyzing tumor cell sensitivity to *in vitro* glucose deprivation we can identify glucose addicted (GA) samples and glucose non-addicted (GNA) ones. Importantly, the GA/GNA status correlated with response to PLT treatment both *in vitro* and *in vivo* and could be affected by prolonged PLT therapy. Even though we are aware of the limited dimension of our patient cohort, our observations suggest that *in vitro* glucose addiction of EOC cells could represent a novel reliable parameter to predict clinical sensitivity to PLT.

As previously mentioned, miRNA, as well as cell metabolism, is one of the most promising fields in cancer research [63]. Moreover, miRNA involvement into cell metabolism has already emerged in several normal and tumor tissues [209,210]. Thereby, miRNA investigation to explain the differences in EOC glucose metabolism has become the second part of our project. To this aim, we firstly attempted to identify the miRNA differentially expressed by GA samples compared to GNA, in both CSC and non-stem cells; indeed, we were also interested in understanding whether the putative miRNA associated to the GA/GNA phenotypes were deregulated in CSC or in the non-stem counterpart as well. Thus, we performed miRNA arrays on the $CD44^+/CD117^+$ CSC and the $CD44^+/CD117^-$ non-stem fraction, *ex vivo* isolated from both GA and GNA patients. However, miRNA signatures did not reveal any miRNA significantly deregulated in GA versus GNA samples, in both CSC and non-stem cells. On the other hand, several miRNA differentially modulated in CSC compared to non-CSC emerged from miRNA expression analysis. We focused our attention on mir-602, whose target casein kinase 1 delta (CSNK1D) has been reported to be involved in breast cancer progression. According to mir-602 up-regulation in cancer stem fraction, the same cells showed the down-regulation of CSNK1D. Similar results were also observed in CSC

and non-CSC isolated from patient-derived xenografts (PDX) obtained by the intraperitoneal injection of human EOC cells into immunodeficient mice. Intriguingly, we observed that the transient inhibition of mir-602 in PDX samples resulted in a decreased expression of stemness-associated genes, such as NANOG, SOX2 and OCT4 (Figure 38). This finding suggests that mir-602 could regulate the stemness properties maintenance. However, since miRNA usually inhibit their targets, the finding that mir-602 is up-regulated in CSC, where NANOG, SOX2 and OCT4 are over-expressed, implies that mir-602 probably does not directly bind to such genes. This evidence is supported by the fact that the 3'-UTR mRNA of these genes does not contain any sequence complementary to the seed region of mir-602. Thus, it is reasonable to hypothesize that mir-602 could indirectly modulate the stemness-associated pathways, probably through a CSNK1D-mediated signaling. Indeed, this kinase has been reported to be involved in cell proliferation and tumor progression, partially because of its activating effect on Wnt/β-catenin signaling, which in turn results in the aberrant growth occurring in carcinogenesis process [211]. These features are usually related to the tumor non-stem bulk, thus speculating that CSNK1D down-regulation could favor stemness properties seems not far-fetched. Therefore, in other terms, we advance that cancer cell stemness pathways could be inhibited by Casein Kinase 1 Delta, whose translation is in turn repressed by mir-602; this implies that the inhibition of mir-602, or the over-expression on CSNK1D, could induce the differentiation of CSC and consequently make these cells more sensitive to chemotherapy regimens (Figure 40). Since it has already been reported that CSC are responsible for tumor recurrence in view of their multidrug resistance [14], targeting mir-602/CSNK1D axis could represent a novel strategy to improve EOC therapeutic treatments.

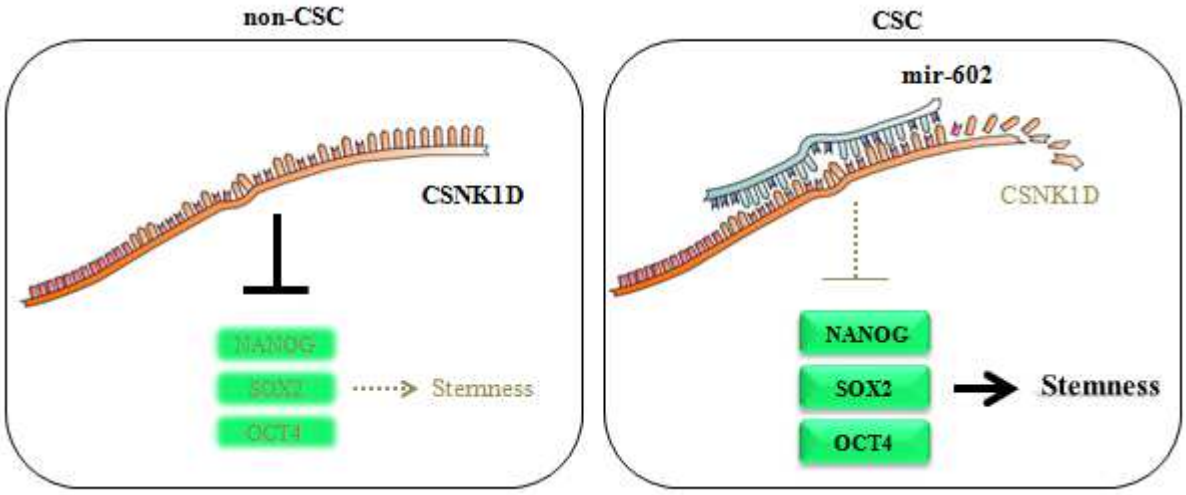

Figure 40. Schematic representation of the supposed stemness regulatory mechanism mediated by mir-602/CSNK1D axis. *In EOC non-stem bulk Casein Kinase 1 Delta (CSNK1D) exhibits its inhibitory function on stemness pathways (left panel). In ovarian CSC, instead, the over-expression of mir-602 leads to the degradation of CSNK1D and consequently to an enhanced expression of the master stemness regulator genes (right panel).*

This speculation could seem partially inconsistent with the findings of Rosenberg and collaborators, who, in addition to their above mentioned results, demonstrated that the inhibition of CSNK1D exhibits potent anti-tumor effects in breast cancer cells [211]. However, these authors did not evaluate the CSC fraction; thus, it is possible that the repression of CSNK1D expression, as well as the currently used anti-cancer strategies, could induce cell death in the malignant non-stem bulk

resulting in rapid tumor regression, but probably selects CSC compartment leading to the relapse of the disease.

