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LIPID METABOLISM: A MEDIATOR OF PHENOTYPIC TRANSFORMATION IN OVARIAN CANCER

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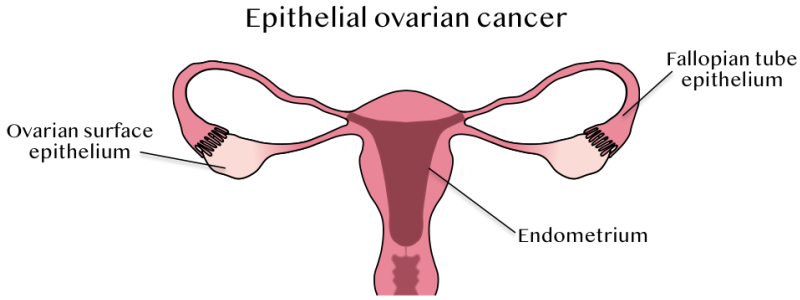
ABSTRACT

Metastatic and chemoresistant disease remains a challenge of ovarian cancer (OVCA), and has been proposed to involve metabolic reprogramming in metastatic and tumour-initiating cells (TIC). Metabolic adaptation to an adipose-rich tumour microenvironment, such as in ovarian cancer metastasis, likely involves crosstalk with adipocytes and upregulated mitochondrial respiration and fatty acid (FA) metabolism. However, the role of this crosstalk and related metabolic alterations in OVCA progression remains poorly understood. Gene expression and enrichment analysis showed that, compared to primary ovarian tumours, omental metastases exhibited upregulated expression of lipid metabolic genes, and enrichment of cellular motility/invasion, growth/proliferation and survival pathways. Additionally, in omental metastases, expression of lipid metabolic genes *FABP4, GPD1,* and *CIDEA* correlated with the expression of cell proliferation genes *GLUL, PODN, APOD, ADIPOQ, DUSP1,* and *FOS,* and high expression of these genes associated with better survival. The role of PGC-1, a master regulator of FA metabolism and mito-biogenesis, was investigated *in-vitro* in an OVCA cell line, SKOV-3, and a cisplatin-selected sub-line, SKOV-3-R, representing tumour progression with TIC properties and lacking in PGC-1α expression. Results showed that, in a PGC-1-independent manner, SKOV-3-R cells increased FA metabolic capabilities evidenced by increased CD36 expression, greater FA-linked OXPHOS coupling efficiency, and unaffected ATP levels in response to the addition of oleic acid and glycolysis inhibition. Although PGC-1α expression did not associate with chemoresistance in the gene expression studies, knockdown of PGC-1α in parental SKOV-3-P cells resulted in increased cisplatin resistance, mitochondrial fragmentation and peri-nuclear clustering, but did not increase TIC-marker expression. Lastly, culturing cells under high- and low glucose conditions with or without oleic acid induced different patterns of mito-organization and PGC-1α levels, indicating the responsiveness of these metabolic properties to the nutrient environment and emphasizing the need for the use of physiologically relevant nutrient conditions when investigating metabolic adaptation in cancer cells. Overall, these data have contributed to the understanding of metabolism in ovarian cancer progression.

INTRODUCTION

Overview of ovarian cancer pathology, classification and treatment

High-grade serous ovarian carcinoma (HGSOC) accounts for 70-80% of ovarian cancer (OVCA) related deaths (Lisio et al., 2019). It is one of four sub-types of epithelial OVCA (EOC) including, serous, mucinous, clear-cell and endometrioid, and is classified based on morphology and tissue architecture. Cytological abnormalities are used as a third degree of stratification to define the tumour grade as low or high. In 2004, a dualistic model of ovarian cancer classification (Fig. 1) was proposed by Kurman and Shih and was officially adopted by the WHO in 2014 (Kurman & Shih, 2016). It divides serous carcinomas into the less and more aggressive type-1 (low-grade) and type-2 (high-grade).



Epithelial ovarian cancer	Type I	Type II
Origin	Mostly from endometriosis or fallopian tubal-related serous borderline ovarian tumours	Mostly from fallopian tube epithelium
Typical genetic alterations in	KRAS, BRAF, PTEN, PIK3CA	TP53, BRCA1/2
Tumours included	e.g. Low-grade serous carcinoma (LGSC), clear cell carcinoma, endometrioid carcinoma	e.g. High-grade serous carcinoma (HGSC)

Fig. 1: Illustration of the dualistic model of ovarian carcinogenesis adapted from Kurman and Shih 2016

This model classifies the tumour based on genetic and molecular properties. Type-1 tumours are typically genomically stable with wild-type tumour protein 53 (TP53) and oncogenic alterations in Ras-mitogen activated protein kinase (RAS-MAPK) and phosphoinositide 3-kinase-protein kinase B (PI3K-AKT) signalling pathways. Conversely, Type-2 tumours are genomically unstable due to aberrations in DNA repair mechanisms and harbour TP53

mutations (Lisio et al., 2019). This was an important step in the identification and characterization of OVCA, which is necessary in the processes of diagnosing and selecting appropriate therapy. However, the mortality rate remains high with 80% of OVCA patients dying within 5 years of diagnosis (American Cancer Society, January 2020).

The high mortality rate of OVCA can be attributed to a number of challenges, such as late detection and relapse into chemoresistant and metastatic disease (Lisio et al., 2019) (Muinao et al., 2018). Early stage OVCA is mainly asymptomatic while; women with advanced disease are more likely to experience symptoms such as bloating, pelvic or abdominal pain, frequent urination and reduced appetite.

Methods of OVCA detection

Currently, transvaginal ultrasonography and serum cancer antigen 125 (CA-125) screening are the most common methods of OVCA detection. CA-125 has played an important role in diagnostic and prognostic approaches; however, effective screening practices for detection, good biomarkers with high sensitivity for early diagnosis, and prognostic biomarkers are needed to combat the high death toll. Ideally, biomarkers should have the potential to detect the disease prior to metastasis, since advanced stage OVCA, despite its high response rate of 80% to surgery and chemotherapy, has a tumour recurrence rate of 60-80% within 6 months to 2 years after treatment (Muinao et al., 2018).

Implications of late OVCA diagnosis and treatment strategies

Late diagnosis of HGSOC contributes substantially to its association with high mortality at a 5-year survival rate of 29.2% as opposed to early stage OVCA, which at stage I and stage II have a much better prognosis of 92.3% and 74.5% respectively. HGSOC disseminates quite easily to the fluid-filled peritoneal cavity, where it implants into organs and tissues to form secondary nodules, in most cases rendering the entire peritoneal cavity full of nodules (Lisio et al., 2019).

These malignant tumours should be treated more aggressively than their less malignant counterparts. However, patients with HGSOC typically receive the same standard of care cytoreductive or debulking surgery, followed by adjuvant cytotoxic chemotherapy as their less malignant counterparts (Lisio et al., 2019). Debulking surgery involves the removal of

all tumorous lesions visible to the eye, and includes the removal of the reproductive organs, the sigmoid colon along with a complete peritonectomy and omentectomy. Residual cancer mass of less than 1 centimeter (cm) is considered a sufficient degree of cytoreduction. Following successful surgery, patients are recommended to undergo adjuvant cytotoxic chemotherapy, which involves intravenous or intraperitoneal administration of platinum-based therapies such as carboplatin and cisplatin, and taxanes such as paclitaxel.

Radiology and serum CA-125 levels are used to periodically monitor response to chemotherapy during and following completion of treatment. Increasing levels of CA-125 are usually indicative of relapse. Approximately 50% of patients with recurring tumours remain responsive to platinum-based therapies, whereas those who are unresponsive may receive alternative therapies such as doxorubicin or topotecan (Lisio et al., 2019). In most cases of advanced OVCA, current standard of practice treatment strategies are not effective in complete eradication of all cancer cells, which can result in chemoresistant residual or recurrent disease. Targeted therapeutic approaches could assist in overcoming these downfalls. To this point, a few promising therapies have been introduced to complement conventional approaches, e.g., poly ADP-Ribose polymerase (PARP) inhibitors targeting DNA repair pathways and anti-angiogenic agents such as bevacizumab and receptor tyrosine kinase (RTK) inhibitor cediranib (Lisio et al., 2019).

Further approaches include improving our understanding of tumour heterogeneity and chemoresistance and metastasis mechanisms in HGSOC. In recent years, metabolic reprogramming of cancer cells, and tumour-initiating cells (TICs) or cancer stem cells (CSCs) are two emerging concepts in understanding chemoresistance and metastasis mechanisms.

Chemoresistance and cancer stem/tumour-initiating cells

Despite the high response rate of HGSOC to conventional therapy, recurrence and acquired resistance frequently occur in these patients. A platinum-free interval (PFI) of less than 6 months is defined as platinum resistance, whereas a PFI of longer than 6 months defines platinum sensitivity. Acquired resistance refers to a failure to respond to subsequent treatment having previously responded to one or more lines of therapy with a PFI longer than 6 months. In HGSOC, acquired resistance often involves increased efflux of drugs, molecular

subtype switching, evasion of apoptosis, and restoration of breast cancer (BRCA) and DNA repair systems, which could confer resistance to multiple drugs (Freimund et al., 2018). Cancer stem cells (CSCs) have been identified in nearly all malignancies (Pastò et al., n.d.), are known to evade chemotherapy (Muinao et al., 2018) and play a role in metastatic disease (Francesco et al., 2018). These cells may self-renew and also give rise to bulk tumour cells thereby earning the name tumour-initiating cells (TICs) (Bezuidenhout & Shoshan, 2019).

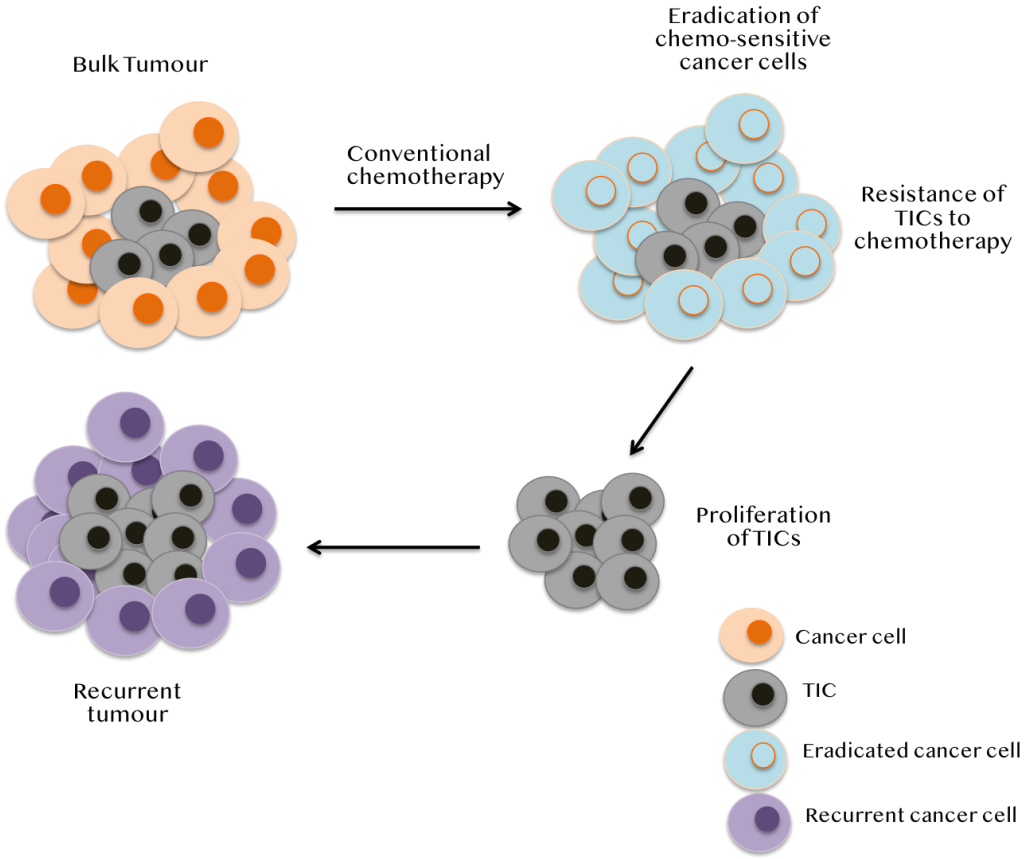


Fig. 2: TIC evasion of chemotherapy and formation of recurrent tumours.

Considering the similarities between the defining properties of CSCs and TICs, often including the increased expression of so-called TIC-markers and the ability to form spheres in stem cell medium, I will henceforth be referring to cells of this nature as TICs.

In recent years the TIC phenotype has evolved to include metabolic reprogramming, which involves the metabolic plasticity of TICs, and their manipulation of mitochondrial metabolism, biogenesis and dynamics to assist in the progression of OVCA, thereby adding

another layer of complexity to the challenge of targeting TICs (Bezuidenhout & Shoshan, 2019).

Metastatic disease

The insidious nature of HGSOC stems from its unique dissemination behavior, which is characterized by infiltration of adjacent organs, and transport by the peritoneal fluid (ascites) throughout the entire peritoneal cavity. This ability to survive in ascites is generally associated with seeding of cancer cells before proliferation in a secondary organ or tissue (Naora & Montell, 2005). Furthermore, OVCA cells preferentially metastasize to the adipose-rich omentum, as evidenced by the fact that the majority of HGSOC patients develop omental metastases, which is associated with increased relapse and poor progression free survival (PFS) (Sun et al. 2018).

An increasing body of evidence points to interactions between OVCA cells and omental adipocytes playing an important role in this dissemination process (Muinao et al., 2018). Recent studies have shown that homing of OVCA cells to the omentum is stimulated by the release of inflammatory adipocytokines such as interleukin-8 (IL-8) and leptin (lep) by omental adipocytes ((Nieman et al., 2011) (Achkar et al., 2019). In turn, OVCA cells induce delipidation of adipocytes leading to the release of free fatty acids (FFAs) and the up-regulation of the expression of fatty acid transporters, cluster of differentiation 36 (CD36) and fatty acid binding protein 4 (FABP4), thus facilitating FFA uptake into the tumour (Ladanyi et al., 2018) (Nieman et al., 2011). Although the fate of these FFAs is poorly understood, evidence indicates the metabolic reprogramming of cancer cells to enable higher rates of mitochondrial fatty acid beta-oxidation (FAO) and lipid droplet (LD) accumulation to provide energy and building blocks for proliferation and tumour growth (Cuello et al., 2018) (Hu et al., 2013) (Ladanyi et al., 2018). Similar results have been obtained from the co-culture of BRCA cells with adipocytes (Balaban et al., 2017) (Wu et al., 2019). Collectively this evidence indicates a facilitative role for crosstalk between adipocytes and OVCA cells in metastasis.

In the next few chapters we aim to shed light on the mechanisms involved in chemoresistance and metastasis conferred by metabolic reprogramming in OVCA cells and TICs, and the role of the adipose-rich omentum therein.

Cancer cell metabolism

The metabolic activities of cancer cells are different compared to normal cells. These altered activities support the acquisition and maintenance of malignancy. It is referred to as metabolic reprogramming when it occurs as a consequence of tumourigenic mutations and/or other factors such as crosstalk with stromal cells. The observation of certain metabolic alterations across many types of cancer has allowed metabolic reprogramming to become a hallmark of cancer (DeBerardinis & Chandel, 2016). However, the underlying mechanisms of reprogramming and their functions in malignancy or how these alterations can be exploited for therapeutic benefit remain largely unknown.

Otto Warburg was the first to demonstrate metabolic reprogramming or metabolic flexibility in cancer cells. Specifically, he showed increased glucose uptake by tumours compared to normal tissues, and that tumours were able to metabolize glucose to lactate even in the presence of sufficient oxygen. He named this process "aerobic glycolysis". In HGSOC tumours, aerobic glycolysis is typically characterized by increased expression of glycolytic enzymes e.g. pyruvate kinase isoform M2 (PKM2), hexokinase II (HKII), lactate dehydrogenase A (LDHA), glucose transporters and glucose uptake (Nayak et al., 2018). In recent years, we have learned considerably more about cancer cell metabolism and the ability of cancer cells to adjust to various mutational and environmental stimuli.

Drivers and consequences of metabolic reprogramming

Metabolic reprogramming is one way in which cancer cells can adapt to mutational and environmental stimuli. It is known to occur due to a single or combination of the following driving events: 1) mutational deregulation of signalling pathways that are frequently disrupted in cancer cells, such as PI3K-AKT mammalian target of rapamycin (mTOR), gain of function MYC, KRas, and TP53, which regulate important processes such as anabolism, catabolism and redox balance, 2) the generation of oncometabolites through mutation of specific metabolic enzymes e.g. mutations in isocitrate dehydrogenase (IDH1)/IDH2 results in an increase in the oncometabolite D-2-hydroxyglutarate (2DHG) in gliomas, and loss-of – function mutations in fumarate hydratase (FH) and the succinate dehydrogenase (SDH) complex results in an increase in oncometabolites fumarate and succinate, or 3) changes in the tumour microenvironment (TME) such as fluctuations in perfusion, e.g., oxygen and nutrient delivery, and crosstalk with stromal cells such as adipocytes in the solid tumour

(DeBerardinis & Chandel, 2016). Understanding more about the consequences of these events and how they contribute to tumour progression could enable targeted metabolic therapeutic approaches.

As per Warburg, cancer cells increase glycolytic flux to provide adequate biomolecule precursors necessary to maintain a high level of proliferation. For many years the understanding of cancer metabolism was restricted to this notion, whereas in recent years evidence indicates that high glycolytic flux does not mean complete shutdown of mitochondrial oxidative phosphorylation (OXPHOS) or sole use of glucose as a substrate. Indeed, mitochondrial respiration and activities are necessary for tumour growth, as OXPHOS is far more efficient at producing ATP than glycolysis, and intermediates from the tricarboxylic acid (TCA) cycle contribute precursors for macromolecule synthesis (Fig.3) (Dar et al., 2017) (DeBerardinis & Chandel, 2016) (Vander Heiden & Deberardinis, 2017). Furthermore, a growing list of cancer cell substrates, including glutamine, FAs, and serine reflects the metabolic flexibility of cancer cells and disputes the rigid dependence on glucose described by Warburg (DeBerardinis et al., 2008) (DeBerardinis et al., 2009) (Zhu & Thompson, 2019).

The high demand for biomass in cancer cells can be satisfied through various pathways and from various sources. In 2016, Hosios et al. quantified the fraction of cell mass derived from different nutrients, using carbon-14 labelled glucose tracing, in rapidly proliferating mammalian cells including various cancer cell lines. They found that although glucose and glutamine were the two most utilized substrates, amino acids were the main contributors to biomass content (Hosios et al., 2016). This indicates that high glycolytic flux is not always the best indicator of cell growth, and that substrates other than glucose play an important role in supplying biomass to support proliferation. In fact, processes such as cata- and anaplerosis, are involved in the generation of biomass from amino acids, and TCA cycle intermediates. Cataplerosis involves the generation of biomass using ATP together with precursors and products of the TCA cycle, e.g., the synthesis of lipids from citrate via de novo lipogenesis. Anaplerosis, on the other hand, involves replenishing TCA cycle intermediates that have been used for biosynthesis, e.g., production of α-ketoglutarate from glutamine and oxaloacetate from pyruvate, and could therefore be a better measure of cell growth. These two reactions involve two well-described metabolic processes involved in tumour progression namely de novo lipogenesis and glutaminolysis.