Based on our results, we regard mir-602/CSNK1D interaction as a possible regulatory mechanism of cell stemness in EOC. To investigate this issue, we intend to down- or up-modulate both mir-602 and CSNK1D in PDX cells, in order to evaluate the effects of these manipulations on cancer stemness features, such as spheroid formation, multidrug resistance and *in vivo* tumorigenic potential. These approaches could clarify the mechanisms behind the mir-602/CSNK1D stemness regulatory axis.

The generation of EOC patient-derived xenografts implies the use of immunodeficient mice to avoid the rejection of human cells from the host immune response. Therefore, this preclinical model does not allow the evaluation of the immune system influence on the tumor growth and on the CSC phenotype. Indeed, it has already been demonstrated that the crosstalk between tumor cells, CSC and immune-infiltrating cells could regulate several stemness-associated programs [225,226], and innumerable mechanisms are exploited by malignant cells and by CSC to escape the immune system recognition [227]. However, the use of a preclinical model is mandatory to perform *in vivo* experiments, such as tumorigenic potential evaluation, as well as several advanced techniques, such as gene silencing. Moreover, to evaluate the effects of our experimental manipulations (for example miRNA and gene silencing), we always compared the characteristics of the manipulated PDX with those of untreated xenografts (control group), so bypassing the bias due to the immunodeficiency of the animals. Thus, the compromise related to the use of an immunodeficient model appears reasonable, and we have confidence in the solidity of the data achievable using PDX.

5. REFERENCES

1. Siegel R, Ma J, Zou Z, Jemal A (2014) Cancer statistics, 2014. CA Cancer J Clin 64: 9-29.
2. Kipps E, Tan DS, Kaye SB (2013) Meeting the challenge of ascites in ovarian cancer: new avenues for therapy and research. Nat Rev Cancer 13: 273-282.
3. Parkin DM (2004) International variation. Oncogene 23: 6329-6340.
4. Goff BA, Mandel L, Muntz HG, Melancon CH (2000) Ovarian carcinoma diagnosis. Cancer 89: 2068-2075.
5. Bast RC, Jr., Hennessy B, Mills GB (2009) The biology of ovarian cancer: new opportunities for translation. Nat Rev Cancer 9: 415-428.
6. Prat J (2012) New insights into ovarian cancer pathology. Ann Oncol 23 Suppl 10: x111-117.
7. Kurman RJ, Shih Ie M (2010) The origin and pathogenesis of epithelial ovarian cancer: a proposed unifying theory. Am J Surg Pathol 34: 433-443.
8. Prat J, Oncology FCoG (2014) Staging classification for cancer of the ovary, fallopian tube, and peritoneum. Int J Gynaecol Obstet 124: 1-5.
9. Puiffe ML, Le Page C, Filali-Mouhim A, Zietarska M, Ouellet V, et al. (2007) Characterization of ovarian cancer ascites on cell invasion, proliferation, spheroid formation, and gene expression in an in vitro model of epithelial ovarian cancer. Neoplasia 9: 820-829.
10. Kim A, Ueda Y, Naka T, Enomoto T (2012) Therapeutic strategies in epithelial ovarian cancer. J Exp Clin Cancer Res 31: 14.
11. Abdullah LN, Chow EK (2013) Mechanisms of chemoresistance in cancer stem cells. Clin Transl Med 2: 3.
12. Tabar V, Studer L (2014) Pluripotent stem cells in regenerative medicine: challenges and recent progress. Nat Rev Genet 15: 82-92.
13. Reya T, Morrison SJ, Clarke MF, Weissman IL (2001) Stem cells, cancer, and cancer stem cells. Nature 414: 105-111.
14. Clevers H (2011) The cancer stem cell: premises, promises and challenges. Nat Med 17: 313-319.
15. Liu HG, Chen C, Yang H, Pan YF, Zhang XH (2011) Cancer stem cell subsets and their relationships. J Transl Med 9: 50.
16. Bonnet D, Dick JE (1997) Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med 3: 730-737.
17. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF (2003) Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A 100: 3983-3988.
18. Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, et al. (2004) Identification of human brain tumour initiating cells. Nature 432: 396-401.
19. Collins CA, Olsen I, Zammit PS, Heslop L, Petrie A, et al. (2005) Stem cell function, self-renewal, and behavioral heterogeneity of cells from the adult muscle satellite cell niche. Cell 122: 289-301.
20. Bapat SA, Mali AM, Koppikar CB, Kurrey NK (2005) Stem and progenitor-like cells contribute to the aggressive behavior of human epithelial ovarian cancer. Cancer Res 65: 3025-3029.
21. Chandler JM, Lagasse E (2010) Cancerous stem cells: deviant stem cells with cancer-causing misbehavior. Stem Cell Res Ther 1: 13.
22. Bjerkvig R, Tysnes BB, Aboody KS, Najbauer J, Terzis AJ (2005) Opinion: the origin of the cancer stem cell: current controversies and new insights. Nat Rev Cancer 5: 899-904.
23. Tabarestani S, Ghafouri-Fard S (2012) Cancer stem cells and response to therapy. Asian Pac J Cancer Prev 13: 5951-5958.
24. Kleffel S, Schatton T (2013) Tumor dormancy and cancer stem cells: two sides of the same coin? Adv Exp Med Biol 734: 145-179.