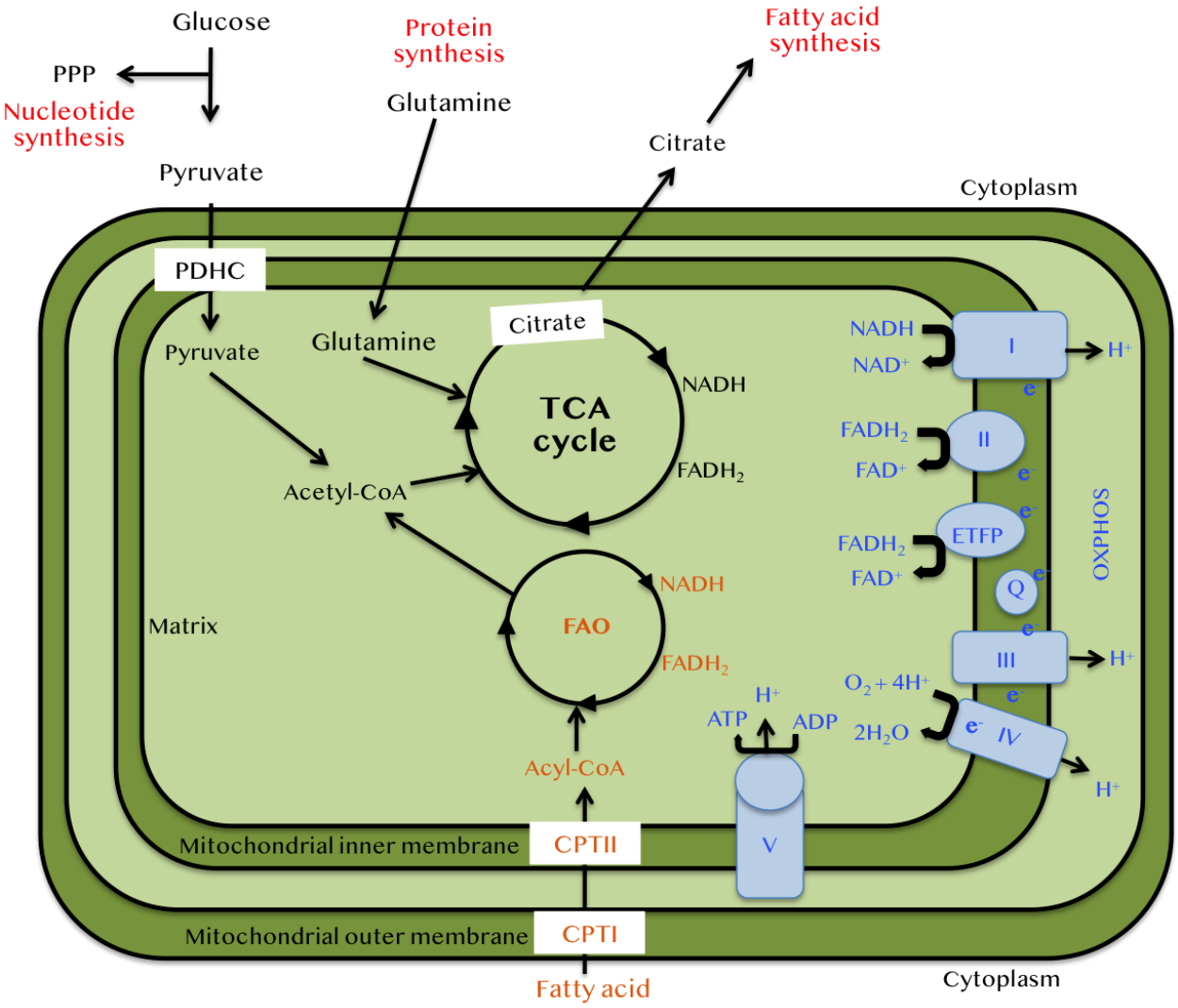


Fig. 3: Overview of cancer cell metabolism

OXPHOS (in blue) consists of two components: 1) the transfer of electrons in the ETC together with the transport of protons (H^+) by respiratory complexes I, III and IV, across the inner mitochondrial membrane to generate a proton gradient, and 2) the use of this electrochemical gradient to generate ATP i.e. chemiosmosis at complex V. OXPHOS, an oxygen consuming process, can be coupled or uncoupled to ATP production, and depends on the level of proton motive force generated. The metabolism of FAs in FAO (in orange) also contributes to the transfer of electrons in the ETC, by providing $FADH_2$ to the ETFP complex and complex II, and NADH to complex I. The metabolism of glucose, glutamine and FA all contribute metabolites to the TCA cycle, and plays a role in nucleotide, protein and FA synthesis respectively to support cancer cell proliferation and growth. Abbreviations: Pentose phosphate pathway (PPP), pyruvate dehydrogenase complex (PDHC), carnitine palmitoyltransferase (CPT) I/II, flavin adenine dinucleotide ($FADH_2$), adenine dinucleotide (NADH), electron-transferring-flavoprotein dehydrogenase (ETFP), fatty acid oxidation (FAO), oxidative phosphorylation (OXPHOS), adenosine triphosphate (ATP), adenosine diphosphate (ADP), FA (fatty acid), electron transport chain (ETC), tricarboxylic acid (TCA).

Alternative biosynthetic pathways

Unlike normal cells which acquire the majority of their FAs from circulation, with the exception of adipocytes and hepatocytes, cancer cells predominantly derive their FAs from de novo lipogenesis through the up-regulation of lipogenic enzymes such as fatty acid synthase (FASN) and acetyl-CoA carboxylase (ACC) (Fig. 3) (Chajès et al., 2006). In addition, cancer cells rely exclusively on exogenous glutamine, the most abundant amino acid in circulation, as their source of reduced nitrogen and carbon (Cluntun et al., 2017). Glutamine can also contribute to de novo lipogenesis through reductive carboxylation of α-ketoglutarate to generate citrate, or through the generation of malate from pyruvate using glutamine-derived α-ketoglutarate i.e. glutaminolysis (Metallo et al., 2013).

Glutaminolysis

Glutaminolysis involves the generation of non-essential amino acids such as glutamate and aspartate to support growth and ATP synthesis, and glutathione to protect against reactive oxygen species (ROS). Furthermore, glutamate and aspartate can be converted to other non-essential amino acids, purines, pyrimidines, and FAs to support tumourigenesis.

"Glutamine addiction" has been described in many cancer cells, and has been shown to contribute to invasivity and migration. Yang et al. reported that highly invasive, and proliferative OVCA cells are more glutamine-dependent and reliant on mitochondrial OXPHOS compared to their low-invasive counterparts (L. Yang et al., 2014). The authors indicated that increased signal transducer and activator of transcription 3 (STAT3) tyrosine phosphorylation by Janus kinases (JAK) RTKs and non-RTKs and the subsequent activation of the STAT3 pathway could be one mechanism by which glutamine confers greater metastatic capability (L. Yang et al., 2014). Furthermore, they reported that high expression of genes involved in glutaminolysis and the TCA cycle correlated with poor survival, whereas increased expression of glycolytic genes associated with better survival in OVCA patients (L. Yang et al., 2014).

Several *in-vitro* studies have indicated that the inhibition of glutaminolysis could be an attractive therapeutic strategy in OVCA treatment. In one study the treatment of OVCA cells with the glutaminase (GLS) inhibitor BPTES resulted in the sensitization of paclitaxel and cisplatin resistant cells, and inhibition through transient knock-down (KD) of GLS1

isoforms, KGA and GAC, in metastatic OVCA cells resulted in sensitization to cisplatin (Masamha & LaFontaine, 2018). Other studies reported that dual inhibition of glycolysis and glutaminolysis, and glutaminolysis and the PI3K/mTOR pathways may be promising therapeutic strategies for the treatment of OVCA (Guo et al., 2016) (Sun et al., 2017).

De novo lipogenesis

Lipid metabolism comprises both anabolic and catabolic processes, and the balance between these two processes controls whether or not cells can derive their energy from FAs instead of glucose (Peixoto & Lima, 2018). The synthesis of FAs (Fig. 4) is anabolic and involves the conversion of acetyl-CoA to malonyl-CoA by FASN, contributing to the storage of lipids in LDs or providing building blocks to support cell proliferation. In contrast, FAO (Fig. 3, 4) is a catabolic process involving the hydrolysis of FAs, from intracellular LDs or from extracellular sources, to produce TCA-cycle intermediates such acetyl-CoA and NADH for the generation of ATP from OXPHOS (Peixoto & Lima, 2018).

Lipogenesis is the production of FAs from acetyl-CoA, with FASN as the main catalyzing enzyme responsible for the NADPH-dependent condensation of acetyl-CoA and malonyl-CoA to produce palmitate (long-chain FA). This cytosolic process depends on mitochondria-derived acetyl-CoA from, e.g., glycolysis. The produced FAs can be esterified to glycerol to synthesize triglycerides, which are packaged within lipid droplets in the cytosol. These lipids may provide one way in which cancer cells can fulfill their demand for increased membrane synthesis during high levels of proliferation. Activation of lipogenesis and increased expression of FASN and other lipogenic enzymes like ACC have been shown to be important in the development and maintenance of the malignant phenotype of OVCA cells (Jiang et al., 2014) (Mukherjee et al., 2012). FASN expression was found to be elevated in high-grade peritoneal metastasis, when compared to normal and benign ovarian tissues and correlates with poor prognosis. In SKOV3 OVCA cells, increased FASN expression promoted colony formation and regulated motility and invasivity. These effects were recapitulated in an intraperitoneal xenograft mouse model where overexpression of FASN stimulated implantation and invasive ability through the induction of epithelial-mesenchymal transition (EMT) (Jiang et al., 2014). Furthermore, FASN-induced lipid droplet accumulation was shown to blunt the anti-tumour activity of T-cells by inhibiting tumour-infiltrating dendritic cells (TIDCs) (Jiang et al., 2018).

In addition, FASN inhibitors such as orlistat and C75 are known to have anti-tumour effects in OVCA and other cancers (Das et al., 2017). Although this evidence implicates *de novo* lipogenesis in OVCA tumour progression, recent reports have indicated that its activation is greatly influenced by the availability of FAs in the tumour microenvironment, thus warranting different therapeutic approaches. Accordingly, the research focus has shifted away from the role of *de novo* lipogenesis in cancer cells to support tumour progression, to the role that stromal cells such as adipocytes in metabolic cross talk with cancer cells might play in this process. In the next section, we will focus on the crosstalk between adipocytes and cancer cells, the metabolic contribution of extracellular FAs to cell proliferation and metastasis, and the role of FAs as a substrate for ATP synthesis.

The metabolic benefit of the adipose rich omentum in tumour progression

The majority of advanced stage HGSOC patients develop omental metastases and recurrent tumours (Sun et al., 2018). The metastatic success of OVCA has been shown to be highly reliant on the adipose-rich TME of the omentum (Tang et al., 2019). This section will focus on the increased availability of lipids in the omentum and their metabolic benefit to support tumour progression, as an alternative to the oncogene-driven *de novo* FA synthesis by cancer cells via glycolysis and glutaminolysis.

Cancer cells are able to facilitate metabolic adaptation allowing the formation of cell aggregates in ascites and survival under hypoxic and nutrient depriving conditions respectively (Cuello et al., 2017). Crosstalk between omental adipocytes and OVCA cells plays a crucial role in homing of OVCA cells to the omentum. Cancer cells stimulate peritumoural adipocytes to undergo delipidation, resulting in a change in the secretome of the adipose tissue and in the dedifferentiation of adipocytes. The adipocyte secretome becomes more inflammatory through the up-regulation of cytokines, adipokines and chemokines such as leptin, interleukin-6 (IL-6) and interleukin-8 (IL-8), which exert a paracrine function on the cancer cells (Duong et al., 2017). Consequently, the migration and proliferation of OVCA cells are promoted by the up-regulation of fatty acid transporters e.g. CD36 and FABP4, which facilitates the uptake of FAs released by adipocytes. Excess FAs can be stored in LDs inside cancer cells, and be released for use in FAO. Fatty acid oxidation, in this case, does not necessarily support ATP production, since FAO has been reported to be uncoupled from ATP production in OVCA cells cultured in an adipose rich environment. Instead, increased

FAO can activate acetyl-CoA carboxylation, the irreversible conversion of acetyl-CoA to malonyl-CoA by ACC (Fig. 4), providing substrates for the biosynthesis of FAs to maintain a state of metabolic remodelling (Cuello et al., 2017).

Leptin, one of the main adipokines secreted by adipose tissue, is known to be pro-inflammatory, pro-angiogenic and pro-tumourigenic, and is involved in various processes contributing to tumour progression in several cancers. Furthermore, it induces the production of other inflammatory cytokines and metalloproteinases promoting tumour invasion and metastasis (Achkar et al., 2019) (Candelaria et al., 2017). Adiponectin on the other hand is typically down-regulated when leptin is increased and has been reported to have a protective role in cancer (Katira & Tan, 2016) (Parida et al., 2019). It has been described as having specific metabolic effects including stimulation of FAO, upregulation of lipid metabolism via activation of the 5' AMP-activated protein kinase (AMPK), p38MAPK, and PPARα pathways, and increased glucose metabolism via upregulation of glucose transporter 4 (GLUT4) (Dalamaga et al., 2012) (Parida et al., 2019).

Clinically, the circulating and intratumoural levels of leptin and adiponectin alone are unlikely to be good predictors of malignancy or clinical outcome, since the up-regulation of leptin is often counteracted by an up-regulation in adiponectin. Thus, both leptin and adiponectin are upregulated in omental metastases when compared to primary ovarian tumours. Therefore, the ratio of leptin:adiponectin has been proposed as a better predictor of malignancy and clinical outcomes (Slomain et al. 2019).

Nieman et al. showed reduced homing of OVCA cells to adipocytes in response to the inhibition of several abundantly secreted cytokines by human omental adipocytes e.g. IL-6, IL-8, and TIMP-1. Furthermore, inhibition of IL-6 and IL-8 receptors resulted in reduced adhesion of fluorescently labelled SKOVip1 OVCA cells to resected human omentum and migration towards omental adipocytes in co-culture (Nieman et al., 2011). They further investigated metabolic alterations in OVCA cells following co-culture with adipocytes, and found an increase in the rate of FAO and the expression of associated proteins, e.g., fatty acid transporter FABP4, the mitochondrial importer of FAs carnitine palmitoyltransferase 1 (CPT1), and increased phosphorylation of the central metabolic sensor AMPK. Consistent with these results, they found increased expression and activation of proteins involved in cancer cell growth and FA metabolism including mTOR, PI3K, total and phosphorylated

ACC, and FABP4 in omental metastases when compared with primary ovarian tumours. Furthermore, inhibition of FABP4 resulted in reduced adipocyte-mediated invasion and lipid accumulation in OVCA cells in co-culture with adipocytes (Nieman et al., 2011). These findings are in line with other reports (Balaban et al., 2017)(Koundouros & Poulogiannis, 2019) (Wang et al., 2017) attributing tumour progression to metabolic reprogramming in the presence of increased extracellular FAs.

The role of increased availability of extracellular fatty acids

Histological examination of solid tumours located at the invasive site consistently shows reduced adipocyte size and number compared to adipocytes associated with the distant site of the tumour (Duong et al., 2017). Furthermore, as detailed below, several membrane-associated FA-binding proteins and transporters are upregulated in cancer cells in an adipocyte-rich TME.

Similar to FABP4, CD36 expression is increased in metastatic omental tumours, and high CD36 expression correlates with greater metastatic potential *in vitro* and *in vivo*, as well as reduced PFS and overall survival (OS) in metastatic HGSOC tumours (Cuello et al., 2018) (Ladanyi et al., 2018) (Wu et al., 2019).

Ladanyi et al. reported that out of several FA transporters up-regulated in OVCA cells cultured with human primary adipocytes, CD36 expression was increased the most independently of the mutational status of TP53, a regulator of lipid metabolism, mutational status, and that this overexpression was linked with increased FA uptake, LD accumulation and cholesterol inside OVCA cells. Co-culture further resulted in the OVCA cell up-regulation of 502 genes including genes associated with lipid metabolism and cholesterol synthesis (p-AMPK, PPARγ, FOXO1, STAT1, HIF-1α), inflammatory cytokines (IL-8), oxidative stress, and the down-regulation of genes involved in FA synthesis and lipogenesis (ACC, SREBPF1/2). Together, this would indicate that in an adipocyte-rich environment OVCA cells reprogrammed their metabolism to reduce or inhibit *de novo* synthesis of lipids, and enhance pathways that facilitate the uptake, usage and storage of extracellular lipids. In addition to playing such a crucial role in FA uptake, CD36 also plays a role in numerous other processes that could be involved in tumour progression such as angiogenesis, cell adhesion and inflammation. Ladanyi et al. described an even wider role for CD36 in OVCA

tumourigenesis and metastasis, by showing that silencing or inhibition of CD36 resulted in reduced colony formation, and reduced adipocyte-stimulated invasion, migration and adhesion of OVCA cells to type-I collagen and laminin, two of the most common extracellular matrix components of the peritoneum (Ladanyi et al., 2018).

TP53 is the best-known tumour suppressor gene and is mutated in 96% of HGSOC cases. More than 80% of mutations in this gene are missense mutations, which do not result in complete loss of protein expression, but in the synthesis of a deficient protein. Accumulating evidence suggests that mutant-TP53 does not only involve the loss of anti-cancer mechanisms, but involves the gain of cancer-promoting features such as metastasis by promoting lipid anabolism (Hu et al., 2013). Hu and colleagues describe roles for TP53 in EMT, adipocytokine (IL-8) stimulated migration of transformed OVCA cells to the omentum, and the up-regulation of FA transporters such as FABP4 to facilitate FA uptake.

The fate of lipids inside cancer cells: lipid droplets and FAO

It is evident that OVCA cells are able to adapt and benefit from the adipocyte-rich TME by stimulating the release of FAs from adipocytes, and increasing the uptake of FAs. However, the fate of these FAs inside the cancer cells remains unclear. As detailed below, recent findings suggest that lipids are essential in oncogenic signalling and supporting the energetic demands of rapidly proliferating and metastatic cells.

LDs are dynamic organelles located in the cytoplasm and are composed of a hydrophobic core of neutral lipids such as triacylglycerol (TAG), cholesteryl esters (CEs) and various proteins, enveloped by a monolayer of phospholipids and covered by structural proteins e.g. perilipin (PLIN) 1 and 4. LDs perform a multitude of functions involving energy metabolism, cell proliferation and apoptosis, and have been shown to accumulate in a variety of cancer cells (Cruz et al., 2020). Increasingly more evidence implicates LDs in tumour progression. Highly proliferating cancer cells have been shown to contain more LDs, suggesting that LDs play a role in this process, and new data associates high LD formation with cell cycle progression (Cruz et al., 2019). In colonic cancer cells, increased LDs stimulated proliferation through the inhibition of forkhead transcription factor 3 (FOXO3) an important factor in suppressing tumour growth (Qi et al., 2013). Cancer aggressiveness, invasion, metastasis and associated poor prognostic outcomes have been tied to the formation of LDs

in breast, ovarian, oral and lung carcinomas, and depended largely on an environment rich in adipocytes and the expression of FA transporters (Cruz et al., 2020). Additionally, current data show that CSCs have high levels of LDs which associate with increased energy demands and the activation of cancer stemness pathways including mainly Wnt/β-catenin (Tirinato et al., 2017), but also NFκB signalling (Cruz et al., 2020).

Several mechanisms of LD formation in cancer have been described, including changes in the expression of genes regulating the uptake of extracellular lipids and *de novo* lipid synthesis, and are affected by environmental conditions such as hypoxia and nutrient availability (Cruz et al., 2020). Lipogenesis plays an important anabolic role in cancer and results in the formation of building blocks for more complex lipids as well as new LDs from which lipids can be mobilized through the action of lipolytic enzymes in a process known as lipolysis. A balance between these two processes is maintained by key regulator of lipid homeostasis SREBP and a sensor of cellular growth mTOR. Increased expression of SREBP1 has been shown to stimulate tumour growth, LD accumulation and the over-expression of lipogenic enzymes (Cruz et al., 2020). The mTOR catalytic complexes mTORC1 and 2 have been shown to be involved in LD formation in cancer (Cruz et al., 2020).

The main neutral lipid found in LDs, TAG, is hydrolysed by three different lipases including adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL), and monoacylglycerol lipase (MAGL), in a sequential manner (Cruz et al., 2020). Additionally, these lipolytic enzymes, responsible for the mobilization of FAs, can also be found in LDs (Cruz et al., 2020). In BRCA Wang et al., 2017 demonstrated the release of FFAs from LDs by ATGL in several tumour cell lines over time, and indicated that ATGL expression correlated with tumour aggressiveness. Cholesteryl esters (CEs), another component of LDs, represent cholesterol in stored form and are synthesized by acyl coenzyme A cholesterol acyltransferase (ACAT). The accumulation of CEs in cancer has been shown to be driven by the loss of PTEN and the upregulation of the PI3K/Akt/mTOR signalling pathway, and is correlated with advanced clinical staging, metastasis and poor survival. Furthermore, inhibition of ACAT significantly reduced cell proliferation, migration and invasion and tumour growth *in-vitro* and *in-vivo* (Cruz et al., 2020). Lipophagy through autophagy is another way in which lipids can be mobilized from LDs, and appears to be a mechanism to increase viability during starvation (Cruz et al., 2020).

FFAs taken up extracellularly, or obtained from lipolysis of lipids in LDs, have been shown to stimulate FAO in cancer cells, thereby contributing to tumour progression. In breast cancer, increased expression of ATGL resulting from co-culture with adipocytes resulted in the release of FFAs from LDs, and a subsequent increase in uncoupled FAO (defined as the oxidation of FAs without the production of ATP) (Wang et al., 2017). CPT1A, a shuttle of FAs into the mitochondrial matrix and the rate-limiting enzyme of FAO, has been shown to be highly expressed in OVCA cell lines, and its overexpression in primary OV tumours correlated with poor OS (Shao et al., 2016). The same report also showed metabolic adaptation of OVCA cells involved greater expression and activity of CPT1A, which resulted in cell cycle progression mediated by increased FAO-derived ATP. Similarly, Nieman et al., 2011 reported increased CPT1 and FABP4 expression, and increased beta-oxidation rates in OVCA cells co-cultured with adipocytes.

Based on this evidence, FAO and LDs represent attractive targets for therapeutic intervention in cancer cells.

TICs and TIC metabolism

The persistence of TICs, a small population of cancer cells with self-renewal and tumour-initiating properties, has been implicated in disease recurrence in OVCA and other cancers. Their ability to become quiescent enables TICs to escape and survive chemotherapy that targets proliferating cells, to later become reactivated by factors in the peritoneal environment, giving rise to new highly resistant tumours. Although the origin of TICs remains unclear, several mechanisms of origin have been described including cell fusion, horizontal gene transfer, mutational stimulation, dedifferentiation of bulk tumour cells under specific conditions, and metabolic reprogramming (Nimmakayala et al., 2019).

The metabolic features of TICs remain poorly understood. However, metabolic flexibility is considered a key feature of TICs, and metabolic reprogramming allows TICs to adapt their biogenetics in a tumour- and TME-specific manner (Pastò et al., 2014) (Anderson et al., 2014). A growing body of evidence indicates greater dependence on OXPHOS and FA metabolism, instead of glycolysis, to support survival and cell proliferation. Accordingly, the relationship between TICs, the adipose-rich omental TME, and alterations in lipid

metabolism prove to be important in understanding the mechanisms involved in the resistant and metastatic nature of HGSOC (Zhao et al., 2019).

Stem cell surface markers or TIC-markers are typically used to identify TICs and include the cluster of differentiation 117 (CD117), CD44, and CD133 across many different cancer types, as well as aldehyde dehydrogenase 1A (ALDH1A) an intracellular protein and another TIC-marker often identified in OVCA (Bezuidenhout & Shoshan, 2019) (Parte et al., 2018). We have previously reported that the chemoresistant SKOV-3-R cell line created by repeated cisplatin treatment of the SKOV-3 OVCA cell line, had gained key TIC properties including an upregulation in the expression of TIC-markers CD117, CD44 and ALDH1A (Gabrielson et al., 2014) (Wintzell et al., 2012). Expression of specific transcription factors regulating embryonic stem cells and developmental pathways, including NANOG, OCT4, SOX2, and MYC are also used for identifying TICs as reviewed in Bezuidenhout & Shoshan, 2019. Although the involvement of these TIC-markers in TIC metabolism remains largely unclear, their expression has been closely associated with OXPHOS and FA metabolism (Li et al., 2017) (Pastò et al., 2014). Furthermore, TICs have been shown to be sensitive to inhibitors of the electron transport chain (ETC) such as rotenone, antimycin A, oligomycin and the diabetic drug metformin, and targeting key lipid metabolic processes such as lipogenesis, lipid uptake, FAO and lipid desaturation has been shown to be effective in eradicating TICs (Yi et al., 2018).

Oxidative metabolism in TICs

In recent years, several studies have reported evidence supporting the preferential use of OXPHOS over glycolysis by TICs. In OVCA, a sub-population of $CD44^+/CD117^+$ TICs, originating from the ascitic effusions of OVCA patients, was found to overexpress key enzymes involved in shuttling pyruvate into the TCA cycle and FA beta-oxidation. When compared to their $CD44^+/CD117^-$ counterparts, these $CD44^+/CD117^+$ cells also demonstrated greater mitochondrial activity evidenced by greater membrane potential and increased sensitivity to ETC inhibitors rotenone, oligomycin, antimycin A, and metformin (Pastò et al., 2014). Furthermore, $CD44^+/CD117^+$ TICs were able to retain their tumour-initiating properties *in vivo* when deprived of glucose, and maintained an OXPHOS dominant metabolic profile whilst entering a quiescent state (Pastò et al., 2014). In lung cancer, a sub-population of urokinase receptor positive ($uPAR^+$) cells or $CD87^+$ TICs were sorted by

fluorescence-activated cell sorting (FACS) and their metabolic profile compared to their non-stem counterparts (Gao et al., 2016). These uPAR^{+} cells preferred to use OXPHOS over glycolysis for ATP production, and inhibition of OXPHOS by oligomycin severely impaired their tumour-initiating and sphere forming abilities (Gao et al., 2016). Similarly, TICs from glioma have shown greater dependence on OXPHOS for ATP production, whilst consuming less glucose and producing less lactate (Vlashi et al., 2011).

Although OXPHOS was coupled to ATP production in the aforementioned studies, mitochondrial oxygen consumption is often increased without the generation of ATP, and uncoupling of OXPHOS and dissipation of the proton gradient is common in cancer. In fact, uncoupling protein 2 (UCP2) is overexpressed in several cancers, but is likely not the only mechanism of uncoupling or dissipation of the proton gradient, since siRNA mediated knock-down of UCP2 did not result in complete abolishment of uncoupling (Pacini & Borziani, 2014). Pathways contributing to biosynthesis such as glutamine metabolism, FA beta-oxidation and pyruvate generated from glycolysis require OXPHOS even in the absence of ATP production. Uncoupled respiration would allow for this scenario.

Lipid metabolism in TICs

In addition to greater dependence on OXPHOS, TICs may rely on lipid metabolism (Fig. 4) for maintaining TIC properties and supporting tumour progression. Specifically, increased FAO, lipid desaturation, LD formation, and *de novo* lipogenesis are reported to be involved in the development and maintenance of TICs (Yi et al., 2018).

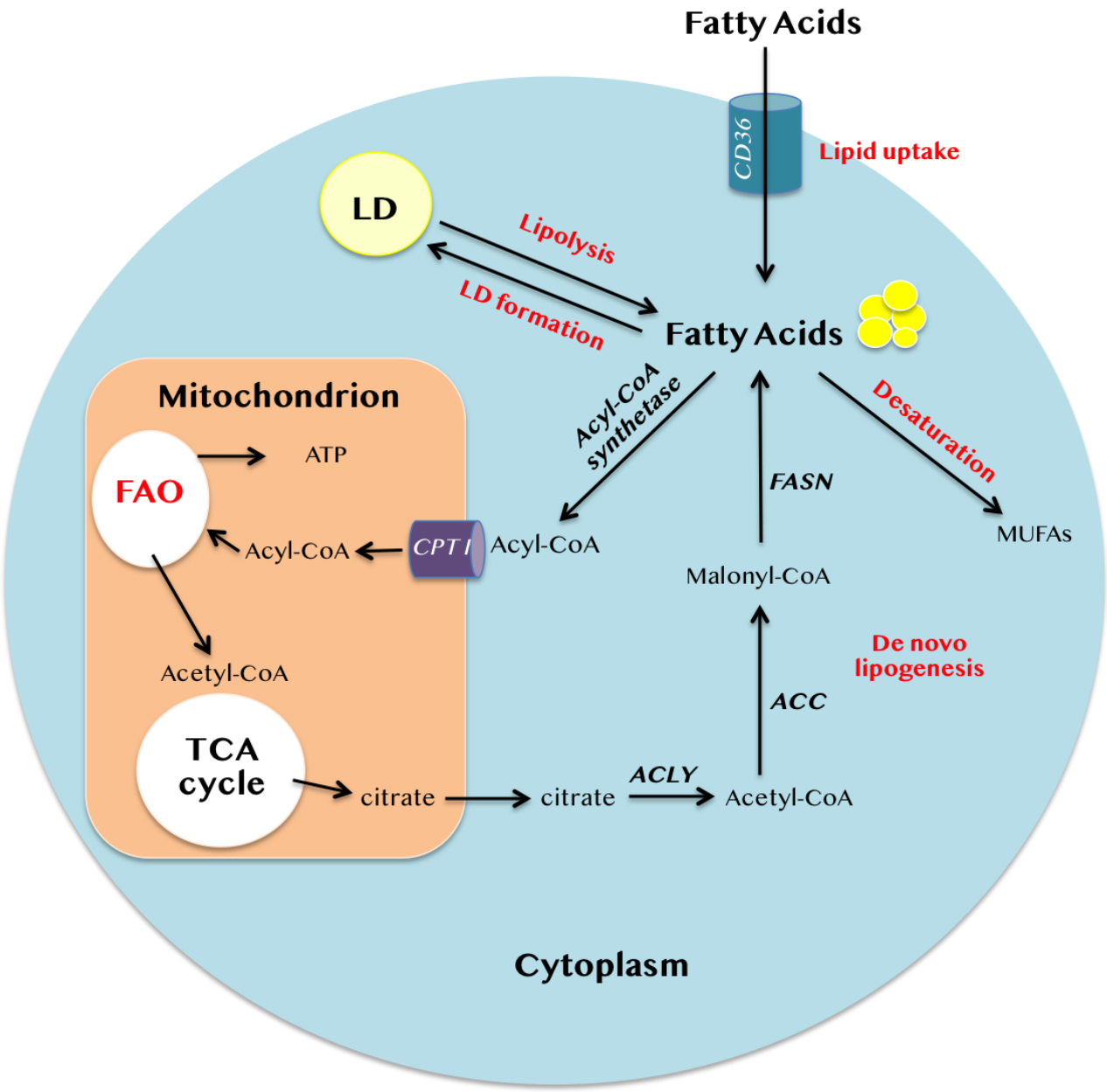


Fig. 4: Lipid metabolism in TICs.

Fatty acids can originate from intracellular (lipolysis of LDs or de novo lipogenesis from citrate) or extracellular (uptake via CD36) sources. These can be metabolized via FAO to produce ATP or new FAs via citrate from the TCA cycle, stored in the form LDs or be desaturated to form unsaturated FAs. Abbreviations: Cluster of differentiation (CD36), lipid droplet (LD), mono-unsaturated fatty acids (MUFAs), fatty acid synthase (FASN), acetyl-CoA carboxylase (ACC), ATP citrate lyase (ACLY), carnitine palmitoyltransferase I (CPT I), fatty acid oxidation (FAO), tricarboxylic acid (TCA), adenosine triphosphate (ATP).

Greater reliance on FAO and less on glycolysis

The activation and upregulation of FAO in TICs have been reported to play a significant role in conserving TIC properties. Chen et al., 2016 used a toll-like receptor 4 (TLR4)/NANOG-dependent and chemoresistant TIC-like (CD133+) cell model capable of inducing hepatocellular carcinoma (HCC) in mice, to show that NANOG-dependent metabolic reprogramming from OXPHOS to FAO is needed to maintain TIC properties including self-renewal, chemoresistance and oncogenesis. Wang et al., 2017 demonstrated that FAO is essential for self-renewal and chemoresistance in BRCA TICs, and that leptin, the leptin receptor and JAK/STAT3 are important role players in this process. Furthermore, they showed that the up-regulation of the FAO rate-limiting enzyme CPT1B is induced by STAT3, and that increased CPT1B expression correlates with poor response to chemotherapy in BRCA patients.

In OVCA, greater desaturation of lipids was found to induce and maintain cancer stemness and tumour initiation capacity in a nuclear factor kappa-B (NF-κB), ALDH1A1, and stearoyl-CoA desaturase-1 (SCD1) dependent manner (Li et al., 2017). In this study, Raman scattering microscopy of single cells and mass spectrometry of extracted lipids was used to show that ovarian $ALDH^{+}/CD133^{+}$ TICs and TIC-enriched spheroids contained significantly more unsaturated lipids compared to $ALDH^{-}/CD133^{-}$ non-TICs and monolayer cultures or OVCA cell lines or primary cells respectively.

Raman spectroscopy revealed that colorectal TICs expressing CD133 contained high levels of LDs when compared to differentiated tumour or normal epithelial cells, and that high LD content conferred greater tumourigenic potential (Tirinato et al., 2015). In BRCA, a sub-population of $CD24^{-}/CD44^{+}/ESA^{+}$ TICs isolated from ductal carcinoma *in-situ* (DCIS) was found to possess greater tumour-initiating ability compared to the corresponding non-TICs, and showed higher expression of lipogenic genes, including a master regulator of lipogenic genes sterol regulatory element-binding protein-1 (SREBP1), enhanced lipogenesis, cell growth and mammosphere formation when compared to non-tumourigenic BRCA cell line MCF10 (Pandey et al., 2013). Furthermore, SREBP1 expression in another DCIS-generating cell line, MCF10AT, significantly increased cell survival *in-vivo* and *in-vitro* (Pandey et al., 2013). In glioblastoma (GBM), Hale et al., 2015, identified a sub-population of $CD113^{+/}$integrin alpha 6^{+} CSCs overexpressing CD36, capable of self-renewal and

possessing tumour initiating capacity. They further reported that high CD36 expression, both at the mRNA and protein level, correlated with poor patient prognosis in glioma and GBM, and that binding of oxidized low-density lipoproteins (LPL), natural ligands of CD36 present in GBM, stimulated cell proliferation in CSCs but not in non-CSCs. In a different study, Yasumoto et al., 2016, used patient-derived glioma stem cells (GSCs) and GSC cell lines to show that activation of de novo lipogenesis and high FASN expression play a major role in the expression of stemness markers CD113, FABP7, SOX2, and nestin, as well as sphere formation and invasivity.

Mitochondrial biogenesis and PGC-1 α in TICs

Functional mitochondria appear to be necessary for maintaining TIC properties, since increased mitochondrial content via the activation of mitochondrial biogenesis is considered a key feature of TICs (Francesco et al. 2018). Furthermore, remodelling of the mitochondrial structure likely accompanies increased OXPHOS and FA metabolism. Several proteins involved in the regulation of mitochondrial biogenesis and structure play an important role in maintaining stemness and functionality (Sancho et al., 2016). One such protein is the master regulator of mitochondrial biogenesis and metabolism peroxisome proliferator-activated receptor gamma co-activator 1 alpha (PGC-1α) (Bost & Kaminski, 2019).

PGC-1α is one of three members of the PGC-1 family, the other two being PGC-1β and PGC-1-related coactivator (PRC). PGC-1α is the most well-known and the first one of the members discovered in brown adipose tissue, an oxidative tissue with a high mitochondrial content focused predominantly on heat production. Its expression is sensitive to specific stimuli, e.g., cold exposure, exercise and nutrient changes, and it plays an important role in several normal biological functions including mitochondrial biogenesis, FAO, FA metabolism and lipogenesis (Bost & Kaminski, 2019). In this way, it has been identified as a metabolic sensor and regulator of oxidative metabolism and mitochondrial biogenesis. Although PGC-1β shares high sequence homology with PGC-1α, and is similarly expressed in oxidative tissues, its expression is not altered in response to external stimuli, and its function, although somewhat overlapping, is limited to maintaining basic mitochondrial properties (Villena, 2015).

PGC-1α expression is regulated by several transcription factors including myocyte enhancer factor-2 (MEF2), activating-transcription factor 2 (ATF2), and the activation of kinases including protein kinase A (PKA) in adipose tissue, and in skeletal muscle; Ca^{2+}/calmodulin-dependent protein kinase IV (CAMKIV), calcineurin A, and p38 mitogen-activated protein kinase (p38 MAPK) (Bost & Kaminski, 2019). PGC-1α activity is regulated by several post-translational modifications including phosphorylation (e.g. AMPK), deacetylation (e.g. SIRT1), acetylation (e.g. GCN5) and methylation (e.g. PRMT1) (Bost & Kaminski, 2019).

PGC-1α interacts with several TFs to modulate specific biological functions, most notably with nuclear respiratory factors 1/2 (NRF1/2), peroxisome proliferator activated receptors α/β (PPARα/β), PPAR-gamma (PPARγ), and estrogen-related receptors α/β (ERRα/β). These are important regulators of the expression of proteins involved in mitochondrial biogenesis (e.g. TFAM, cytochrome c oxidase subunits), with the PPARs playing a prominent role in FAO (e.g. CD36) and UCP1 expression. Other TFs interacting with PGC-1α in regulation of triglyceride metabolism, lipoprotein secretion, CPT1 expression and lipogenesis include farnesoid X receptor (FXR), liver X receptor α/β (LXRα/β), SREBP1/2 and thyroid hormone receptor β (TRβ), respectively (Bost & Kaminski, 2019).

Given the prominent role of PGC-1α in the regulation of oxidative metabolism and mitochondrial biogenesis, and its ability to respond to environmental and physiological stimuli, it is not surprising that PGC-1α plays a role in cancer. In BRCA cell lines and primary and metastatic BRCA tumours, MitoTracker staining was used to identify a subpopulation of CSCs with high mitochondrial mass, increased capacity to form mammospheres, tumour initiation and paclitaxel resistance (Farnie et al., 2015). In invasive breast tumours, PGC-1α expression and activity was found to correlate with increased mitochondrial biogenesis, OXPHOS and enhanced migration and invasivity, and overexpression of PGC-1α was found to be necessary for maintaining CSC properties (Jiang et al., 2003) (Luca et al., 2015). Sancho et al., 2015, reported that pancreatic $CD113^{+}$ CSCs from spheres were more oxidative, showed increased mitochondrial mass and expressed more PGC-1α compared to their highly glycolytic and adherent non-CSC counterparts. In these cells, increased expression of PGC-1α and inhibition of c-MYC, i.e., the MYC/PGC-1α ratio played a key role in the dependency on OXPHOS metabolism and metformin-sensitivity. Further metabolic characterization of these CSCs also revealed the up-regulation of genes involved in lipid metabolism including CPT1 and FABP1 (Sancho et al., 2015).

However, a more tumour-suppressive role for PGC-1α has also been indicated. We have for instance earlier reported that compared to the parental SKOV-3 cells, the chemoresistant TIC-like subclone SKOV-3-R cells expressed no or very low levels of PGC-1α and had 50% more mitochondrial mass (Gabrielson et al., 2014) (Wintzell et al., 2012).

Mitochondrial biogenesis and PGC-1α clearly play an important role in maintaining stemness properties and facilitating tumour progression. Understanding more about the genesis of mitochondria and the role of PGC-1 or other related regulators of this process could assist in targeting TICs.

Mitochondrial dynamics in TICs

Mitochondrial dynamics involves coordinated cycling between two processes called fission and fusion, and facilitates mitochondrial biogenesis. Fission is characterized by the division of one mitochondrion to produce two daughter mitochondria and involves dynamin related protein 1 (DRP1) and adapter proteins including mitochondrial fission protein 1 (FIS1) and mitochondrial fission factor (MFF). The fusion machinery, including mitofusin1 and 2 (MFN1/2) and optic atrophy 1 (OPA1), regulates the merging of two mitochondria into one (Tilokani et al., 2018) (Senft & Ronai, 2016). Mitochondrial shape and intracellular organization/networking are not only regulated by fission and fusion proteins, but include the movement of mitochondria along microtubules which are regulated by mitochondrial Rho GTPases 1 and 2 (MIRO1/2), Milton proteins, and the motor proteins kinesin and dynein (Senft & Ronai, 2016).

In brain and breast TICs, high expression of dynamin-related protein 1 (DRP1) and related mitochondrial fission were found to support TIC properties and correlated with poor prognosis (Katajisto et al., 2015) (Xie et al., 2015). Comparison of nasopharyngeal carcinoma (NPC) TICs to their non-TIC counterparts revealed that metabolic reprogramming is necessary in maintaining TIC properties including chemo-and radioresistance and metastatic capacity (Shen et al., 2015). These TICs displayed greater reliance on glycolysis for ATP production and subtle reorganization of mitochondria from being widely distributed throughout the cell to peri-nuclear. Although mitochondrial respiratory function was reduced, mitochondrial biogenesis was active, and expression of related genes e.g. TFAM and PGC-1α, mtDNA, membrane potential and mitochondrial respiratory enzyme subunits was higher

compared to parental cells. Furthermore, the authors showed that small globular and simple tubular shapes (fragmented mitochondria) were more representative of mitochondrial shape and networking in these TICs, whereas branched networks of elongated mitochondria (fused mitochondria) were reduced (Shen et al., 2015).

Several anti-mitochondrial pharmacological drugs are being tested *in-vitro*, *in-vivo*, as well as in preclinical and clinical studies for their action against TICs (Francesco et al., 2018). In fact, several FDA-approved antibiotics, known to inhibit mitochondrial biogenesis and translation, such as doxycycline and azithromycin have been shown to effectively eradicate TICs in patients with MALT lymphoma (Ferreri et al., 2006) and non-small cell lung tumours (Farnie et al., 2015). These and several other FDA-approved antibiotics targeting mitochondrial biogenesis as an 'off target' effect, e.g. tigecycline and pyrvinium pamoate, have been shown effective in eliminating TICs in twelve cancer cell lines including ovarian (SKOV3), breast (MCF7), and gliobastoma (U-87 MG) (Lamb et al., 2015). In BRCA, the diabetic drug metformin, a complex I inhibitor, was found to selectively target TICs *in-vitro* and when administered in combination with doxorubicin reduced tumour mass and prevented tumour recurrences in a xenograft mouse model (Lee et al., 2014) (Hirsch et al. 2009). In OVCA, metformin inhibited growth and proliferation of TICs *in-vitro*, an effect that was additive when used in combination with cisplatin. Similarly, metformin inhibited TIC sphere formation *in-vitro* and in patient tumours, and enhanced the cytotoxic effects of cisplatin in cell line xenografts (Shank et al. 2012).

Although a relationship between mitochondrial dynamics, tumour progression and other pathologies exists, no consistent connection has been established between mitochondrial dynamics/shape and mitochondrial function.

Obesity in cancer

Many studies have shown that high adiposity correlates with EOC risk, including a meta-analysis of 47 epidemiological studies with 25 157 EOC patients that revealed an increased risk of developing EOC with higher body mass index (BMI) and body weight (Tang et al., 2019). The chronic inflammatory environment present in obesity correlates with higher EOC risk and has been shown to influence cancer cell behaviour. Overweight and obese OVCA patients have worse clinical outcomes than their leaner counterparts, and despite similar

cancer driver gene expression patterns have worse prognoses when tumours from these patients express high levels of obesity and lipid metabolism related genes, e.g. CD36 and TFG-β (Tang et al., 2019). Furthermore, obesity has been shown to drive ectopic LD accumulation in non-adipose tissues in metabolic disease such as insulin-resistance and in cancer (Cruz et al., 2020).

Summary and knowledge gaps

Ovarian cancer is a complex disease. It consists of several subtypes and is characterized by a multitude of genetic and molecular properties, oncogenic mutations, and metabolic alterations making treatment all the more difficult. In depth characterization of OVCA and investigation of the mechanisms involved in OVCA development and progression has led to better categorization, diagnosis and treatment of the disease. Although targeted therapy in conjunction with standard-of-care debulking surgery and adjuvant cytotoxic chemotherapy have produced promising outcomes, chemoresistant and metastatic disease remain two of the major challenges of OVCA. The substantial contribution of these two factors to poor patient outcomes and disease progression has largely been attributed to homing of OVCA cells to the adipose-rich omental TME and the resistance of TICs to conventional chemotherapy. Increased availability of extracellular FAs from adipocytes activates metabolic reprogramming of OVCA cells and TICs to facilitate survival and support cell proliferation. As described in detail in the background section, multiple reports implicate metabolic switching from glycolysis to OXPHOS with greater reliance on FAO, as one of the driving factors in adapting to the adipose-rich metastatic site. Despite promising results from several FDA-approved drugs targeting OXPHOS and lipid metabolism both pre-clinically and clinically, much about the metabolic differences between primary ovarian tumours and omental metastases and their association with patient outcomes remains unknown. A better understanding of tumour metabolism could improve the development of treatment strategies and patient prognosis. Furthermore, the as yet undefined metabolic properties of TICs add to the challenge of targeting these elusive cells. The roles PGC-1 might play in regulating OXPHOS and lipid metabolism as well as TIC-ness motivate further investigation into related mechanisms. It is not known whether PGC-1 isoforms affects the expression of TIC-markers, nor processes related to FA metabolism or mitochondrial respiration and networking/organization in cancer cells. Nor is it known how different growth conditions might affect these metabolic and mitochondrial properties.