25. Diehn M, Majeti R (2010) Metastatic cancer stem cells: an opportunity for improving cancer treatment? Cell Stem Cell 6: 502-503.
26. Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, et al. (2008) The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell 133: 704-715.
27. Liao WT, Ye YP, Deng YJ, Bian XW, Ding YQ (2014) Metastatic cancer stem cells: from the concept to therapeutics. Am J Stem Cells 3: 46-62.
28. Tilly JL, Rueda BR (2008) Minireview: stem cell contribution to ovarian development, function, and disease. Endocrinology 149: 4307-4311.
29. Zhang S, Balch C, Chan MW, Lai HC, Matei D, et al. (2008) Identification and characterization of ovarian cancer-initiating cells from primary human tumors. Cancer Res 68: 4311-4320.
30. Pilotto G, Pasto A, Bellio C, Ciminale V, Silic-Benussi M, et al. (2014) Cancer stem cells from epithelial ovarian cancer patients privilege oxidative phosphorylation, and resist glucose deprivation. Oncotarget 5: 4305-4319.
31. Burgos-Ojeda D, Rueda BR, Buckanovich RJ (2012) Ovarian cancer stem cell markers: prognostic and therapeutic implications. Cancer Lett 322: 1-7.
32. Baba T, Convery PA, Matsumura N, Whitaker RS, Kondoh E, et al. (2009) Epigenetic regulation of CD133 and tumorigenicity of CD133+ ovarian cancer cells. Oncogene 28: 209-218.
33. Curley MD, Therrien VA, Cummings CL, Sergent PA, Koulouris CR, et al. (2009) CD133 expression defines a tumor initiating cell population in primary human ovarian cancer. Stem Cells 27: 2875-2883.
34. Silva IA, Bai S, McLean K, Yang K, Griffith K, et al. (2011) Aldehyde dehydrogenase in combination with CD133 defines angiogenic ovarian cancer stem cells that portend poor patient survival. Cancer Res 71: 3991-4001.
35. Landen CN, Jr., Goodman B, Katre AA, Steg AD, Nick AM, et al. (2010) Targeting aldehyde dehydrogenase cancer stem cells in ovarian cancer. Mol Cancer Ther 9: 3186-3199.
36. Hu L, McArthur C, Jaffe RB (2010) Ovarian cancer stem-like side-population cells are tumourigenic and chemoresistant. Br J Cancer 102: 1276-1283.
37. Kobayashi Y, Seino K, Hosonuma S, Ohara T, Itamochi H, et al. (2011) Side population is increased in paclitaxel-resistant ovarian cancer cell lines regardless of resistance to cisplatin. Gynecol Oncol 121: 390-394.
38. Hosonuma S, Kobayashi Y, Kojo S, Wada H, Seino K, et al. (2011) Clinical significance of side population in ovarian cancer cells. Hum Cell 24: 9-12.
39. Cao L, Shao M, Schilder J, Guise T, Mohammad KS, et al. (2012) Tissue transglutaminase links TGF-beta, epithelial to mesenchymal transition and a stem cell phenotype in ovarian cancer. Oncogene 31: 2521-2534.
40. Motohara T, Masuko S, Ishimoto T, Yae T, Onishi N, et al. (2011) Transient depletion of p53 followed by transduction of c-Myc and K-Ras converts ovarian stem-like cells into tumor-initiating cells. Carcinogenesis 32: 1597-1606.
41. Luo L, Zeng J, Liang B, Zhao Z, Sun L, et al. (2011) Ovarian cancer cells with the CD117 phenotype are highly tumorigenic and are related to chemotherapy outcome. Exp Mol Pathol 91: 596-602.
42. Chen D, Zhang Y, Wang J, Chen J, Yang C, et al. (2013) MicroRNA-200c overexpression inhibits tumorigenicity and metastasis of CD117+CD44+ ovarian cancer stem cells by regulating epithelial-mesenchymal transition. J Ovarian Res 6: 50.
43. Cheng W, Liu T, Wan X, Gao Y, Wang H (2012) MicroRNA-199a targets CD44 to suppress the tumorigenicity and multidrug resistance of ovarian cancer-initiating cells. FEBS J 279: 2047-2059.
44. Kwon MJ, Shin YK (2013) Regulation of ovarian cancer stem cells or tumor-initiating cells. Int J Mol Sci 14: 6624-6648.

45. Tomao F, Papa A, Rossi L, Strudel M, Vici P, et al. (2013) Emerging role of cancer stem cells in the biology and treatment of ovarian cancer: basic knowledge and therapeutic possibilities for an innovative approach. J Exp Clin Cancer Res 32: 48.
46. Schilder RJ, Sill MW, Lee RB, Shaw TJ, Senterman MK, et al. (2008) Phase II evaluation of imatinib mesylate in the treatment of recurrent or persistent epithelial ovarian or primary peritoneal carcinoma: a Gynecologic Oncology Group Study. J Clin Oncol 26: 3418-3425.
47. Chen K, Huang YH, Chen JL (2013) Understanding and targeting cancer stem cells: therapeutic implications and challenges. Acta Pharmacol Sin 34: 732-740.
48. Iorio MV, Croce CM (2016) Commentary on microRNA Fingerprint in Human Epithelial Ovarian Cancer. Cancer Res 76: 6143-6145.
49. Whitworth JM, Londono-Joshi AI, Sellers JC, Oliver PJ, Muccio DD, et al. (2012) The impact of novel retinoids in combination with platinum chemotherapy on ovarian cancer stem cells. Gynecol Oncol 125: 226-230.
50. McAuliffe SM, Morgan SL, Wyant GA, Tran LT, Muto KW, et al. (2012) Targeting Notch, a key pathway for ovarian cancer stem cells, sensitizes tumors to platinum therapy. Proc Natl Acad Sci U S A 109: E2939-2948.
51. Borovski T, De Sousa EMF, Vermeulen L, Medema JP (2011) Cancer stem cell niche: the place to be. Cancer Res 71: 634-639.
52. Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144: 646-674.
53. Ambros V (2004) The functions of animal microRNAs. Nature 431: 350-355.
54. Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116: 281-297.
55. Bartel DP (2009) MicroRNAs: target recognition and regulatory functions. Cell 136: 215-233.
56. Fabian MR, Sonenberg N, Filipowicz W (2010) Regulation of mRNA translation and stability by microRNAs. Annu Rev Biochem 79: 351-379.
57. Friedman RC, Farh KK, Burge CB, Bartel DP (2009) Most mammalian mRNAs are conserved targets of microRNAs. Genome Res 19: 92-105.
58. Lewis BP, Burge CB, Bartel DP (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 120: 15-20.
59. Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB (2003) Prediction of mammalian microRNA targets. Cell 115: 787-798.
60. Ellwanger DC, Buttner FA, Mewes HW, Stumpflen V (2011) The sufficient minimal set of miRNA seed types. Bioinformatics 27: 1346-1350.
61. Krek A, Grun D, Poy MN, Wolf R, Rosenberg L, et al. (2005) Combinatorial microRNA target predictions. Nat Genet 37: 495-500.
62. Garzon R, Marcucci G, Croce CM (2010) Targeting microRNAs in cancer: rationale, strategies and challenges. Nat Rev Drug Discov 9: 775-789.
63. Lages E, Ipas H, Guttin A, Nesr H, Berger F, et al. (2012) MicroRNAs: molecular features and role in cancer. Front Biosci (Landmark Ed) 17: 2508-2540.
64. Rodriguez A, Griffiths-Jones S, Ashurst JL, Bradley A (2004) Identification of mammalian microRNA host genes and transcription units. Genome Res 14: 1902-1910.
65. Cai X, Hagedorn CH, Cullen BR (2004) Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. RNA 10: 1957-1966.
66. Weber MJ (2005) New human and mouse microRNA genes found by homology search. FEBS J 272: 59-73.
67. Kim YK, Kim VN (2007) Processing of intronic microRNAs. EMBO J 26: 775-783.
68. Baskerville S, Bartel DP (2005) Microarray profiling of microRNAs reveals frequent coexpression with neighboring miRNAs and host genes. RNA 11: 241-247.
69. Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T (2001) Identification of novel genes coding for small expressed RNAs. Science 294: 853-858.