AIMS

We therefore aimed to identify a metabolism-specific gene expression category with potential prognostic value, in HGSOC tumours. Similarly, and based on a small Swedish cohort of omental metastases, and an Italian cohort of omental, sigmoid and diaphragm metastases, we also aimed to contribute to understanding the cellular metabolism of omental metastases and how it relates to various patient outcomes.

We further aimed to investigate, in an *in-vitro* study of TIC-like OVCA cells, the effects of PGC-1 expression on the expression of TIC-markers and genes related to lipid metabolism and mitochondrial biogenesis, cisplatin resistance, ATP production, mitochondrial OXPHOS coupling efficiency, networking and organization and LD accumulation. In the same in-vitro model, we aimed to examine the effects of different nutrient conditions on these metabolic and mitochondrial properties. Lastly, we aimed to examine the associations between the expression of PGC-1 isoforms and mitochondria-related genes in the same Swedish cohort of primary ovarian tumours and omental metastases.

METHODS AND MATERIALS

Karolinska Institute (KI) cohort

Patients and tumour specimens

All clinical material was collected after informed consent from the patient. The studies have been approved by the regional ethics committee in Stockholm, Sweden and have been conducted according to the Declaration of Helsinki's principles. Primary ovarian and metastatic omental tumour tissue samples were collected from 37 patients (mean age=65 years) with pathologically confirmed ovarian cancer from 2013-2016. Inclusion criteria were HGSOC, Stage IA-IVB disease according to the International Federation of Gynaecology and Obstetrics (FIGO), and availability of clinical data and tumour tissue samples. All tumours were collected during the debulking surgery, embedded in optimal cutting temperature compound (OCT) and stored at -80 degrees Celsius.

Following initial histopathological evaluation of the tumours by a pathologist, 10 µM-thick sections were stained for hematoxylin and eosin (H&E) and the tumour content evaluated. Sections with more than 60% tumour cells were included (Fig 6). Since the focus of the study was metabolic/mitochondria-related tumour cell gene expression, the sections were also evaluated for the contamination of adipocytes. The average number of adipocyte nuclei/mm^2 tissue was counted, and the percentage of adipocytes per total area of tissue calculated. Sections with more than 12% adipocytes per total area were excluded (Fig. 6). We did not evaluate the sections for other cell types, such as immune cells, as it was not the focus of this study.

RNA extraction and evaluation of purity and integrity

The OCT was removed and 10µM-thick tumour sections approximating 20-30 mg of tissue were collected and used to extract RNA (Qiagen Mini RNeasy-Plus kit, Cat No./ID: 74136). Initial RNA concentrations and purity were determined using the Nanodrop, followed by more thorough quality control checks using the 2100 Agilent Bioanalyzer and the Agilent RNA 6000 Pico Kit (Cat No. 5067-1513) to assess RNA integrity, concentration and purity. Samples with RNA integrity (RIN) values lower than 8 were excluded.

cDNA library preparation and RNA sequencing

Total RNA was couriered to Novogene in Hong Kong, China, for sequencing. All RNA was preamplified using the SMART-Seq™ v4 Ultra™ Low Input RNA kit for sequencing

(Clontech). Amplified ds-cDNA was then purified with AMPure XP beads and quantified with Qubit. cDNA was sheared using the Covaris system, and sheared fragments end-repaired, A-tailed and ligated to sequencing primers. Fragments of 200 bp were selected before PCR enrichment was performed. Library concentrations were then determined using a Qubit 2.0 fluorometer (Life Technologies), the insert size checked with the Agilent 2100 and finally quantified using quantitative PCR. Sequencing was performed with the Illumina PE150 machine. Raw data from the Illumina was transformed into sequenced reads by base calling, and quality assessment of the data and mapping to the reference genome was performed.

The gene expression level was determined by counting the reads that map to genes or exons, where the read count is proportional to the actual gene expression levels, the gene length and the sequencing depth. The expected number of Fragments Per Kilobase of transcript sequence per Millions base pairs sequenced (FPKM) was used to ensure that the gene expression levels estimated from different genes and experiments are comparable. HTSeq software (union mode) was used to analyze gene expression levels. An FPKM value of 0.1 or 1 is set as the threshold for determining the expression of a gene or not. Differential gene expression and enrichment (Gene Ontology and KEGG pathway) analysis was performed. Enrichment analysis of differentially expressed genes (DEGs) allows assessment of which biological function or pathways significantly associate with DEGs. Gene Ontology (GO, http://www.geneontology.org/) annotates genes to biological processes, molecular functions and cellular components, whereas Kyoto Encyclopedia of Genes and Genomes (KEGG) annotates genes to pathway level.

Based on these analyses we established 2 gene categories: mitochondria associated DEGs (MAD) and lipid metabolism, lipid droplet and inflammation associated DEGs (LMID) comprised of 13 and 56 genes respectively (Fig. 9). A further 7 categories were established, to assess the prognostic value of LMID and MAD. These categories, comprised of both DEGs and non-DEGs, are named "*mixed mitochondria-associated or MMA"*, "*lipid metabolism*", "*lipid particle organization*", "*triglyceride metabolism*", "*composition of lipid particles*", "*regulation of lipid metabolism*", and "*glycolysis/ gluconeogenesis*".

Statistical analysis

The statistical software SPSS was used to perform Student's t-tests, to evaluate differences in gene expression between primary tumours and metastases. Graphs were generated using GraphPad Prism version 9.0. As a preliminary test of the relationships between clinical outcomes and gene expression; SPSS software was used to perform Chi-squared tests on samples in the KI-cohort to evaluate relationships between high and low levels of expression of specific genes and survival or clinical resistance. To construct heatmaps and correlation matrices, FPKM count data for genes of interest were extracted for both primary tumours and metastases, and plotted using GraphPad Prism version 9.0. Pearson correlation analysis was used to test for relationships in gene expression.

Pan-cancer dataset

Data retrieval and application of gene categories

Pan-cancer RNA-sequencing data were accessed from NIH genomic data commons (GDC) database (https://gdc.cancer.gov) along with matching patient clinico-pathological information. All data quality control, normalisation and gene level counts were performed by the PanCanAtlas investigators as described in the original publication (Hoadley et al. 2018). The data were subsequently subsetted to include ovarian cancer patients only, N = 304 in total.

In order to assess how expression of lipid metabolism and mitochondrial genes influence ovarian cancer patient survival, we applied 8 of the 9 gene categories to this data using previously defined methodology (Desmedt et al. 2008). Only 8 categories were established at the time of this analysis. Briefly, for each category, annotated genes were extracted from the ovarian cancer dataset and summed column-wise, resulting in a category score for every tumour. This continuous variable was then divided into tertiles of low intermediate and high expression as described in the original publication.

Survival analysis and feature selection

Kaplan-Meier analysis was performed on the ovarian cancer samples of the Pan-cancer (TCGA-derived) dataset using the *survival* (version 3.1 - 8) and *survplot* (0.0.7) R packages to compare gene category tertiles with OS as the clinical endpoint. Feature selection was

performed as part of a Cox proportional hazards regression model with genes from the "*gene selection*" category only using the "stepAIC" function of the *MASS* (7.3 - 51.5) package with the direction set to "backward". All analyses were run within the R-statistical programming environment version 3.6.2 (R Core Team 2019).

METABRIC and TEX datasets

Data retrieval and application of gene categories

METABRIC and TEX RNA-sequencing data were accessed from the European Genome-phenome Archive (EGA) database (http://github.com/cclab-brca) and ClinicalTrials.gov database ((http://www.clinicaltrials.gov/ct2/show/NCT01433614) along with matching patient clinico-pathological information. All data quality control, normalisation and gene level counts were performed by the METABRIC and TEX investigators as described in the original publications (Pereira et al., 2016) (Tobin et al., 2015). The data were subsequently subsetted to include primary BRCA patients (N=1971), and metastatic BRCA patients (N=109).

In order to assess how expression of GDP1, CIDEA, and FABP4 genes influence BRCA patient survival, we applied these genes to this data using previously defined methodology (Desmedt et al. 2008). Briefly, annotated genes were extracted from the BRCA datasets and summed column-wise, resulting in a gene score for every tumour. This continuous variable was then divided into tertiles of low intermediate and high expression as described in the original publication.

Survival analysis and feature selection

Kaplan-Meier analysis was performed on the BRCA samples of the METABRIC (primary BRCA) and TEX (metastatic BRCA) datasets using the *survival* (version 3.1 - 8) and *survplot* (0.0.7) R packages to compare gene category tertiles with breast cancer specific survival (BCSS) and relapse to death (years) as the clinical endpoints in primary BRCA tumours and metastatic BRCA tumours respectively. Feature selection was performed as part of a Cox proportional hazards regression model with genes from the "*gene selection*" category only using the "stepAIC" function of the *MASS* (7.3 - 51.5) package with the

direction set to “backward”. All analyses were run within the R-statistical programming environment version 3.6.2 (R Core Team 2019).

University of Bologna (UNIBO) cohort

Patients and tumour specimens

All clinical material was collected after informed consent from the patient, and according to internal review board protocols. The studies have been conducted within the frame of the Mitochondria in Progression of Endometrial and Ovarian cancer (MIPEO) study, approved by the regional ethics committee in Bologna, Italy, and conducted according to the Declaration of Helsinki’s principles. Primary ovarian and metastatic omental, diaphragm and sigmoid tumour tissue samples were collected from 23 patients (mean age=65 years) with pathologically confirmed ovarian cancer from 2016-2019. Inclusion criteria were high-grade serous ovarian cancer, Stage IA-IVB disease according to the International Federation of Gynaecology and Obstetrics (FIGO), and availability of clinical data and tumour tissue samples. All tumours were collected during debulking surgery, and stored at -80 degrees Celsius.

Following initial histopathological evaluation of the tumours by a pathologist, 10 µM-thick sections were stained for hematoxylin and eosin (H&E) and the tumour content evaluated. The sections were also evaluated for the contamination of adipocytes. The average number of adipocyte nuclei/mm^2 tissue was counted, and the percentage of adipocytes per total area of tissue calculated. Samples with more than 12% adipocytes and less than 60% tumour content were excluded.

RNA extraction and evaluation of purity and integrity

Approximately 20-30 mg of tissue was used to extract total RNA (Qiagen Mini RNeasy-Plus kit, Cat No./ID: 74136). Initial RNA concentrations and purity were determined using the Nanodrop.

cDNA preparation and RT-PCR

Each 300ng RNA/sample was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Life Technologies). Quantitative Real-Time PCR (qRT-PCR) was performed, using TaqMan Fast Advanced PCR Master Mix (Cat No. 4444556) and TaqMan Array 96-well Fast Custom plates (Cat No. 4413263), on the Applied Biosystems 7500 Fast Real-Time PCR System using the following cycling conditions: 95^{o}C for 20 sec, 40 cycles of 95^{o}C for 3 sec and 60^{o}C for 30 sec. Each reaction was performed in duplicate. The relative expression was normalized to the peptidylprolyl isomerase A (PPIA) ovarian cancer housekeeping gene, and the gene expression was calculated using the 2-ΔΔCT-method.

Statistical analysis

Fold gene expression data was log10 transformed and plotted using the statistical software R. SPSS statistical software was used to calculate differences between groups. One-way ANOVA with a Tukey's posthoc test was used for multiple comparisons of continuous data. A p-value < 0.05 was considered significant.

In-vitro studies

Cell culture

The chemoresistant SKOV-3-R cell line, created by repeated cisplatin treatment of the SKOV-3 ovarian cancer cell line, had lost PGC-1α expression and gained key properties of cancer stem cells, or tumor-initiating cells (TICs) (Gabrielson et al., 2014; Wintzell et al., 2012). As SKOV-3 cells originate from patient ascites, they survive in suspension and represent progression from a primary tumor; being metastatic they are accordingly often used for xenografting. Together, the increased chemoresistance and motility, the expression of TIC markers such as CD117 and ALDH1A, and the ability to form spheres in stem cell medium (Wintzell et al., 2012) indicate that SKOV-3-R cells represent yet another progression step.

The cell lines SKOV-3-P and SKOV-3-R were cultured in RPMI-1640 medium (Standard Medium; SM; Hyclone; GE Healthcare), containing 11 mM glucose and supplemented with 2 mM L-glutamine, 10% FBS, 1% Penstrep, at 37°C and 5% CO2. Low glucose medium 1 (LGM1) (1 mM glucose, 2 mM glutamine 0.1% FBS, 1% Penstrep) and low glucose medium 2 (LGM2) (1 mM glucose, 2 mM glutamine 10% FBS, 1% Penstrep) were prepared using

Dulbecco's Modified Eagle's Medium (DMEM) (#D5030; Sigma Aldrich). 100 µM Oleic acid-BSA (bovine serum albumin) conjugate (#O3008; Sigma Aldrich) and 10 mM 2-deoxy-D-glucose (2DG; glycolysis inhibitor) (#D6134; Sigma Aldrich) was added to LGM1 or LGM2 to generate LGM1/LGM2 + OA and LGM1/LGM2 + OA + 2DG.

SRB assay

Using sulforhodamine B (SRB; Sigma-Aldrich) dye, total remaining cellular protein was assessed colorimetrically at t=0 and after the indicated treatments, in order to evaluate fold growth and IC50 values for cisplatin treatments. Cells were seeded in triplicate in 96-well plates (3000/5000 cells/well in 200 µl of SM). Following overnight incubation, the cells were treated for 72 h with a range of concentrations in the indicated culture media. The supernatants were then removed; cells were gently rinsed with PBS before precipitation and staining of the cellular proteins according to manufacturer´s instructions.

Transfection of siRNA

SKOV-3-P and SKOV-3-R were seeded in 6-well plates (250 000 cells/well in 2 mL of SM). Following overnight incubation, the cells were transfected using VIROMER® GREEN (Lypocalyx), according to manufacturer's instructions. The next day the cells were washed with PBS, fresh medium was added, and siRNAs, all at 2.8 µM final concentration. Three different siRNAs were used for PPARGC1A (PGC-1α) (Ambion), four for PPARGC1B (PGC-1β) (Qiagen), and one scrambled siRNA (Ambion) as control. Transfection efficiency over several experiments: KD of PGC-1α in P cells reduced expression by 70%, and KD of PGC-1β resulted in a 61% and 25% reduction in P and R cells respectively.

RNA extraction

Following 24 or 48 h siRNA treatment, cells were harvested using 500 µl of TrypLE (Gibco), and the pellet washed with PBS before extraction. RNA was extracted using the RNeasy Plus Mini Kit (Qiagen), and quantified using NanoDrop.

Quantitative Real-Time PCR

300ng RNA/sample was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Life Technologies). Quantitative Real-Time PCR (qRT-PCR) was performed, using TaqMan Fast Universal PCR Master Mix and various TaqMan Gene Expression Assays, on the Bio-Rad CFX96 Real-Time PCR System using the following cycling conditions: 95^{o}C for 20 sec, 40 cycles of 95^{o}C for 3 sec and 60^{o}C for 30 sec. Each reaction was performed in duplicate with two biological replicates. The relative expression

was normalized to GUSB and the fold gene expression was calculated using the 2-ΔΔCT-method.

Confocal microscopy

The fluorescent dyes MitoTracker™ Red CMXRos 579/599 (Thermo Fisher Scientific), and BODIPY 493/503 (#790389; Sigma Aldrich) were used to detect mitochondria and neutral lipids, respectively, in fixed cells. SKOV-3-P and SKOV-3-R cells were seeded onto coverslips in 6-well plates (100 000 R cells and 180 000 P cells/well in 2 ml SM). Following overnight incubation cells were treated with low glucose medium 1 (LGM1) (1 mM glucose, 2 mM glutamine 0.1% FBS, 1% Penstrep), or LGM1 + 100 µM oleic acid for 72 h. After treatment, cells were washed with 1x PBS, stained and fixed according manufacturer's instructions. The VECTASHIELD® Mounting Medium with DAPI (VECTOR laboratories) was used to mount the slides, and the Leica TCS SP5 confocal microscope used to capture the images.

ATP assay

The ATP fluorometric assay kit (#MAK190; Sigma Aldrich) was used to determine the ATP concentration following various treatments, and is based on the formation of a fluorometric product from the phosphorylation of glycerol that is proportional to the amount of ATP present. SKOV-3-P and SKOV-3-R cells were seeded in 6-well plates (300 000 cells/well in 2 ml SM). Following overnight incubation, cells were washed with PBS and treated with SM, LGM1, LGM1 + 100 µM OA or LGM1 + OA 100 µM + 10 mM 2-deoxy-D-glucose (2DG; glycolysis inhibitor) (#D6134; Sigma Aldrich) for 72 h. Samples were prepared according to manufacturer's instructions.

High-resolution respirometry

The O2K oxygraph (Oroboros Instruments, Innsbruck), a high-resolution respirometer (HRR) was used to assess mitochondrial oxygen consumption in permeabilized SKOV-3-P and SKOV-3-R cells in the presence of fatty acid oxidation (FAO) substrates following various treatments.

SKOV-3-P and SKOV-3-R cells were seeded in 6-well plates (2 x 300 000 cells/well in 2 ml SM). Following overnight incubation, cells were washed with PBS and treated with either SM, LGM1, LGM1 + 100 µM OA or LGM1 + 100 µM OA + 10 mM 2DG for 72 h. A separate set of experiments was performed using SKOV-3-P and SKOV-3-R cells transfected

with siRNA (scrambled, PPARGC1A, PPARGC1B) as previously described. Following treatment, all samples were prepared for Oxygraph analysis in the same manner. Cells were washed with PBS and trypsinized. RPMI was added to neutralize the trypsin and cells were collected in a 15 ml centrifuge tube and pelleted at 1500 rpm for 4 min at 25°C. The supernatant was carefully removed and the pellet resuspended in 2.2 ml of MiR05 respiration medium (0.5 mM EGTA, 3 mM $MgCl_2.6H_2O$, 60 mM lactobionic acid, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 100 mM D-sucrose and 1 g/l BSA), before insertion into the oxygraph chamber. A customized substate-uncoupler-inhibitor-titration (SUIT) protocol was used to measure oxygen (O_2) consumption in various respiratory states. The O_2 range during experiment was maintained at 250-80 nmol/ml. O_2 was injected into the gas phase in the chamber (reoxygenation) when appropriate. Mitochondrial coupling control states were determined with the sequential titration of substrates as listed in the optimized SUIT protocol.

SUIT protocol:

1) Cells
2) Oxygen range (250-80 nmol/ml)
3) ROUTINE respiration (intact cell – mitochondrial respiration using endogenous substrates)
4) Digitonin (Dig) 8.1 mM stock into chamber – 4.1 µM f.c. (permeabilization of plasma membrane)
5) Malate (M) 0.4 M stock – 2 mM f.c.
6) Octanoyl carnitine (Oct) 100 mM stock – 0.2 µM f.c.
7) ADP (D) 0.4 M stock – 5 mM f.c.(optimal conc. evaluated 5 mM f.c. determined)
8) Cytochrome c (c) 4 mM stock – 10 µM f.c.
9) FCCP uncoupler (U) 1 mM stock – 0.5-5 µM f.c.
10) Antimycin (Ama) 5 mM stock – 2.5 µM f.c.

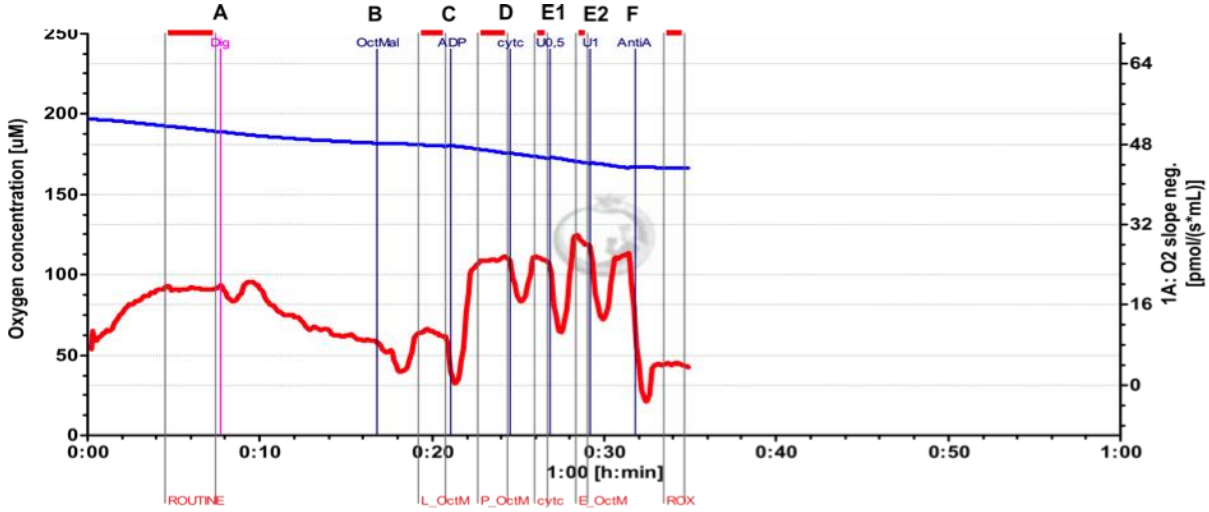


Fig. 5: Representative trace of the SUIT protocol. (A) *Digitonin,* ***(B)*** *Octanoyl carnitine + Malate,* ***(C)*** *ADP,* ***(D)*** *cytochrome c,* ***(E1, E2)*** *FCCP,* ***(F)*** *Antimycin A. O2 slope neg. (oxygen flux) (red line). Oxygen concentration (blue line).*

The cytochrome c control factor (FCFc) was calculated for all samples as a measure of outer mitochondrial membrane integrity. An FCFc of <10% was considered normal, i.e., the outer mitochondrial membrane was not damaged and cytochrome c was not leaking into the inter-membrane space. Furthermore, for the analysis, data was normalized to protein content (mg/ml) determined by the BCA protein determination method after retrieval of the full contents of the chambers (2 ml of sample) after each experiment.