70. Lau NC, Lim LP, Weinstein EG, Bartel DP (2001) An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. Science 294: 858-862.
71. Lee RC, Ambros V (2001) An extensive class of small RNAs in Caenorhabditis elegans. Science 294: 862-864.
72. Lee Y, Kim M, Han J, Yeom KH, Lee S, et al. (2004) MicroRNA genes are transcribed by RNA polymerase II. EMBO J 23: 4051-4060.
73. Altuvia Y, Landgraf P, Lithwick G, Elefant N, Pfeffer S, et al. (2005) Clustering and conservation patterns of human microRNAs. Nucleic Acids Res 33: 2697-2706.
74. Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, et al. (2005) MicroRNA expression profiles classify human cancers. Nature 435: 834-838.
75. Zhou X, Ruan J, Wang G, Zhang W (2007) Characterization and identification of microRNA core promoters in four model species. PLoS Comput Biol 3: e37.
76. Lee Y, Ahn C, Han J, Choi H, Kim J, et al. (2003) The nuclear RNase III Drosha initiates microRNA processing. Nature 425: 415-419.
77. Gregory RI, Chendrimada TP, Shiekhattar R (2006) MicroRNA biogenesis: isolation and characterization of the microprocessor complex. Methods Mol Biol 342: 33-47.
78. Han J, Lee Y, Yeom KH, Kim YK, Jin H, et al. (2004) The Drosha-DGCR8 complex in primary microRNA processing. Genes Dev 18: 3016-3027.
79. Han J, Lee Y, Yeom KH, Nam JW, Heo I, et al. (2006) Molecular basis for the recognition of primary microRNAs by the Drosha-DGCR8 complex. Cell 125: 887-901.
80. Berezikov E, Chung WJ, Willis J, Cuppen E, Lai EC (2007) Mammalian mirtron genes. Mol Cell 28: 328-336.
81. Murchison EP, Hannon GJ (2004) miRNAs on the move: miRNA biogenesis and the RNAi machinery. Curr Opin Cell Biol 16: 223-229.
82. Lund E, Dahlberg JE (2006) Substrate selectivity of exportin 5 and Dicer in the biogenesis of microRNAs. Cold Spring Harb Symp Quant Biol 71: 59-66.
83. Krol J, Sobczak K, Wilczynska U, Drath M, Jasinska A, et al. (2004) Structural features of microRNA (miRNA) precursors and their relevance to miRNA biogenesis and small interfering RNA/short hairpin RNA design. J Biol Chem 279: 42230-42239.
84. Khvorova A, Reynolds A, Jayasena SD (2003) Functional siRNAs and miRNAs exhibit strand bias. Cell 115: 209-216.
85. Schwarz DS, Hutvagner G, Du T, Xu Z, Aronin N, et al. (2003) Asymmetry in the assembly of the RNAi enzyme complex. Cell 115: 199-208.
86. Rana TM (2007) Illuminating the silence: understanding the structure and function of small RNAs. Nat Rev Mol Cell Biol 8: 23-36.
87. Schwarz DS, Zamore PD (2002) Why do miRNAs live in the miRNP? Genes Dev 16: 1025-1031.
88. Okamura K, Chung WJ, Lai EC (2008) The long and short of inverted repeat genes in animals: microRNAs, mirtrons and hairpin RNAs. Cell Cycle 7: 2840-2845.
89. Pratt AJ, MacRae IJ (2009) The RNA-induced silencing complex: a versatile gene-silencing machine. J Biol Chem 284: 17897-17901.
90. MacRae IJ, Ma E, Zhou M, Robinson CV, Doudna JA (2008) In vitro reconstitution of the human RISC-loading complex. Proc Natl Acad Sci U S A 105: 512-517.
91. Mourelatos Z, Dostie J, Paushkin S, Sharma A, Charroux B, et al. (2002) miRNPs: a novel class of ribonucleoproteins containing numerous microRNAs. Genes Dev 16: 720-728.
92. Meister G, Landthaler M, Peters L, Chen PY, Urlaub H, et al. (2005) Identification of novel argonaute-associated proteins. Curr Biol 15: 2149-2155.
93. Stark A, Lin MF, Kheradpour P, Pedersen JS, Parts L, et al. (2007) Discovery of functional elements in 12 Drosophila genomes using evolutionary signatures. Nature 450: 219-232.
94. Orom UA, Nielsen FC, Lund AH (2008) MicroRNA-10a binds the 5'UTR of ribosomal protein mRNAs and enhances their translation. Mol Cell 30: 460-471.

95. Eiring AM, Harb JG, Neviani P, Garton C, Oaks JJ, et al. (2010) miR-328 functions as an RNA decoy to modulate hnRNP E2 regulation of mRNA translation in leukemic blasts. Cell 140: 652-665.
96. Beitzinger M, Meister G (2010) Preview. MicroRNAs: from decay to decoy. Cell 140: 612-614.
97. Khraiwesh B, Arif MA, Seumel GI, Ossowski S, Weigel D, et al. (2010) Transcriptional control of gene expression by microRNAs. Cell 140: 111-122.
98. Gonzalez S, Pisano DG, Serrano M (2008) Mechanistic principles of chromatin remodeling guided by siRNAs and miRNAs. Cell Cycle 7: 2601-2608.
99. Kim DH, Saetrom P, Snove O, Jr., Rossi JJ (2008) MicroRNA-directed transcriptional gene silencing in mammalian cells. Proc Natl Acad Sci U S A 105: 16230-16235.
100. Mraz M, Pospisilova S (2012) MicroRNAs in chronic lymphocytic leukemia: from causality to associations and back. Expert Rev Hematol 5: 579-581.
101. Musilova K, Mraz M (2015) MicroRNAs in B-cell lymphomas: how a complex biology gets more complex. Leukemia 29: 1004-1017.
102. Calin GA, Dumitru CD, Shimizu M, Bichi R, Zupo S, et al. (2002) Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proc Natl Acad Sci U S A 99: 15524-15529.
103. Volinia S, Calin GA, Liu CG, Ambs S, Cimmino A, et al. (2006) A microRNA expression signature of human solid tumors defines cancer gene targets. Proc Natl Acad Sci U S A 103: 2257-2261.
104. Calin GA, Ferracin M, Cimmino A, Di Leva G, Shimizu M, et al. (2005) A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. N Engl J Med 353: 1793-1801.
105. Garzon R, Volinia S, Liu CG, Fernandez-Cymering C, Palumbo T, et al. (2008) MicroRNA signatures associated with cytogenetics and prognosis in acute myeloid leukemia. Blood 111: 3183-3189.
106. Yanaihara N, Caplen N, Bowman E, Seike M, Kumamoto K, et al. (2006) Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. Cancer Cell 9: 189-198.
107. Iorio MV, Ferracin M, Liu CG, Veronese A, Spizzo R, et al. (2005) MicroRNA gene expression deregulation in human breast cancer. Cancer Res 65: 7065-7070.
108. Wang H, Garzon R, Sun H, Ladner KJ, Singh R, et al. (2008) NF-kappaB-YY1-miR-29 regulatory circuitry in skeletal myogenesis and rhabdomyosarcoma. Cancer Cell 14: 369-381.
109. Mott JL, Kobayashi S, Bronk SF, Gores GJ (2007) mir-29 regulates Mcl-1 protein expression and apoptosis. Oncogene 26: 6133-6140.
110. Xiong Y, Fang JH, Yun JP, Yang J, Zhang Y, et al. (2010) Effects of microRNA-29 on apoptosis, tumorigenicity, and prognosis of hepatocellular carcinoma. Hepatology 51: 836-845.
111. Zhao JJ, Lin J, Lwin T, Yang H, Guo J, et al. (2010) microRNA expression profile and identification of miR-29 as a prognostic marker and pathogenetic factor by targeting CDK6 in mantle cell lymphoma. Blood 115: 2630-2639.
112. Bonci D, Coppola V, Musumeci M, Addario A, Giuffrida R, et al. (2008) The miR-15a-miR-16-1 cluster controls prostate cancer by targeting multiple oncogenic activities. Nat Med 14: 1271-1277.
113. Bottoni A, Piccin D, Tagliati F, Luchin A, Zatelli MC, et al. (2005) miR-15a and miR-16-1 down-regulation in pituitary adenomas. J Cell Physiol 204: 280-285.
114. Schetter AJ, Leung SY, Sohn JJ, Zanetti KA, Bowman ED, et al. (2008) MicroRNA expression profiles associated with prognosis and therapeutic outcome in colon adenocarcinoma. JAMA 299: 425-436.
115. Johnson SM, Grosshans H, Shingara J, Byrom M, Jarvis R, et al. (2005) RAS is regulated by the let-7 microRNA family. Cell 120: 635-647.

116. Takamizawa J, Konishi H, Yanagisawa K, Tomida S, Osada H, et al. (2004) Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. Cancer Res 64: 3753-3756.
117. Yu F, Yao H, Zhu P, Zhang X, Pan Q, et al. (2007) let-7 regulates self renewal and tumorigenicity of breast cancer cells. Cell 131: 1109-1123.
118. Akao Y, Nakagawa Y, Naoe T (2006) let-7 microRNA functions as a potential growth suppressor in human colon cancer cells. Biol Pharm Bull 29: 903-906.
119. Yang N, Kaur S, Volinia S, Greshock J, Lassus H, et al. (2008) MicroRNA microarray identifies Let-7i as a novel biomarker and therapeutic target in human epithelial ovarian cancer. Cancer Res 68: 10307-10314.
120. Motoyama K, Inoue H, Nakamura Y, Uetake H, Sugihara K, et al. (2008) Clinical significance of high mobility group A2 in human gastric cancer and its relationship to let-7 microRNA family. Clin Cancer Res 14: 2334-2340.
121. Garzon R, Heaphy CE, Havelange V, Fabbri M, Volinia S, et al. (2009) MicroRNA 29b functions in acute myeloid leukemia. Blood 114: 5331-5341.
122. Sampson VB, Rong NH, Han J, Yang Q, Aris V, et al. (2007) MicroRNA let-7a down-regulates MYC and reverts MYC-induced growth in Burkitt lymphoma cells. Cancer Res 67: 9762-9770.
123. Lujambio A, Calin GA, Villanueva A, Ropero S, Sanchez-Cespedes M, et al. (2008) A microRNA DNA methylation signature for human cancer metastasis. Proc Natl Acad Sci U S A 105: 13556-13561.
124. Chan JA, Krichevsky AM, Kosik KS (2005) MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells. Cancer Res 65: 6029-6033.
125. Cimmino A, Calin GA, Fabbri M, Iorio MV, Ferracin M, et al. (2005) miR-15 and miR-16 induce apoptosis by targeting BCL2. Proc Natl Acad Sci U S A 102: 13944-13949.
126. He L, Thomson JM, Hemann MT, Hernando-Monge E, Mu D, et al. (2005) A microRNA polycistron as a potential human oncogene. Nature 435: 828-833.
127. Costinean S, Zanesi N, Pekarsky Y, Tili E, Volinia S, et al. (2006) Pre-B cell proliferation and lymphoblastic leukemia/high-grade lymphoma in E(mu)-miR155 transgenic mice. Proc Natl Acad Sci U S A 103: 7024-7029.
128. Ma L, Teruya-Feldstein J, Weinberg RA (2007) Tumour invasion and metastasis initiated by microRNA-10b in breast cancer. Nature 449: 682-688.
129. Tavazoie SF, Alarcon C, Oskarsson T, Padua D, Wang Q, et al. (2008) Endogenous human microRNAs that suppress breast cancer metastasis. Nature 451: 147-152.
130. Boutla A, Delidakis C, Tabler M (2003) Developmental defects by antisense-mediated inactivation of micro-RNAs 2 and 13 in Drosophila and the identification of putative target genes. Nucleic Acids Res 31: 4973-4980.
131. Hutvagner G, Simard MJ, Mello CC, Zamore PD (2004) Sequence-specific inhibition of small RNA function. PLoS Biol 2: E98.
132. Weiler J, Hunziker J, Hall J (2006) Anti-miRNA oligonucleotides (AMOs): ammunition to target miRNAs implicated in human disease? Gene Ther 13: 496-502.
133. Krutzfeldt J, Rajewsky N, Braich R, Rajeev KG, Tuschl T, et al. (2005) Silencing of microRNAs in vivo with 'antagomirs'. Nature 438: 685-689.
134. Choi WY, Giraldez AJ, Schier AF (2007) Target protectors reveal dampening and balancing of Nodal agonist and antagonist by miR-430. Science 318: 271-274.
135. Ebert MS, Neilson JR, Sharp PA (2007) MicroRNA sponges: competitive inhibitors of small RNAs in mammalian cells. Nat Methods 4: 721-726.
136. Xiao J, Yang B, Lin H, Lu Y, Luo X, et al. (2007) Novel approaches for gene-specific interference via manipulating actions of microRNAs: examination on the pacemaker channel genes HCN2 and HCN4. J Cell Physiol 212: 285-292.