Statistical analysis

SPSS software, R, or GraphPad Prism version 9.0 was used for statistical analysis**.** Independent Student´s t-test or Pearson correlation for comparisons between two groups, or One-way ANOVA with a Tukey's posthoc test was used for multiple comparisons of continuous data. A p-value < 0.05 was considered significant.

RESULTS

Tumour metabolism and patient prognosis

Evidence suggests a role for lipid metabolism, mitochondria and obesity related genes in OVCA tumour progression. A better understanding of tumour metabolism, as reflected in the expression of metabolism-related genes in primary tumours and metastases from HGSOC patients and their association with PFS and OS, could improve the selection of treatment strategies and patient prognosis.

Similar clinical characteristics in KI and UNIBO cohorts

A total of 18 and 23 HGSOC patients were included in the cohorts from KI and UNIBO respectively. All patients had a mean age of 65 years. All or almost all patients (94% KI-cohort, 100% UNIBO-cohort) were diagnosed with late stage (FIGO stage > IIIA) metastatic disease. Patient height (cm) and weight (kg) were collected prior to surgery, and body mass index (BMI) calculated and used to categorize patients into lean (BMI≤25) or overweight/obese (BMI ≥ 25). All patients were subject to follow-up to monitor clinical resistance (CR), defined as relapse within 6 months after chemotherapy, and survival.

Clinical data are presented in Table 1. The associations between metastasis, CR, OS and BMI were tested in both cohorts, with the exception of survival in the cohort from UNIBO since 100% of the patients are still alive and the earliest diagnosis dates back to 2016 (4 years ago). Clinically resistant patients were less likely to survive (data not shown; p=0.001). Patients with metastases at diagnosis were not more likely (p=0.95) to become clinically resistant. Patients with a BMI ≥ 25.0 (overweight/obese) were not more prone to metastasis (p=0.41), CR (p=0.15), or shorter survival (p=0.85). Nor did metastatic patients show shorter survival (p=0.41).

Table 1: Clinical data in two cohorts of high-grade serous ovarian cancer.

	N (%) KI cohort	N (%) UNIBO cohort
Total number of patients	18 (100)	23 (100)
FIGO stage ≥ IIIA	17 (94)	23 (100)
Patients with metastases at diagnosis	14 (78)	23 (100)
Relapse within 6 months after chemotherapy	5 (27)	1 (4,3)
Patients still alive	9 (50)	23 (100)
BMI ≥ 25 (overweight/obese)	7 (39)	9 (39)
Primary ovarian tumours	n=9	n=22
Metastatic omental tumours	n=12	n=16
Metastatic sigmoid colon tumours	NA	n=8
Metastatic diaphragm tumours	NA	n=12

NA: not applicable

Adipocytes detected in the TME of omental metastases but not primary tumours

The TME of omental metastases, unlike that of primary ovarian tumours, is known to be rich in adipocytes (Alagumuthu et al., 2006), and adipocytes in direct contact with cancer cells at the invasive site are known to release FAs (Duong et al., 2017) (Wu et al., 2019). Based on visual inspection of all H&E stained tumour sections, only omental metastases were infiltrated with adipocytes (Fig. 6), confirming the difference in adipocyte content in the TMEs of primary ovarian tumours and omental metastases. Additionally, intratumoural or tumour-associating adipocytes were smaller in size compared to adipocytes distal to the tumour (Fig. 6). This is in line with previous reports on adipocyte size and number in which the histological examination of solid tumours located at the invasive site consistently showed reduced adipocyte size and number compared to adipocytes associated with the distant site of the tumour (Duong et al., 2017). Furthermore, it corresponds with reports on the upregulation of FA transporter FABP4 in OVCA cells associating with adipocytes in omental metastases to increase the uptake of FAs (Nieman et al., 2011).

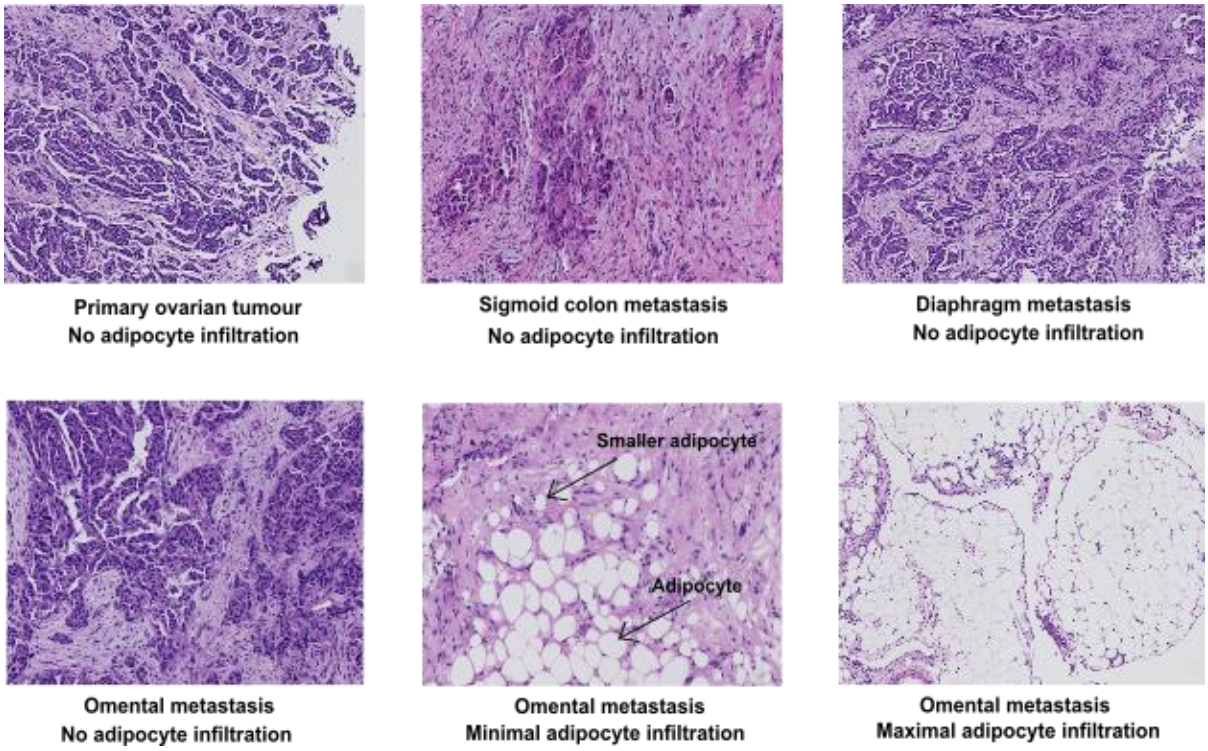


Fig.6: Hematoxylin and Eosin staining of tumour sections. *Primary ovarian tumours and omental, diaphragm, and sigmoid colon metastases from both the KI- and UNIBO-cohorts were evaluated for tumour and adipocyte content. Images captured using Hamamatsu NanoZoomer Slide Scanner at 40x magnification. Images featured were digitally enlarged.*

Lipid metabolic gene expression upregulated in omental metastases

To evaluate differences in the expression of specific genes between tumours with distinct TMEs, we compared gene expression and enrichment of pathways between primary ovarian tumours and omental metastases. Indeed, differential expression analysis of the RNA-seq data from the KI-cohort revealed an up-regulation of 472 genes in omental metastases when compared to primary ovarian tumours (Fig.7A). Based on the previously reported involvement of genes associated with mitochondrial FAO in OVCA tumour progression and clinical outcomes (Nieman et al., 2011) (Shao et al., 2016), we expected elevated expression of mitochondria-associated (MAD) genes in omental metastases. However, very few MAD-genes were differentially expressed (Fig. 7B) and those that were, were downregulated predominantly in omental metastases, including genes involved in apoptosis, respiratory chain complex assembly and activity, mitochondrial membranes, and transcription of mitochondrial genes.

In line with an adipose-rich TME, a number of differentially expressed genes (DEGs) were identified in omental metastases including genes associated with lipid metabolism (FA transport, lipolysis, lipogenesis, and LD formation), adipocytokines and related receptors (e.g. LEP, ADIPOQ, CXCR2), as well as other inflammatory cyto- and chemokines (e.g. PF4, IL7R, CFD, SAA1) (Fig. 7B; LMID category). Similarly, Gene Ontology and KEGG pathway analysis revealed the enrichment of a number of biological processes (BP), molecular functions (MF), cellular compartments (CC) and KEGG pathways in omental metastases. These included lipid metabolic processes, lipid droplet formation, PPAR-signalling, FA degradation, adipocytokine signalling, cell growth/proliferation, cell adhesion, extracellular matrix organization, cell motility, and inflammatory/immune processes (Fig. 7C). The gene expression profile and enrichment of processes observed in omental metastases are clearly different to that of primary ovarian tumours, and most likely reflect variations in their TMEs. Based on our analysis, omental metastases from the KI-cohort could be characterized as proliferative tumours with enhanced lipid metabolic capabilities.

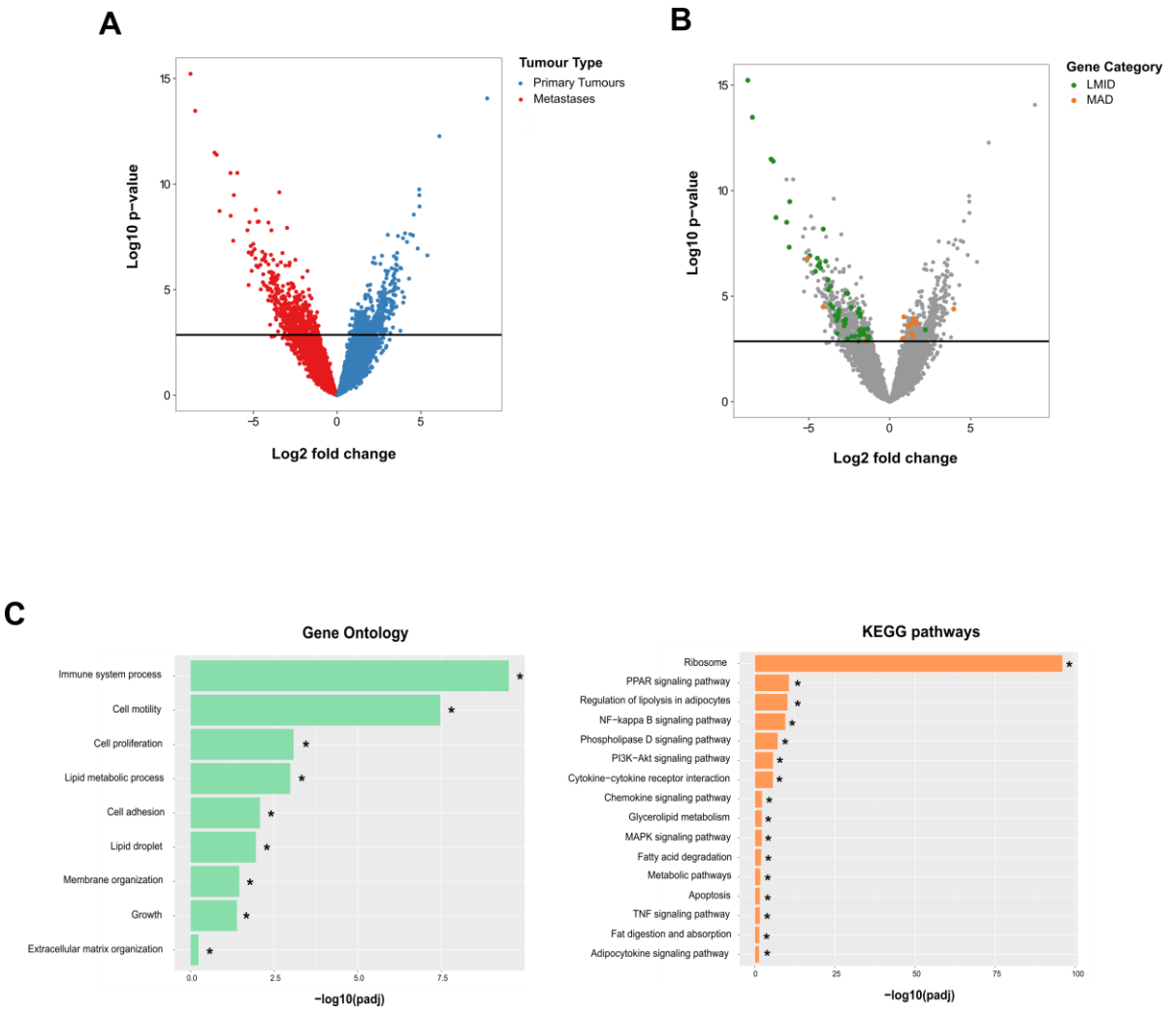


Fig. 7: Differential gene expression and enrichment in primary tumours and metastases from HGSOC patients. ***(A)*** *Differential expression between primary ovarian tumours (blue; right) (n=9) and omental metastases (red; left) (n=12) from the KI-cohort.* ***(B)*** *LMID category (green; left) and MAD category (orange; right); each dot represents a gene.* ***(C)*** *Selected Gene Ontology terms and KEGG pathways enriched in omental metastases (n=12) when compared to primary ovarian tumours (n=9) from the KI-cohort: data represented as –log10 of adjusted p-values for comparisons between primary tumours and metastases. * P<0.05 indicates significant differences.*

Low expression of genes from the LMID-category associated with better OS in primary tumours

While differential expression and enrichment findings further outline differences between primary ovarian tumours and omental metastases, the relationships between gene expression and patient outcomes could provide important information for the selection of treatment strategies and prognosis in HGSOC patients. We established two gene categories, firstly the lipid metabolism, inflammatory/immune-related, and adipocytokine associated DEGs or

LMID category (56 genes) (Fig. 8) and secondly, the mitochondria-associated DEGs or *MAD category* (13 genes) (Fig. 8). Some of the genes in LMID and MAD are already known to be associated to clinical outcomes, e.g., CD36, CXCR2/IL-8R, COX7B and AIFM2, whereas others are novel, i.e., have not been reported in this regard, e.g., CIDEA, CFD, TFB2M and FASTKD3. A further 7 related categories comprised of both DEGs and non-DEGs were established independently of the above findings, to test the prognostic value of LMID and MAD in clinical outcomes.

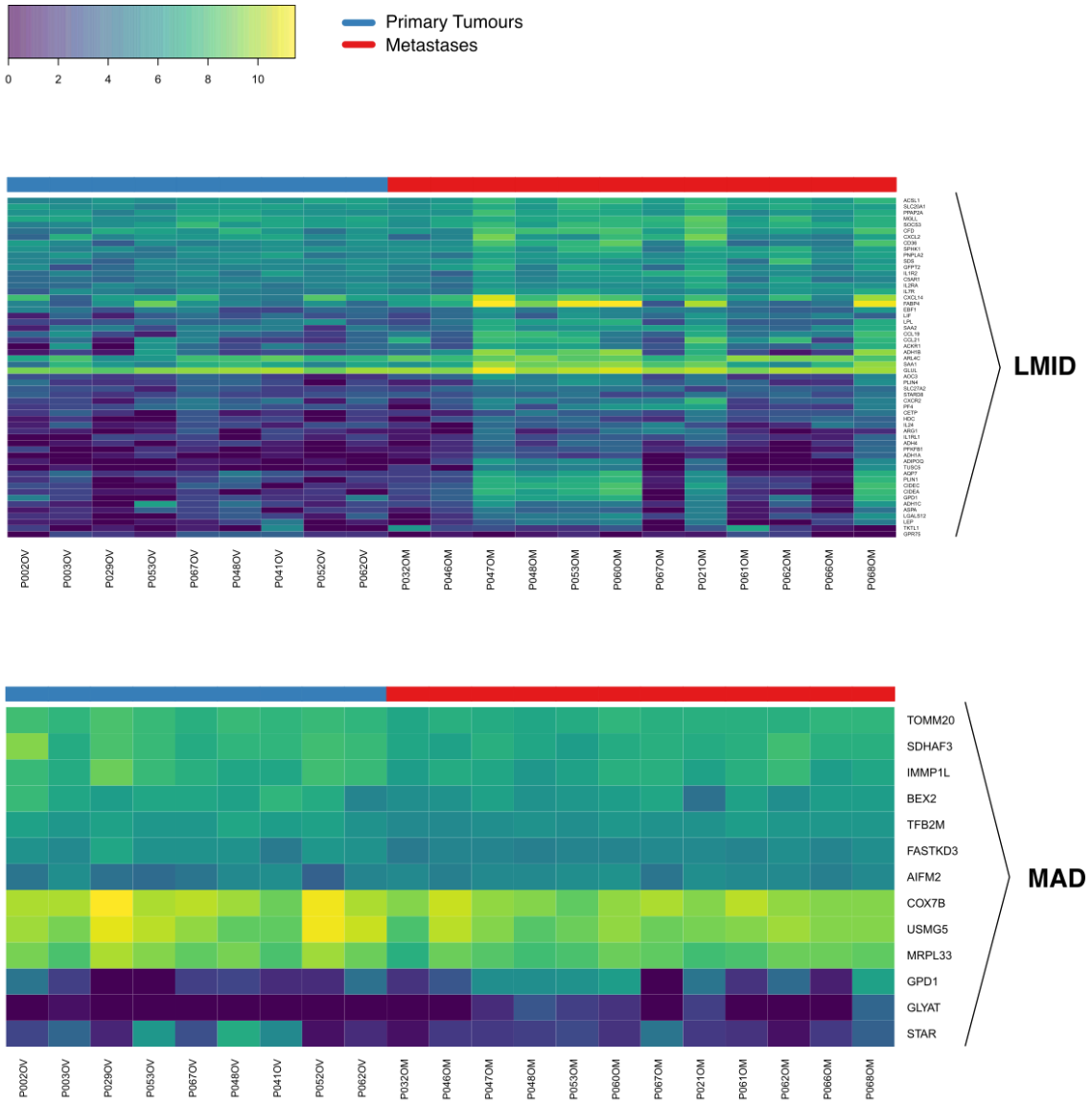


Fig. 8: Heatmaps of DEGs in primary tumours and metastases. *LMID category (56 genes) and MAD category (13 genes) in primary tumours (blue bar) (n=9) and omental metastases (red bar) (n=12) from the KI-cohort.*

The prognostic values of the 9 gene categories were tested in the cohort of primary tumours and omental metastases from KI. However, no associations were found which is likely attributable to the small sample size. As such, the Pan-cancer dataset with primary ovarian tumour samples (N=304) was interrogated. Results showed that patients with lower expression of genes in the *LMID category* survived significantly longer (Fig. 9). No associations were detected between gene expression of genes in the *MAD category* (Fig. 9) or any of the other 7 gene categories and patient outcomes (data not shown).

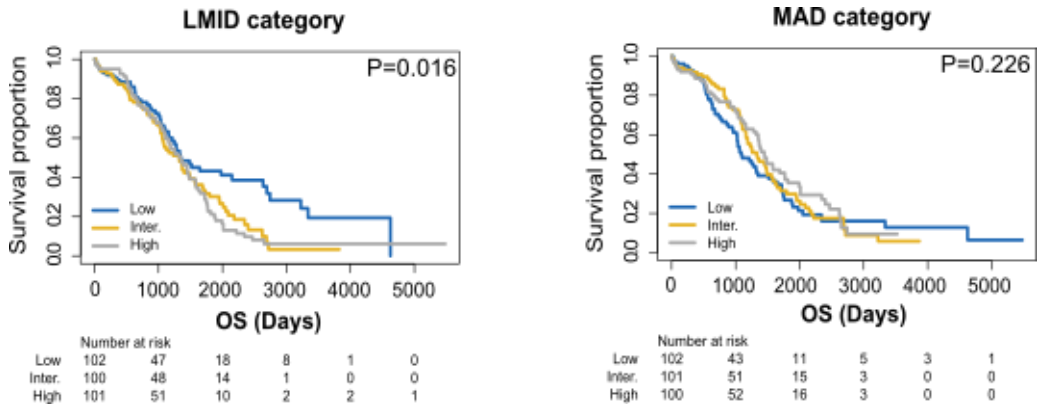


Fig. 9: Comparative analysis of overall survival (OS) and the expression of genes from the *LMID* and *MAD* categories. *Analysis of three levels of gene expression (low, intermediate, and high) obtained after stratification of RNA expression microarray data of LMID and MAD genes in primary ovarian tumours from the Pan-cancer dataset (N=304). Feature selection was performed as part of a Cox proportional hazards regression model. P<0.05 indicates significant difference.*

High expression of FABP4, GPD1, and CIDEA in omental metastases associated with better survival

A further 3 genes involved in lipid metabolism, including FABP4, cell death-inducing DFFA-like effector A (CIDEA), and glycerol-3-phosphate dehydrogenase 1 (GPD1), were selected from the *LMID category*. The selection was based on their association with survival in the Pan-cancer dataset of primary ovarian tumours (Fig. 9), upregulation in omental metastases (Fig. 10A), and known functions in lipid metabolic processes that could support tumour progression. Upregulated mRNA expression of FABP4, CIDEA, and GPD1 in omental metastases was confirmed (Fig. 10B) in a cohort from UNIBO consisting of primary ovarian tumours and omental, sigmoid colon, and diaphragm metastases. These genes were

also upregulated in distant diaphragm metastases when compared to primary ovarian tumours (Fig. 10B), but not in sigmoid colon metastases (Fig. 10B).