137. Loya CM, Lu CS, Van Vactor D, Fulga TA (2009) Transgenic microRNA inhibition with spatiotemporal specificity in intact organisms. Nat Methods 6: 897-903.
138. Michelfelder S, Trepel M (2009) Adeno-associated viral vectors and their redirection to cell-type specific receptors. Adv Genet 67: 29-60.
139. Aagaard L, Rossi JJ (2007) RNAi therapeutics: principles, prospects and challenges. Adv Drug Deliv Rev 59: 75-86.
140. Saito Y, Liang G, Egger G, Friedman JM, Chuang JC, et al. (2006) Specific activation of microRNA-127 with downregulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells. Cancer Cell 9: 435-443.
141. Lujambio A, Ropero S, Ballestar E, Fraga MF, Cerrato C, et al. (2007) Genetic unmasking of an epigenetically silenced microRNA in human cancer cells. Cancer Res 67: 1424-1429.
142. Hackanson B, Bennett KL, Brena RM, Jiang J, Claus R, et al. (2008) Epigenetic modification of CCAAT/enhancer binding protein alpha expression in acute myeloid leukemia. Cancer Res 68: 3142-3151.
143. Garzon R, Pichiorri F, Palumbo T, Visentini M, Aqeilan R, et al. (2007) MicroRNA gene expression during retinoic acid-induced differentiation of human acute promyelocytic leukemia. Oncogene 26: 4148-4157.
144. Roccaro AM, Sacco A, Chen C, Runnels J, Leleu X, et al. (2009) microRNA expression in the biology, prognosis, and therapy of Waldenstrom macroglobulinemia. Blood 113: 4391-4402.
145. Galm O, Herman JG, Baylin SB (2006) The fundamental role of epigenetics in hematopoietic malignancies. Blood Rev 20: 1-13.
146. Di Leva G, Croce CM (2013) The Role of microRNAs in the Tumorigenesis of Ovarian Cancer. Front Oncol 3: 153.
147. Mezzanzanica D, Bagnoli M, De Cecco L, Valeri B, Canevari S (2010) Role of microRNAs in ovarian cancer pathogenesis and potential clinical implications. Int J Biochem Cell Biol 42: 1262-1272.
148. van Jaarsveld MT, Helleman J, Berns EM, Wiemer EA (2010) MicroRNAs in ovarian cancer biology and therapy resistance. Int J Biochem Cell Biol 42: 1282-1290.
149. Iorio MV, Visone R, Di Leva G, Donati V, Petrocca F, et al. (2007) MicroRNA signatures in human ovarian cancer. Cancer Res 67: 8699-8707.
150. Yang D, Sun Y, Hu L, Zheng H, Ji P, et al. (2013) Integrated analyses identify a master microRNA regulatory network for the mesenchymal subtype in serous ovarian cancer. Cancer Cell 23: 186-199.
151. Zhang L, Volinia S, Bonome T, Calin GA, Greshock J, et al. (2008) Genomic and epigenetic alterations deregulate microRNA expression in human epithelial ovarian cancer. Proc Natl Acad Sci U S A 105: 7004-7009.
152. Nam EJ, Yoon H, Kim SW, Kim H, Kim YT, et al. (2008) MicroRNA expression profiles in serous ovarian carcinoma. Clin Cancer Res 14: 2690-2695.
153. Yang H, Kong W, He L, Zhao JJ, O'Donnell JD, et al. (2008) MicroRNA expression profiling in human ovarian cancer: miR-214 induces cell survival and cisplatin resistance by targeting PTEN. Cancer Res 68: 425-433.
154. Eitan R, Kushnir M, Lithwick-Yanai G, David MB, Hoshen M, et al. (2009) Tumor microRNA expression patterns associated with resistance to platinum based chemotherapy and survival in ovarian cancer patients. Gynecol Oncol 114: 253-259.
155. Hu X, Macdonald DM, Huettner PC, Feng Z, El Naqa IM, et al. (2009) A miR-200 microRNA cluster as prognostic marker in advanced ovarian cancer. Gynecol Oncol 114: 457-464.
156. Marchini S, Cavalieri D, Fruscio R, Calura E, Garavaglia D, et al. (2011) Association between miR-200c and the survival of patients with stage I epithelial ovarian cancer: a retrospective study of two independent tumour tissue collections. Lancet Oncol 12: 273-285.