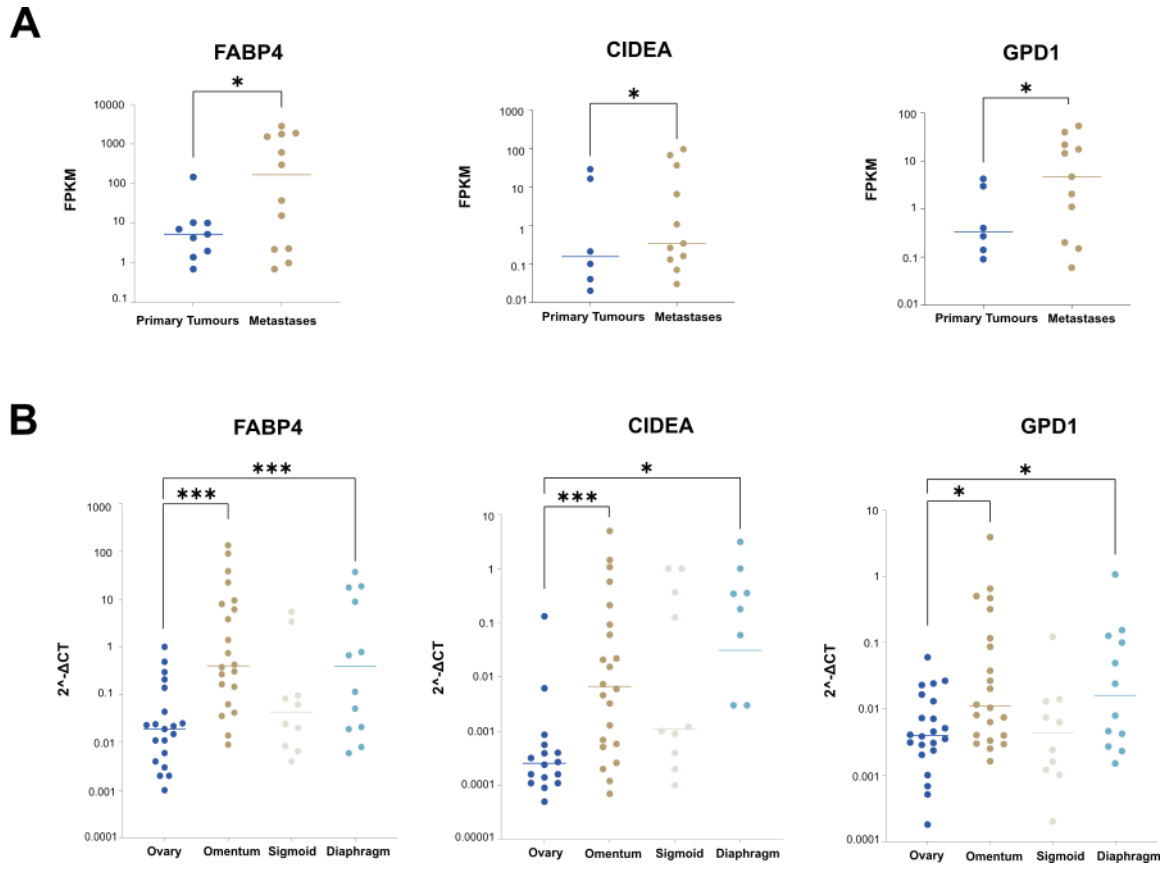


Fig. 10: GPD1, FABP4 and CIDEA in primary tumours and metastases. ***(A)*** *mRNA expression of GPD1, CIDEA and FABP4 in primary ovarian tumours (n=9) and omental metastases (n=12); data from the KI cohort represented as FPKM (fragments per kilobase of exon per million fragments mapped) and plotted on log10 scale.* ***(B)*** *Patient-matched primary ovarian tumours (n=19) and omental metastases (n=14), and metastases from sigmoid (n=7) and diaphragm (n=11); data from the UNIBO-cohort and represented as mean 2^-ΔCT and plotted on log10 scale. *P<0.05 indicates significant difference.*

FABP4 is involved in the uptake of FAs, while GPD1 and CIDEA are involved in lipid synthesis and LD formation, respectively. The NAD-dependent GPD1 protein located in the outer mitochondrial membrane converts glucose-derived dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate (G-3-P) and NAD+, which upon acylation produces triglycerides. Additionally, glycerol-3-phosphate together with glycerol form the backbone for lipid biosynthesis (Zhou et al., 2017). These phospholipids perform as major structural components of cell membranes (Pavlova & Thompson, 2016). The PPAR/PGC-1 regulated

CIDEA protein, typically known for its involvement in apoptosis, has been shown to play a role in energy metabolism and lipid droplet formation (Yonezawa et al., 2011).

Low expression of FAPB4, CIDEA, and GPD1 associated with better survival in the Pan-cancer dataset of primary ovarian tumours (Fig. 9A). We therefore evaluated the relationships between individual FABP4, CIDEA, and GPD1 expression and survival in primary tumours (Pan-cancer dataset and KI-cohort) and in omental metastases (KI-cohort). No associations were detected between GPD1, CIDEA, FABP4, and survival in primary tumours from the Pan-cancer dataset (data not shown) or from the KI-cohort (Fig. 11), while high levels of FABP4, GPD1, and CIDEA expression in omental metastases from the KI-cohort associated with a greater number of patients surviving (Fig. 11). No associations were detected between the expression of these lipid metabolic genes and CR in either primary tumours or metastases (data not shown) from the KI-cohort and Pan-cancer dataset.

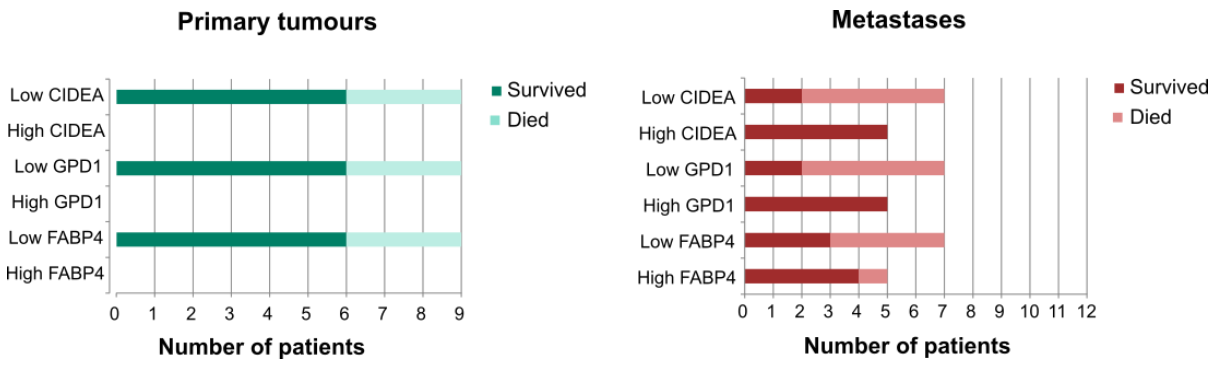


Fig. 11: FABP4, GPD1, CIDEA and survival in the KI cohort. *Preliminary evaluation of survival in two levels of gene expression (low and high) obtained after stratification of mRNA expression data of FABP4, CIDEA, and GPD1 genes in primary ovarian tumours (n=9) and omental metastases (n=12) from the KI-cohort. Stratification: Mean FPKM expression was used as cut-off value for high and low expression. Represents the number of patients that survived/died out of the total number of patients with either low or high expression in respective tumours. No significant associations detected.*

To evaluate if the observed expression patterns are unique to OVCA and metastases, we interrogated larger datasets of primary and metastatic breast cancer (BRCA) datasets METABRIC (N=1971) and TEX (N=109), respectively. Similar to omental metastases, primary BRCA tumours also reside in an adipose-rich TME, whereas BRCA metastases do not (Balaban et al., 2017) (Wu et al., 2019). In line with our findings in omental metastases

from the KI-cohort, we determined that high expression of FABP4, CIDEA, and GPD1 correlated with better OS in primary BRCA tumours (Fig. 12A), but not in BRCA metastases (Fig. 12B). This supports the idea that expression of lipid metabolic genes plays a role in predicting OS, and that this relationship involves interaction between an adipose-rich TME and associated cancer cells.

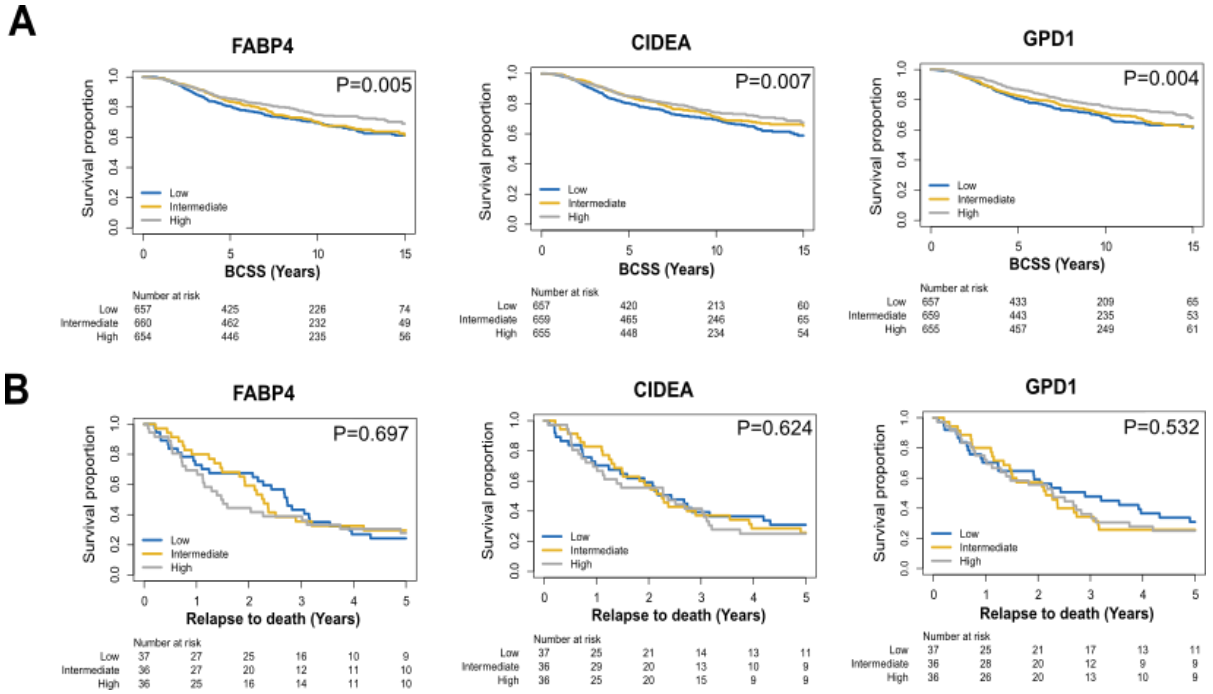


Fig. 12: Comparative analysis of BRCA survival and the expression of lipid metabolic genes. *Analysis of three levels of gene expression (low, intermediate and high) obtained after stratification of RNA expression data of FABP4, CIDEA, and GPD1 in* ***(A)*** *primary BRCA tumours (N=1971) from the METABRIC dataset and* ***(B)*** *metastatic BRCA tumours (N=109) from the TEX dataset. Feature selection was performed as part of a Cox proportional hazards regression model. P<0.05 indicates significant difference.*

Expression of TIC-marker, lipid metabolic, and adipocytokine genes correlated in primary tumours and omental metastases

The inflammatory and adipose-rich nature of the omental TME and the described supportive roles of TIC-markers and lipid metabolism in tumour progression (Duong et al., 2017) (Yi et al., 2018), prompted investigation of the relationships between TIC-marker, lipid metabolic, and adipocytokine gene expression, and their associations with survival.

In the cohort from KI, primary ovarian tumours and omental metastases did not differ in their expression of TIC-markers ALDH1A and CD44 (Fig. 13A). However, a greater proportion of the metastases expressed high levels of these TIC-markers (Fig. 13B) when compared to primary tumours. In the same cohort, ADIPOQ expression was significantly elevated in omental metastases (Fig. 13C) compared to primary tumours, and predominantly metastases expressed high levels of this adipocytokine (Fig. 13D). Although no significant differences were detected in the expression of LEP between primary tumours and metastases (Fig. 13C), 5 out of 6 metastases expressed high levels of LEP (Fig. 13D). Furthermore, no difference was detected in the LEP: ADIPOQ ratio between primary tumours and metastases (data not shown).

Several positive correlations were detected between specific lipid metabolic, adipocytokine, and TIC-marker genes in both primary tumours (Fig. 13E) and omental metastases (Fig. 13F) from the KI-cohort. In particular, FABP4, GPD1, and CIDEA expression correlated with CD36 (FA transport), as well as LEP and ADIPOQ expression in omental metastases (Fig. 13F); and FABP4 expression correlated with CPT1A expression (Fig. 13E) in primary tumours. Furthermore, the expression of TIC-markers CD44 and ALDH1A, respectively, correlated with ACADL (FAO) (Fig. 13E) in primary tumours, and with CD36, CPT1A (FA transport), ACADL, and ADIPOQ (Fig. 13F) in omental metastases.

No relationships were identified between LEP and ADIPOQ, and ALDH1A and CD44 expression, respectively, and survival in primary ovarian tumours (Pan-cancer dataset and KI-cohort) nor in omental metastases (KI-cohort) (data not shown).

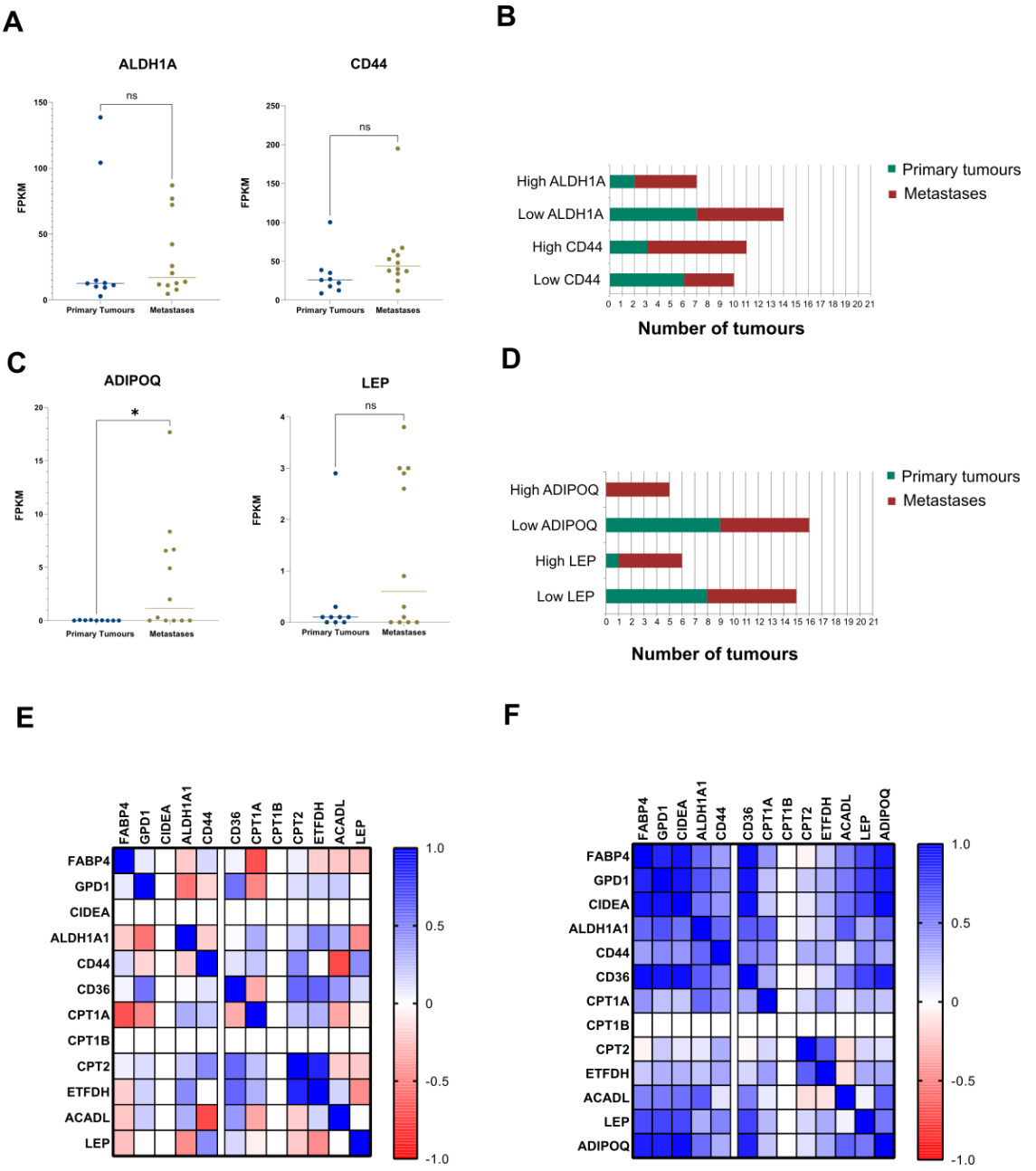


Fig. 13: TIC-marker and adipocytokines gene expression, and correlation with lipid metabolic genes. ***(A)*** *mRNA expression of ALDH1A and CD44, and* ***(C)*** *ADIPOQ and LEP in primary ovarian tumours (n=9) and omental metastases (n=12); data from the KI cohort represented as FPKM (fragments per kilobase of exon per million fragments mapped). *P<0.05 indicates a significant difference* ***(B)***, ***(D)*** *Comparative analysis of two levels of gene expression (low and high) obtained after stratification of mRNA expression data of* ***(B)*** *ALDH1A and CD44, and* ***(D)*** *ADIPOQ and LEP genes in primary ovarian tumours (n=9) and omental metastases (n=12) from the KI-cohort. Stratification: Mean FPKM expression was used as cut-off value for high and low expression. No significant associations detected.* ***(E)***, ***(F)*** *Correlation between the expression of genes: ALDH1A, CD44, FA transporters (CD36, FABP4, CPT1A/B, CPT2), FA metabolism enzymes (ETFDH, ACADL, GPD1, CIDEA), and adipocytokines (LEP, ADIPOQ), in* ***(E)*** *primary ovarian tumours (n=9) and* ***(F)*** *omental metastases (n=12) from the KI-cohort; data represented as Pearson correlation coefficient (-1 to 1).*

The expression of genes involved in cell proliferation correlated with lipid metabolic and TIC-marker genes, and survival in omental metastases

Our results suggest either a tumour suppressive role for FABP4, GPD1, and CIDEA or a better response to treatment. Omental metastases in the KI cohort were enriched for cell proliferation/growth and survival pathways (Fig. 7C), with the possible support of lipid metabolism. We therefore further evaluated the expression of 30 genes involved in cell proliferation (according to the GO term cell proliferation) in primary tumours and metastases, and their relationship with lipid metabolic and TIC-marker genes, and survival in the KI-cohort.

Of the 30 proliferative genes, 6 genes (GLUL, APOD, PODN, ADIPOQ, DUSP1, and FOS) were found to be both upregulated in omental metastases (Fig. 14A) and to correlate with lipid metabolic and TIC-marker gene expression in omental metastases (Fig. 14A), but not primary tumours (Fig.14B). Tumourigenic and tumour progressive roles have been described for GLUL, APOD, ADIPOQ, DUSP1, and FOS in OVCA (Denkert et al., 2002) (Fan et al., 2018) (Feng et al., 2018) (Li et al., 2017) (Mahner et al., 2008) (Shen et al., 2016) (Vázquez et al., 2000) (Yin et al., 2016) and for PODN in glioma (Geng et al., 2020). In line with the prognostic roles of FABP4, GPD1, and CIDEA in omental metastases from the KI-cohort, preliminary survival analysis indicated that high levels of GLUL, APOD, PODN, ADIPOQDUSP1, and FOS associated with better survival in omental metastases (Fig. 15). In contrast, low levels of APOD, PODN, ADIPOQ, DUSP1, and FOS associated with better survival in primary tumours (Fig. 15).

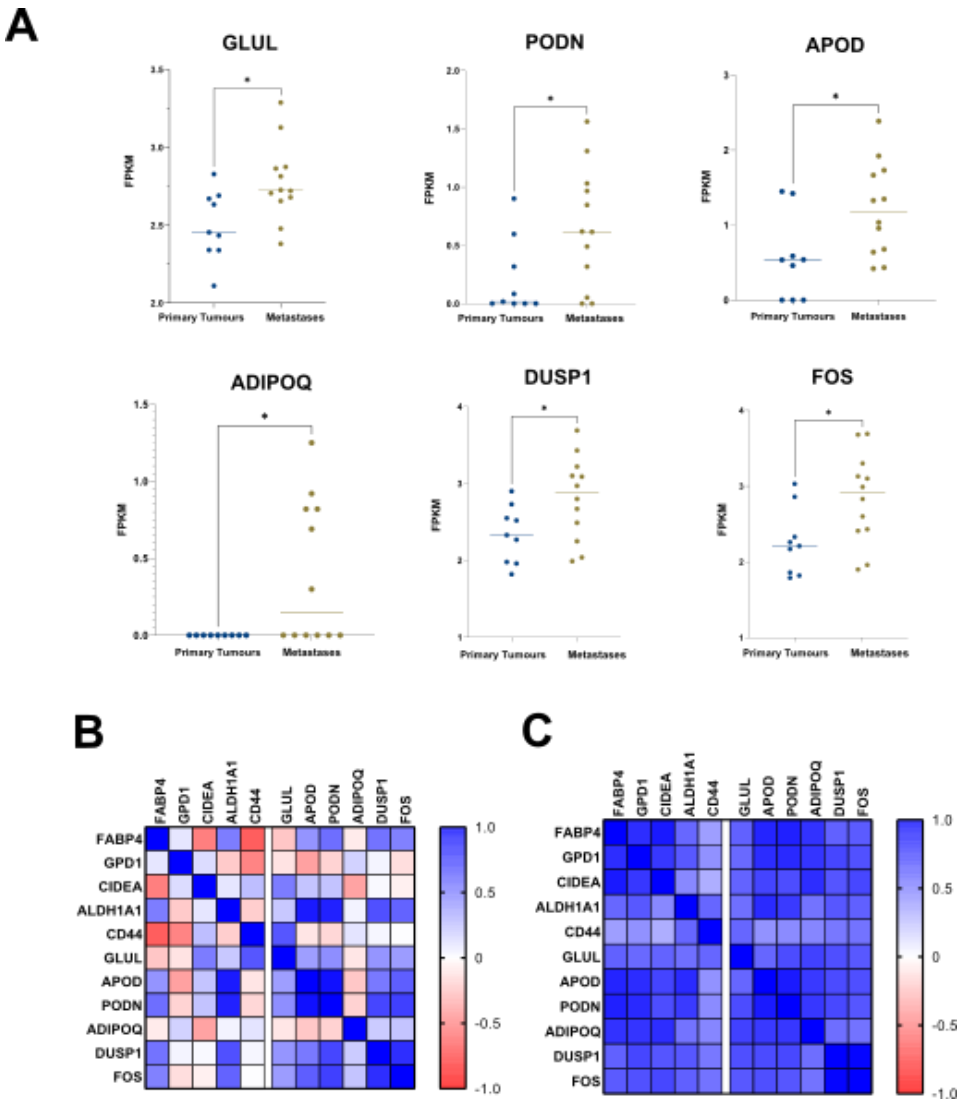


Fig. 14: Expression of genes involved in cell proliferation, and their relationship with lipid metabolic and TIC-marker genes. ***(A)*** *mRNA expression of GLUL, APOD, PODN, ADIPOQ, DUSP1, and FOS genes in primary ovarian tumours (n=9) and omental metastases (n=12); data from the KI cohort plotted as FPKM (fragments per kilobase of exon per million fragments mapped)(log10). *P<0.05 indicates a significant difference.* ***(B)****,* ***(C)*** *Correlation between the expression of genes: TIC-markers (ALDH1A, CD44), lipid metabolism (FABP4, GPD1, CIDEA), and selected genes involved in cell proliferation and/or cell death in* ***(B)*** *primary ovarian tumours (n=9) and* ***(C)*** *omental metastases (n=12) from the KI-cohort; data represented as Pearson correlation coefficient (-1 to 1).*

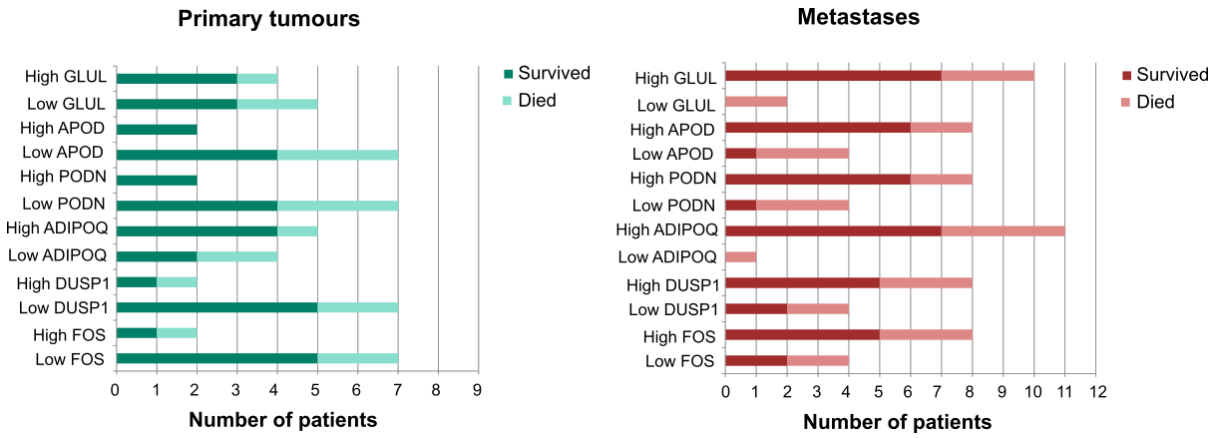


Fig. 15: Expression of genes involved in cell proliferation, and survival in the KI cohort. *Preliminary evaluation of survival in two levels of gene expression (low and high) obtained after stratification of mRNA expression data of GLUL, APOD, PODN, ADIPOQ, ITGA5, DUSP1, and FOS genes in primary ovarian tumours (n=9) and omental metastases (n=12) from the KI-cohort. Stratification: Mean FPKM expression was used as cut-off value for high and low expression. Represents the number of patients that survived/died out of the total number of patients with either low or high expression in respective tumours. No significant associations detected.*

In summary, cancer-associated adipocytes (CAAs) appeared smaller in size compared to those adipocytes distal to the invasive site. Compared to primary ovarian tumours, omental metastases in the cohort from KI were characterized by the upregulation of genes and the enrichment of KEGG pathways and GO terms involved in lipid metabolism, cell growth/proliferation, cell migration/invasion and inflammatory/immune processes. Very few mitochondria-associated genes were differentially expressed, and then mostly downregulated in omental metastases.

Lipid metabolic genes FABP4, GPD1 and CIDEA were upregulated in omental metastases, when compared to primary ovarian tumours from the KI-cohort. A similar pattern was confirmed in omental and diaphragm metastases in the UNIBO-cohort, but not in sigmoid metastases. Also in the KI-cohort, high expression of FABP4, GPD1, and CIDEA in the omental metastases associated with better OS, but not in primary ovarian tumours. Additionally, in BRCA tumours from the METABRIC dataset high expression of FABP4, GPD1 and CIDEA correlated with better OS in primary BRCA tumours, while no correlation was detected in BRCA metastases from the TEX dataset.

In the KI-cohort, a greater proportion of metastases expressed high levels of TIC-markers ALDH1A and CD44 and adipocytokines ADIPOQ and LEP than primary tumours. In the same cohort, correlations were detected between adipocytokine (LEP and ADIPOQ), TIC-

marker (ALDH1A and CD44) and lipid metabolic gene expression predominantly in metastases. No relationships were detected between LEP/ADIPOQ nor ALDH1A/CD44 and survival in either primary tumours or omental metastases.

Seven genes involved in cell proliferation, GLUL, PODN, APOD, ADIPOQ, DUSP1, and FOS, were upregulated in omental metastases from the KI-cohort, and also correlated with the expression of lipid metabolic and TIC-marker genes. Additionally, high expression of these cell proliferation genes associated with better survival in metastases, whereas low expression associated with better survival in primary tumours.

Overall, the upregulation of lipid metabolic genes in omental metastases and its relationship with the expression of cell proliferation genes and survival, most likely involves interactions between cancer cells and other cells/factors, such as adipocytes and adipocytokines, present in an adipose-rich TME.

TIC metabolism and mitochondria in OVCA tumour progression

Progression from primary tumor to metastasis via a proposed TIC stage is thought to involve increased metabolic plasticity, or adaptation to growth conditions (Bezuidenhout & Shoshan, 2019). An increased dependence on FA metabolism has been noted in several *in-vitro* and *in-vivo* models of cancer progression (Nieman et al., 2011) (Pascual et al., 2017) (Wu et al., 2019). It is not known whether PGC-1α or β, a master regulator of oxidative metabolism, FAO and mitochondrial dynamics, affect expression of TIC markers, nor processes related to FA metabolism in TICs, nor how different nutrient conditions affect expression. Using the OVCA cell model consisting of SKOV-3-P cells (hereafter P-cells) and TIC-like SKOV-3-R cells (hereafter R-cells) lacking PGC-1α expression (Wintzell et al., 2012), and gene expression and enrichment data from primary ovarian tumours and omental metastases (KI-cohort), we investigated the roles of the PGC-1 isoforms in chemoresistance, TIC-ness and metabolism.

Knock-down of PGC-1α induced cisplatin resistance

To assess the involvement of the two main PGC-1 isoforms, particularly PGC-1α, a known metabolic modulator in cancer (Bost & Kaminski, 2019), in cisplatin resistance and TIC-marker expression, we transiently reduced the expression of both isoforms using siRNA-mediated knock-down (KD). The very low expression of PGC-1α in R-cells was confirmed (Fig. 16A), while expression of the PGC-1β isoform was found to be maintained (Fig. 16A). Similarly, advanced-stage ovarian cancer tumours in the KI-cohort of primary ovarian and metastatic omental tumours expressed no or very low levels of PGC-1α, and PGC-1β mRNA expression was higher relative to PGC-1α (Fig.16B). To examine whether the loss of PGC-1α expression contributes to chemoresistance and/or TIC-marker expression in R-cells, P-cells were treated with siRNA for PGC-1α. This resulted in a 5.8-fold increase in cisplatin resistance (Table 2), whereas expression of TIC-markers ALDH1A and CD117 was not affected (Fig. 17). By contrast, KD of PGC-1β had no effect on resistance (Table 2) or TIC-marker expression in either cell line (data not shown). It is therefore likely that loss of PGC-1α plays a role in cisplatin resistance but not other TIC properties in our model.

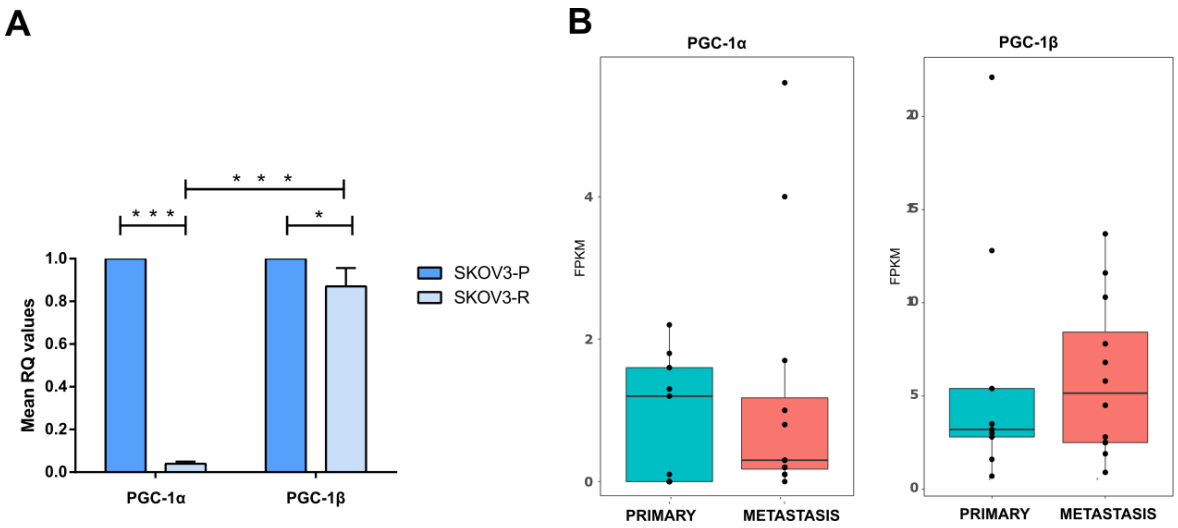


Fig. 16: PGC-1α and -β mRNA expression
(A) *PGC-1α and -β expression in P-and R-cells. Data are based on mean relative quantification (RQ) values relative to P-cells; STDEV; n=3.* ***(B)*** *PGC-1α and -β expression in primary tumours and omental metastases from HGSOC patients. Data represented as FPKM (fragments per kilobase of exon per million fragments mapped). Primary ovarian tumours n= 9, omental metastases n=12. *P<0.05 indicates significant differences.*

Table 2: Effect of PGC-1 KD on cisplatin sensitivity in P- and R-cells
Cisplatin IC_{50} (µM) over 72h in P- and R-cells. Data are based on mean cisplatin IC50, n=3 (P_control, R_control), n=4 (P_scrambled, P_PGC-1α siRNA), n=2 (R_scrambled, R_PGC-1β siRNA).

	Control	Scrambled	PGC-1α siRNA	PGC-1β siRNA
P-cells	2	10	58	7
R-cells	10	23	NA	21

NA: not applicable

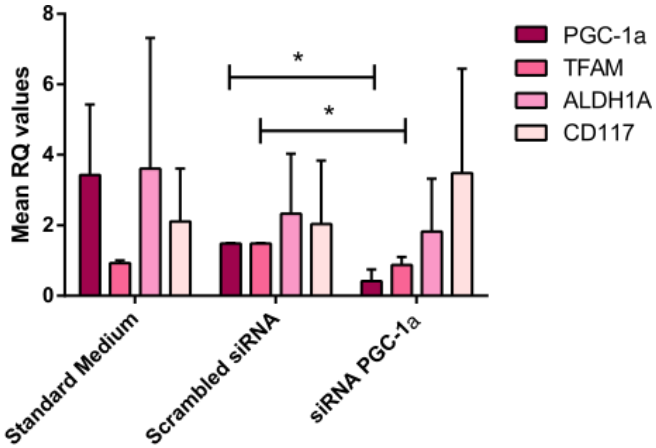


Fig. 17:Effect of PGC-1α KD in P cells. *mRNA expression of PGC-1α, TFAM and TIC-marker was assessed. Data are based on mean relative quantification (RQ) values ± STDEV, n=3. *P<0.05.*

However, in the cohort of tumours from KI no relationship between CR, TIC-marker expression and the PGC-1 isoforms was found (Fig. 18A). Hypothesizing that upregulation of PGC-1β might compensate for decreased levels of PGC-1α, the ratio of the isoforms (β:α) was calculated, but was found to not correlate with resistance (Fig. 18A,C) or TIC marker expression (Fig.18B,C).

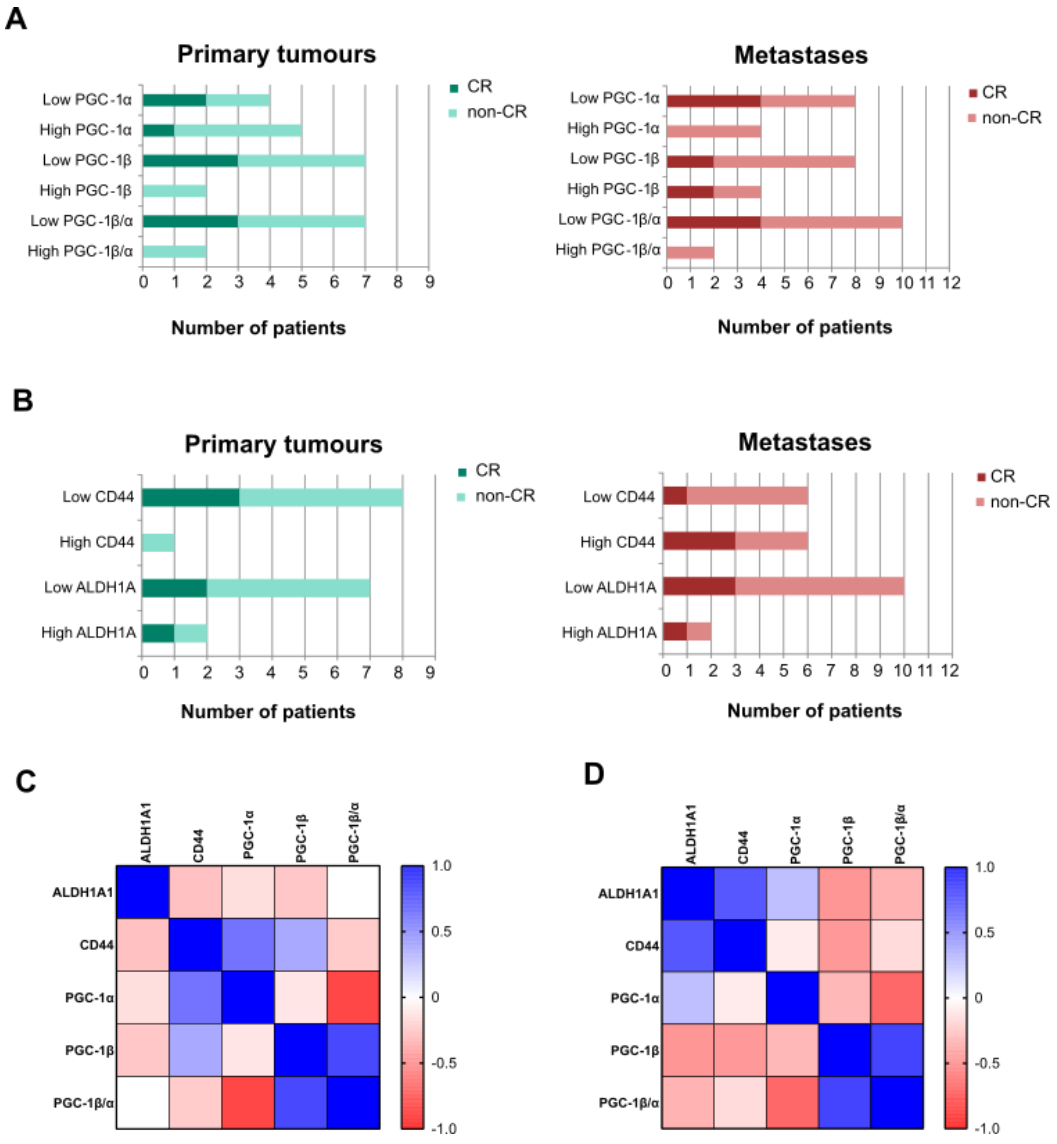


Fig. 18: Clinical resistance, PGC-1 and TIC-marker expression. ***(A)***, ***(B)*** *Evaluating the number of clinically resistant (CR) and non-CR patients in two levels of gene expression (low and high) obtained after stratification of mRNA expression data of* ***(A)*** *PGC-1isoforms and PGC-1β/α ratios, and* ***(B)*** *TIC-markers CD44 and ALDH1A, in primary ovarian tumours (n=9) and omental metastases (n=12) expressing high or low PGC-1 levels and PGC-1β/α ratios. No significant associations detected.* ***(C)***, ***(D)*** *Correlation between the expression of genes: PGC-1isoforms, PGC-1β/α ratios, and TIC-markers (ALDH1A, CD44) in* ***(C)*** *primary ovarian tumours (n=9) and* ***(D)*** *omental metastases (n=12) from the KI-cohort; data represented as Pearson correlation coefficient (-1 to 1). No significant associations detected.*

Invasion and metastasis associated with TIC-marker expression

In order to determine if the expression of PGC-1 isoforms could be involved in the invasion and metastatic process, we investigated the relationship between the expression of PGC-1 and markers of invasivity and metastasis in primary tumours and omental metastases from the KI-cohort. Here, in line with reports on TICs and metastatic potential (Bezuidenhout & Shoshan, 2019; Pascual et al., 2017), gene expression data from the KI cohort indicated no relationships between the expression of PGC-1, markers of invasivity, and metastasis (data not shown). By contrast, tumours expressing high levels of the TIC-markers ALDH1A and CD44 were found to be predominantly metastases (see earlier Fig. 13B), and were enriched for processes related to migration and invasion, such as extracellular matrix organization and cell motility (see earlier Fig. 7C). Furthermore, genes known to be involved in invasion were upregulated in omental metastases, including matrix metalloproteinase (MMP) 2 and MMP19 (Fig. 19A), and significant correlations were detected between MMP19, CD44, and PGC-1α in primary tumours (Fig. 19B), and MMP19, ALDH1A, and CD44 in metastases (Fig.19C). Additionally, ALDH1A expression significantly correlated with the FA transporter CD36 in omental metastases (see earlier Fig. 13F), but not in primary tumours (see earlier Fig. 13E). This FA transporter plays a major role in the metastatic potential of OVCA cells (Ladanyi et al., 2018) (Pascual et al., 2017). However, no relationships could be established between PGC-1, FA transporters and metastasis (data not shown).