157. Bagnoli M, Canevari S, Califano D, Losito S, Maio MD, et al. (2016) Development and validation of a microRNA-based signature (MiROvaR) to predict early relapse or progression of epithelial ovarian cancer: a cohort study. Lancet Oncol 17: 1137-1146.
158. Taylor DD, Gercel-Taylor C (2008) MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. Gynecol Oncol 110: 13-21.
159. Resnick KE, Alder H, Hagan JP, Richardson DL, Croce CM, et al. (2009) The detection of differentially expressed microRNAs from the serum of ovarian cancer patients using a novel real-time PCR platform. Gynecol Oncol 112: 55-59.
160. Kan CW, Hahn MA, Gard GB, Maidens J, Huh JY, et al. (2012) Elevated levels of circulating microRNA-200 family members correlate with serous epithelial ovarian cancer. BMC Cancer 12: 627.
161. Vaksman O, Hetland TE, Trope CG, Reich R, Davidson B (2012) Argonaute, Dicer, and Drosha are up-regulated along tumor progression in serous ovarian carcinoma. Hum Pathol 43: 2062-2069.
162. Leskela S, Leandro-Garcia LJ, Mendiola M, Barriuso J, Inglada-Perez L, et al. (2011) The miR-200 family controls beta-tubulin III expression and is associated with paclitaxel-based treatment response and progression-free survival in ovarian cancer patients. Endocr Relat Cancer 18: 85-95.
163. Vecchione A, Belletti B, Lovat F, Volinia S, Chiappetta G, et al. (2013) A microRNA signature defines chemoresistance in ovarian cancer through modulation of angiogenesis. Proc Natl Acad Sci U S A 110: 9845-9850.
164. Kong F, Sun C, Wang Z, Han L, Weng D, et al. (2011) miR-125b confers resistance of ovarian cancer cells to cisplatin by targeting pro-apoptotic Bcl-2 antagonist killer 1. J Huazhong Univ Sci Technolog Med Sci 31: 543-549.
165. Wang YQ, Guo RD, Guo RM, Sheng W, Yin LR (2013) MicroRNA-182 promotes cell growth, invasion, and chemoresistance by targeting programmed cell death 4 (PDCD4) in human ovarian carcinomas. J Cell Biochem 114: 1464-1473.
166. Liu N, Zhou C, Zhao J, Chen Y (2012) Reversal of paclitaxel resistance in epithelial ovarian carcinoma cells by a MUC1 aptamer-let-7i chimera. Cancer Invest 30: 577-582.
167. Cochrane DR, Howe EN, Spoelstra NS, Richer JK (2010) Loss of miR-200c: A Marker of Aggressiveness and Chemoresistance in Female Reproductive Cancers. J Oncol 2010: 821717.
168. Prislei S, Martinelli E, Mariani M, Raspaglio G, Sieber S, et al. (2013) MiR-200c and HuR in ovarian cancer. BMC Cancer 13: 72.
169. Wu G, Liu A, Zhu J, Lei F, Wu S, et al. (2015) MiR-1207 overexpression promotes cancer stem cell-like traits in ovarian cancer by activating the Wnt/beta-catenin signaling pathway. Oncotarget 6: 28882-28894.
170. Wu Q, Guo R, Lin M, Zhou B, Wang Y (2011) MicroRNA-200a inhibits CD133/1+ ovarian cancer stem cells migration and invasion by targeting E-cadherin repressor ZEB2. Gynecol Oncol 122: 149-154.
171. Nam EJ, Lee M, Yim GW, Kim JH, Kim S, et al. (2012) MicroRNA profiling of a CD133(+) spheroid-forming subpopulation of the OVCAR3 human ovarian cancer cell line. BMC Med Genomics 5: 18.
172. Warburg O, Wind F, Negelein E (1926) The metabolism of tumors in the body.
173. Warburg O (1956) On respiratory impairment in cancer cells. Science 124: 269-270.
174. Fowler JS, Ido T (2002) Initial and subsequent approach for the synthesis of 18FDG. Semin Nucl Med 32: 6-12.
175. Bardella C, Pollard PJ, Tomlinson I (2011) SDH mutations in cancer. Biochim Biophys Acta 1807: 1432-1443.
176. Dang L, White DW, Gross S, Bennett BD, Bittinger MA, et al. (2009) Cancer-associated IDH1 mutations produce 2-hydroxyglutarate. Nature 462: 739-744.

177. Christofk HR, Vander Heiden MG, Harris MH, Ramanathan A, Gerszten RE, et al. (2008) The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth. Nature 452: 230-233.
178. Semenza GL, Roth PH, Fang HM, Wang GL (1994) Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. J Biol Chem 269: 23757-23763.
179. Semenza GL (2012) Hypoxia-inducible factors in physiology and medicine. Cell 148: 399-408.
180. Kwee SA, Hernandez B, Chan O, Wong L (2012) Choline kinase alpha and hexokinase-2 protein expression in hepatocellular carcinoma: association with survival. PLoS One 7: e46591.
181. Guo W, Qiu Z, Wang Z, Wang Q, Tan N, et al. (2015) MiR-199a-5p is negatively associated with malignancies and regulates glycolysis and lactate production by targeting hexokinase 2 in liver cancer. Hepatology 62: 1132-1144.
182. Flavahan WA, Wu Q, Hitomi M, Rahim N, Kim Y, et al. (2013) Brain tumor initiating cells adapt to restricted nutrition through preferential glucose uptake. Nat Neurosci 16: 1373-1382.
183. Upadhyay M, Samal J, Kandpal M, Singh OV, Vivekanandan P (2013) The Warburg effect: insights from the past decade. Pharmacol Ther 137: 318-330.
184. Xintaropoulou C, Ward C, Wise A, Marston H, Turnbull A, et al. (2015) A comparative analysis of inhibitors of the glycolysis pathway in breast and ovarian cancer cell line models. Oncotarget 6: 25677-25695.
185. Zhang XD, Deslandes E, Villedieu M, Poulain L, Duval M, et al. (2006) Effect of 2-deoxy-D-glucose on various malignant cell lines in vitro. Anticancer Res 26: 3561-3566.
186. Szokoloczi O, Schwab R, Petak I, Orfi L, Pap A, et al. (2005) TT232, a novel signal transduction inhibitory compound in the therapy of cancer and inflammatory diseases. J Recept Signal Transduct Res 25: 217-235.
187. Madhok BM, Yeluri S, Perry SL, Hughes TA, Jayne DG (2011) Targeting glucose metabolism: an emerging concept for anticancer therapy. Am J Clin Oncol 34: 628-635.
188. Herter FP, Weissman SG, Thompson HG, Jr., Hyman G, Martin DS (1961) Clinical experience with 6-aminonicotinamide. Cancer Res 21: 31-37.
189. Rapisarda A, Uranchimeg B, Scudiero DA, Selby M, Sausville EA, et al. (2002) Identification of small molecule inhibitors of hypoxia-inducible factor 1 transcriptional activation pathway. Cancer Res 62: 4316-4324.
190. Kim SH, Lee GM (2007) Down-regulation of lactate dehydrogenase-A by siRNAs for reduced lactic acid formation of Chinese hamster ovary cells producing thrombopoietin. Appl Microbiol Biotechnol 74: 152-159.
191. Flynn P, Wongdagger M, Zavar M, Dean NM, Stokoe D (2000) Inhibition of PDK-1 activity causes a reduction in cell proliferation and survival. Curr Biol 10: 1439-1442.
192. Wen S, Zhu D, Huang P (2013) Targeting cancer cell mitochondria as a therapeutic approach. Future Med Chem 5: 53-67.
193. Hirsch HA, Iliopoulos D, Tsichlis PN, Struhl K (2009) Metformin selectively targets cancer stem cells, and acts together with chemotherapy to block tumor growth and prolong remission. Cancer Res 69: 7507-7511.
194. Kim TH, Suh DH, Kim MK, Song YS (2014) Metformin against cancer stem cells through the modulation of energy metabolism: special considerations on ovarian cancer. Biomed Res Int 2014: 132702.
195. Stuart SD, Schauble A, Gupta S, Kennedy AD, Keppler BR, et al. (2014) A strategically designed small molecule attacks alpha-ketoglutarate dehydrogenase in tumor cells through a redox process. Cancer Metab 2: 4.

196. Moserle L, Indraccolo S, Ghisi M, Frasson C, Fortunato E, et al. (2008) The side population of ovarian cancer cells is a primary target of IFN-alpha antitumor effects. Cancer Res 68: 5658-5668.
197. Nardo G, Favaro E, Curtarello M, Moserle L, Zulato E, et al. (2011) Glycolytic phenotype and AMP kinase modify the pathologic response of tumor xenografts to VEGF neutralization. Cancer Res 71: 4214-4225.
198. Indraccolo S, Tisato V, Agata S, Moserle L, Ferrari S, et al. (2006) Establishment and characterization of xenografts and cancer cell cultures derived from BRCA1 -/- epithelial ovarian cancers. Eur J Cancer 42: 1475-1483.
199. Ricci F, Bernasconi S, Porcu L, Erba E, Panini N, et al. (2013) ALDH enzymatic activity and CD133 positivity and response to chemotherapy in ovarian cancer patients. Am J Cancer Res 3: 221-229.
200. Quah BJ, Parish CR (2012) New and improved methods for measuring lymphocyte proliferation in vitro and in vivo using CFSE-like fluorescent dyes. J Immunol Methods 379: 1-14.
201. Lyons AB (2000) Analysing cell division in vivo and in vitro using flow cytometric measurement of CFSE dye dilution. J Immunol Methods 243: 147-154.
202. Boulares AH, Yakovlev AG, Ivanova V, Stoica BA, Wang G, et al. (1999) Role of poly(ADP-ribose) polymerase (PARP) cleavage in apoptosis. Caspase 3-resistant PARP mutant increases rates of apoptosis in transfected cells. J Biol Chem 274: 22932-22940.
203. Phadngam S, Castiglioni A, Ferraresi A, Morani F, Follo C, et al. (2016) PTEN dephosphorylates AKT to prevent the expression of GLUT1 on plasmamembrane and to limit glucose consumption in cancer cells. Oncotarget.
204. Wieman HL, Horn SR, Jacobs SR, Altman BJ, Kornbluth S, et al. (2009) An essential role for the Glut1 PDZ-binding motif in growth factor regulation of Glut1 degradation and trafficking. Biochem J 418: 345-367.
205. Galluzzi L, Senovilla L, Vitale I, Michels J, Martins I, et al. (2012) Molecular mechanisms of cisplatin resistance. Oncogene 31: 1869-1883.
206. Huang Y, Sadee W (2006) Membrane transporters and channels in chemoresistance and -sensitivity of tumor cells. Cancer Lett 239: 168-182.
207. Vlashi E, Lagadec C, Vergnes L, Matsutani T, Masui K, et al. (2011) Metabolic state of glioma stem cells and nontumorigenic cells. Proc Natl Acad Sci U S A 108: 16062-16067.
208. Vander Heiden MG (2011) Targeting cancer metabolism: a therapeutic window opens. Nat Rev Drug Discov 10: 671-684.
209. Chan B, Manley J, Lee J, Singh SR (2015) The emerging roles of microRNAs in cancer metabolism. Cancer Lett 356: 301-308.
210. Vienberg S, Geiger J, Madsen S, Dalgaard LT (2016) MicroRNAs in Metabolism. Acta Physiol (Oxf).
211. Rosenberg LH, Lafitte M, Quereda V, Grant W, Chen W, et al. (2015) Therapeutic targeting of casein kinase 1delta in breast cancer. Sci Transl Med 7: 318ra202.
212. Pinato DJ, Graham J, Gabra H, Sharma R (2013) Evolving concepts in the management of drug resistant ovarian cancer: dose dense chemotherapy and the reversal of clinical platinum resistance. Cancer Treat Rev 39: 153-160.
213. Kinose Y, Sawada K, Nakamura K, Kimura T (2014) The role of microRNAs in ovarian cancer. Biomed Res Int 2014: 249393.
214. Vander Heiden MG, Cantley LC, Thompson CB (2009) Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science 324: 1029-1033.
215. Jin L, Huo Y, Zheng Z, Jiang X, Deng H, et al. (2014) Down-regulation of Ras-related protein Rab 5C-dependent endocytosis and glycolysis in cisplatin-resistant ovarian cancer cell lines. Mol Cell Proteomics 13: 3138-3151.

216. Catanzaro D, Gaude E, Orso G, Giordano C, Guzzo G, et al. (2015) Inhibition of glucose-6-phosphate dehydrogenase sensitizes cisplatin-resistant cells to death. Oncotarget 6: 30102-30114.
217. Davidson SM, Papagiannakopoulos T, Olenchock BA, Heyman JE, Keibler MA, et al. (2016) Environment Impacts the Metabolic Dependencies of Ras-Driven Non-Small Cell Lung Cancer. Cell Metab 23: 517-528.
218. Matassa DS, Amoroso MR, Lu H, Avolio R, Arzeni D, et al. (2016) Oxidative metabolism drives inflammation-induced platinum resistance in human ovarian cancer. Cell Death Differ 23: 1542-1554.
219. Kimmelman AC (2011) The dynamic nature of autophagy in cancer. Genes Dev 25: 1999-2010.
220. Rebecca VW, Amaravadi RK (2016) Emerging strategies to effectively target autophagy in cancer. Oncogene 35: 1-11.
221. Sui X, Chen R, Wang Z, Huang Z, Kong N, et al. (2013) Autophagy and chemotherapy resistance: a promising therapeutic target for cancer treatment. Cell Death Dis 4: e838.
222. Ge R, Liu L, Dai W, Zhang W, Yang Y, et al. (2016) Xeroderma Pigmentosum Group A Promotes Autophagy to Facilitate Cisplatin Resistance in Melanoma Cells through the Activation of PARP1. J Invest Dermatol 136: 1219-1228.
223. Yang Z, Klionsky DJ (2010) Eaten alive: a history of macroautophagy. Nat Cell Biol 12: 814-822.
224. Stuart GC, Kitchener H, Bacon M, duBois A, Friedlander M, et al. (2011) 2010 Gynecologic Cancer InterGroup (GCIG) consensus statement on clinical trials in ovarian cancer: report from the Fourth Ovarian Cancer Consensus Conference. Int J Gynecol Cancer 21: 750-755.
225. Maccalli C, Volontè A, Cimminiello C, Parmiani G (2014) Immunology of cancer stem cells in solid tumors. A review. Eur J Cancer 50: 649-655.
226. Blaylock RL (2015) Cancer microenvironment, inflammation and cancer stem cells: A hypothesis for a paradigm change and new targets in cancer control. Surg Neurol Int: 6-92.
227. Schreiber RD, Old LJ, Smyth MJ (2011) Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. Science 331: 1565-1570.