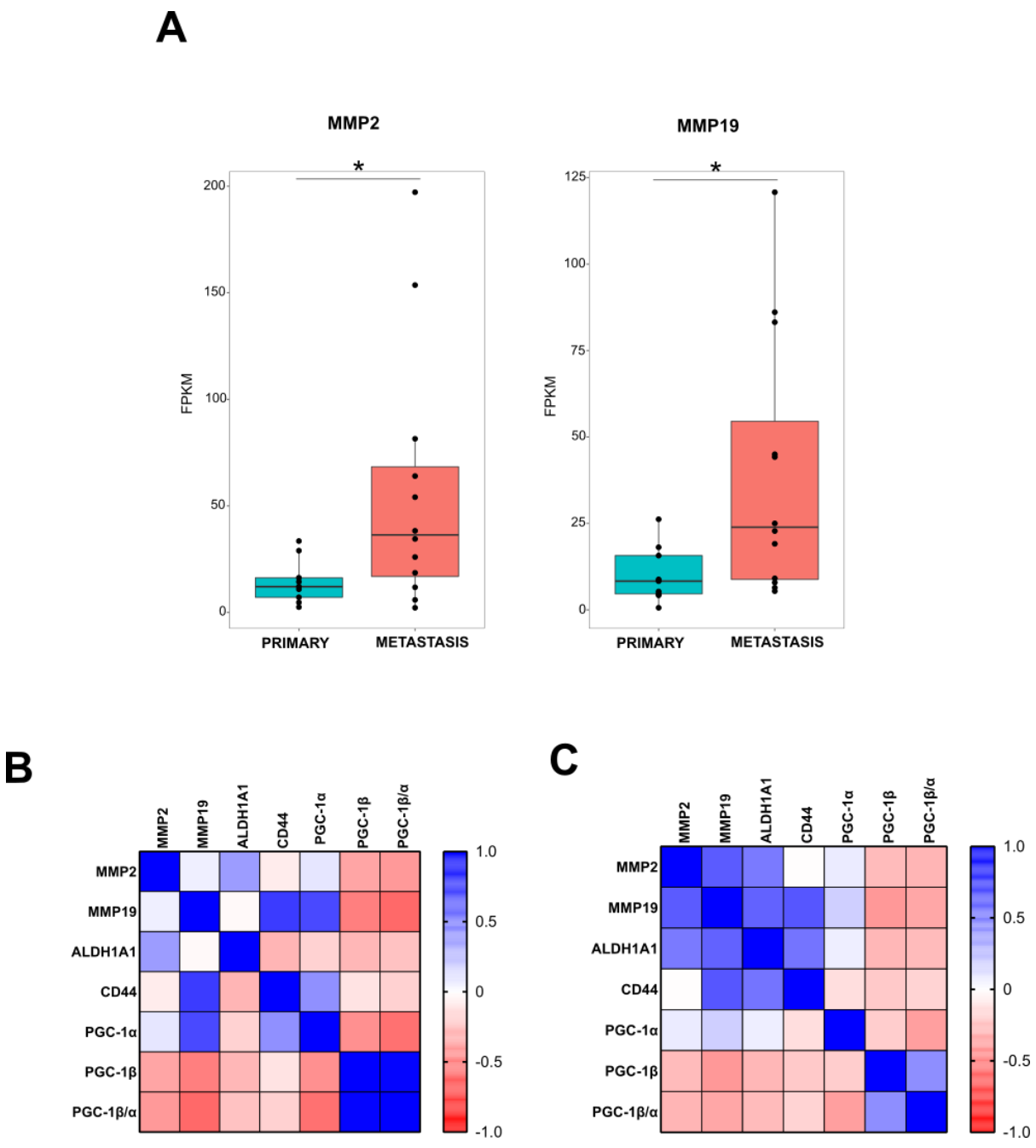


Fig. 19: Expression of invasivity markers, PGC-1, TIC-markers, and tumour type. ***(A)*** *MMP2 and MMP19 expression in primary tumours (n=9) and omental metastases (n=12) from HGSOC patients. Data represented as FPKM (fragments per kilobase of exon per million fragments mapped). *P<0.05 indicates a significant difference.* ***(B)***, ***(C)*** *Correlation between the expression of genes: MMP2/9, TIC-markers, PGC-1isoforms, and PGC-1β/α ratios, in* ***(B)*** *primary ovarian tumours (n=9) and* ***(C)*** *omental metastases (n=12) from the KI-cohort; data represented as Pearson correlation coefficient (-1 to 1).*

Knock-down of PGC-1α induced fragmented peri-nuclear mitochondria

Since KD of PGC-1α, a key regulator of mitochondrial biogenesis, induced cisplatin resistance in P-cells we investigated if this resistance could be related to mitochondrial

alterations. P-cell mitochondria appeared as filamentous networks throughout the entire cell, while in R-cells fragmented clusters of mitochondria were predominantly peri-nuclear (Fig. 20). KD of PGC-1α in P-cells resulted in peri-nuclear clustering and fragmentation of mitochondria, mirroring the effect of a lack of PGC-1α in R-cells (Fig. 20), whereas KD of PGC-1β had no effect. Peri-nuclear clustering may be typical for TICs (Shen et al., 2015), but little is known about the physiological role of this organization and networking of mitochondria. Increased reactive species (ROS) produced by peri-nuclear mitochondria has been shown to affect transcriptional activation of survival pathways in response to stress (Al-Mehdi et al., 2012). However, no differences were found in ROS and antioxidant levels between P- and R-cells (data not shown). We suggest that PGC-1α might play a role in cisplatin resistance that is related to mitochondrial organization and networking.

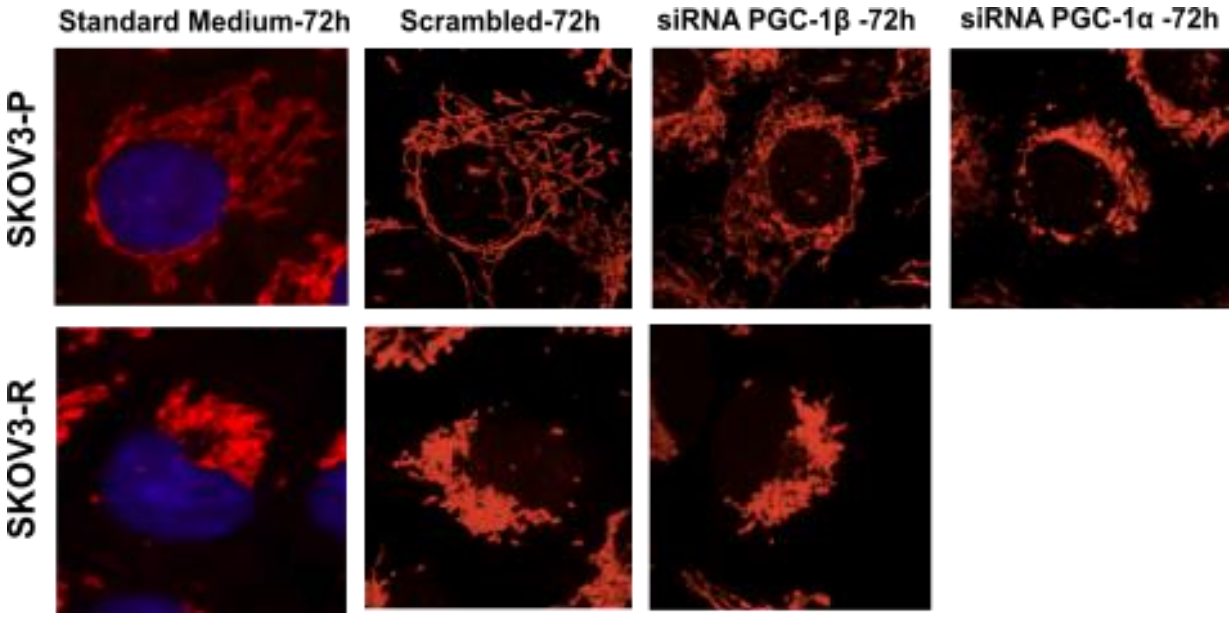


Fig. 20: Mitochondrial organization and networking. *P- and R-cells cultured in standard medium: 11 mM glucose, 2 mM glutamine, 10% FBS. Mitochondria (red), and nuclei (blue). Scrambled = scrambled siRNA sequence representing negative control n=2 or 4.*

In the KI-cohort, no relationships could be established between CR, the expression of PGC-1 isoforms, TIC-markers and genes involved in fission, fusion and mitochondrial transport (data not shown). However, to evaluate the effect of PGC-1 KD on mitochondrial dynamics and transport, functional assessment of related proteins would likely be more informative. Nevertheless, mitochondrial dynamics is known to be sensitive to environmental stressors

such as repeated cisplatin treatment and can adjust accordingly to promote cell survival (Gomes et al., 2011) (Molina et al., 2009).

Greater FA metabolic capabilities did not involve PGC-1α

Given the effect of reduced PGC-1α expression on resistance and mitochondrial organization and networking in P-cells, we compared P- and R-cells regarding expression of mitochondria-associated genes known to be regulated by PGC-1α, namely TFAM (transcription factor A, mitochondrial) a key activator of mitochondrial transcription, and ATP5B (ATP synthase F1 subunit beta) a subunit of the mitochondrial ATP synthase and mitochondrial function, as well as mitochondrial respiration/ATP producing capabilities.
R-cells expressed significantly less TFAM and ATP5B than P-cells (Fig. 21A). The reduction in TFAM expression following PGC-1α and β KD confirmed that its regulation involves PGC-1α and is independent of PGC-1β (Fig. 21B) in our model.

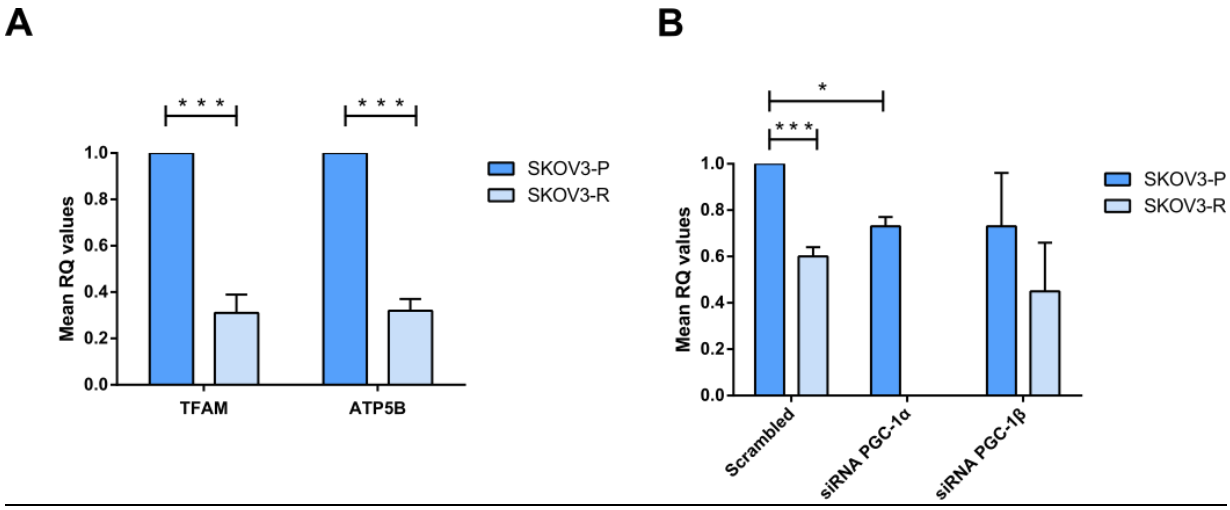


Fig. 21: mRNA expression of mito-associated genes in P- and R-cells. ***(A)*** *TFAM and ATP5B expression. Shown are mean RQ ± STDEV values relative to SKOV3-P (n=3). *P<0.05 indicates significant differences.* ***(B)*** *Effect of PGC-1 KD on TFAM expression in P-and R-cells. (Mean RQ values; STDEV; n=3 or n=2). Scrambled siRNA represents negative control. *P<0.05 indicates significant differences.*

Despite lower expression of TFAM and ATP5B in R-cells, routine respiration using endogenous substrates was no different between P-and R-cells (Fig. 22A). Instead, FA-linked OXPHOS coupling efficiency (OCE) was greater in R-cells compared to P-cells (Fig. 22B). OCE is determined by the equation 1 - (LEAK/OXPHOS), where OCE=1 represents a fully coupled system and OCE=0 a fully uncoupled system. A higher OCE value is therefore

representative of a system with greater ATP producing potential. Thus, when given FA substrates R-cells were more coupled to ATP production.

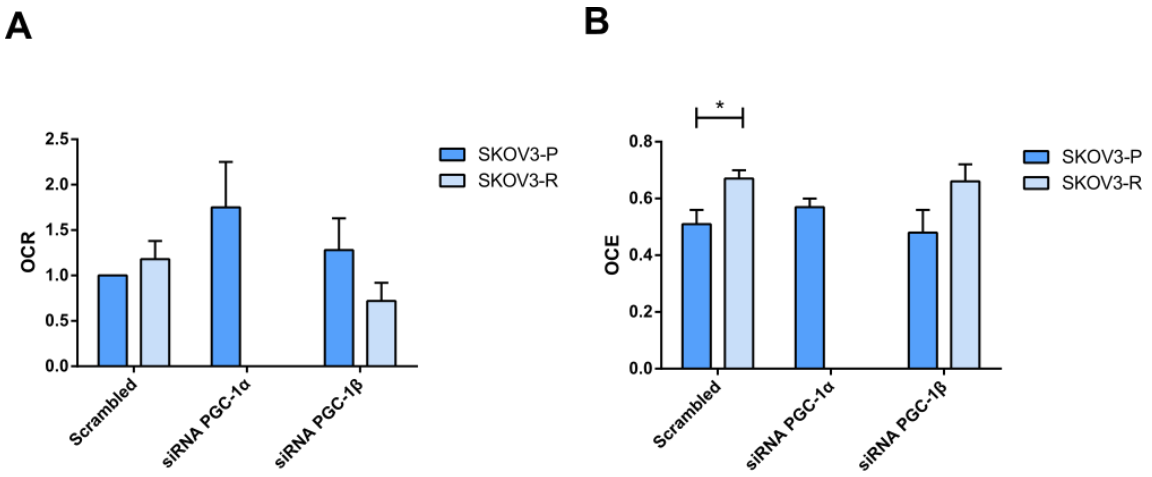


Fig. 22: Effect of PGC-1 KD on mitochondrial respiration in P- and R-cells. ***(A)*** *Routine respiration* ***(B)*** *OCE.* ***OCR*** *(oxygen consumption rate) and OCE (OXPHOS coupling efficiency) were normalized to protein content and expressed relative to P_scrambled Mean, STDEV, n=3. Scrambled = scrambled siRNA sequence represents negative control. *P<0.05 indicates a significant difference.*

Results of KD of PGC-1α/β in P- and R-cells indicated that neither isoform plays a role in mitochondrial respiration in our model (Fig. 22). This is in contrast to what is known about PGC-1α, and the inducing effect it has on mitochondrial OXPHOS (Bost & Kaminski, 2019). It is possible that other major cancer related pathways such as MAPK, PI3K/AKT could play a role in reprogramming mitochondrial function and dynamics, on which cancer cells rely (Senft & Ronai, 2016). Indeed, KEGG pathway analysis revealed enrichment of both MAPK and PI3K/AKT pathways (Project 1, Fig. 7C) in metastases when compared to primary tumours. Furthermore, no relationships were detected between the expression of PGC-1 isoforms, TFAM, ATP5B, mitochondrial respiratory complexes, and CR (data not shown). However, in line with our observations in R-cells, ALDH1A expression also negatively correlated with TFAM in omental metastases (Fig.23).

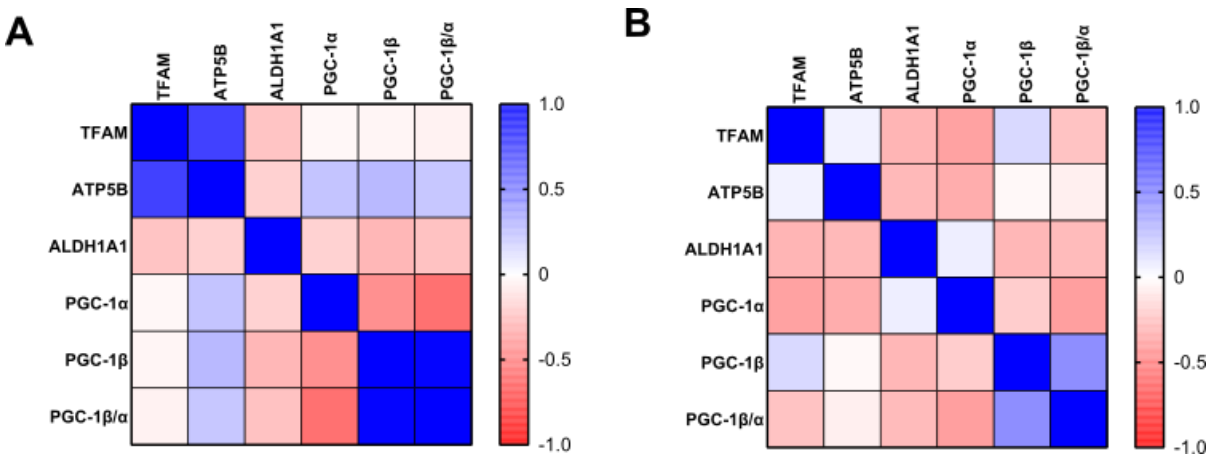


Fig. 23: Correlation of PGC-1 isoforms, TFAM, ATP5B, and ALDH1A expression. ***(A)***, ***(B)*** *Correlation between the expression of genes: TFAM, ATP5B, TIC-markers, PGC-1isoforms, and PGC-1β/α ratios, in* ***(A)*** *primary ovarian tumours (n=9) and* ***(B)*** *omental metastases (n=12) from the KI-cohort; data represented as Pearson correlation coefficient (-1 to 1).*

Considering the fact that R-cells possessed greater ATP producing capability when given FAs as a substrate, we wanted to determine if this corresponded to greater FA uptake and how it related to the fate of FAs inside the cell, i.e., storage vs. energy production. We therefore compared FA transporter expression, LD accumulation, and ATP levels between P- and R-cells. Compared to P-cells, R-cells expressed 6.5-fold more of the FA transporter CD36 (Fig. 24), and did not accumulate lipid droplets when cultured in SM (Fig. 25), suggesting that R-cells are more likely to hydrolyze FAs to produce energy than store it. To assess this, we measured total ATP produced by P- and R- cells cultured in different nutrient conditions. Compared to R-cells, ATP levels in P-cells were more sensitive to the inhibition of glycolysis by 2DG in LGM1+OA and were not able to use OA to produce ATP (Fig. 26A), or sustain growth (Fig. 26B). This together with the fact that R-cells were more coupled to ATP production when given FA substrates, suggests that compared to P-cells, R-cells could be more efficient at metabolizing FAs when needed.

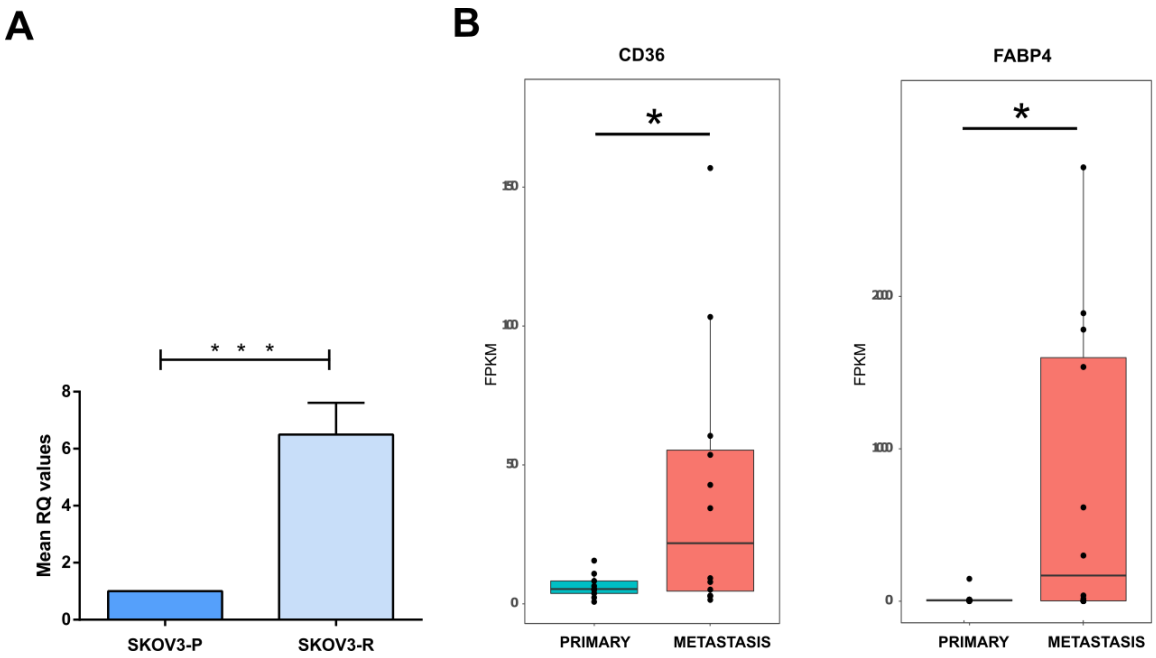


Fig. 24: Expression of fatty acid transporters. ***(A)*** *CD36 mRNA expression in P and R cells. Mean RQ values (STDEV, n=3) expressed relative to SKOV3-P. *P-value <0.05 indicates a significant difference.* ***(B)*** *CD36 and FABP4 expression in primary tumours and omental metastases from HGSOC patients. Data represented as FPKM (fragments per kilobase of exon per million fragments mapped). Primary ovarian tumours n= 9, Metastatic omental tumours n=12. *P<0.05 indicates a significant difference.*

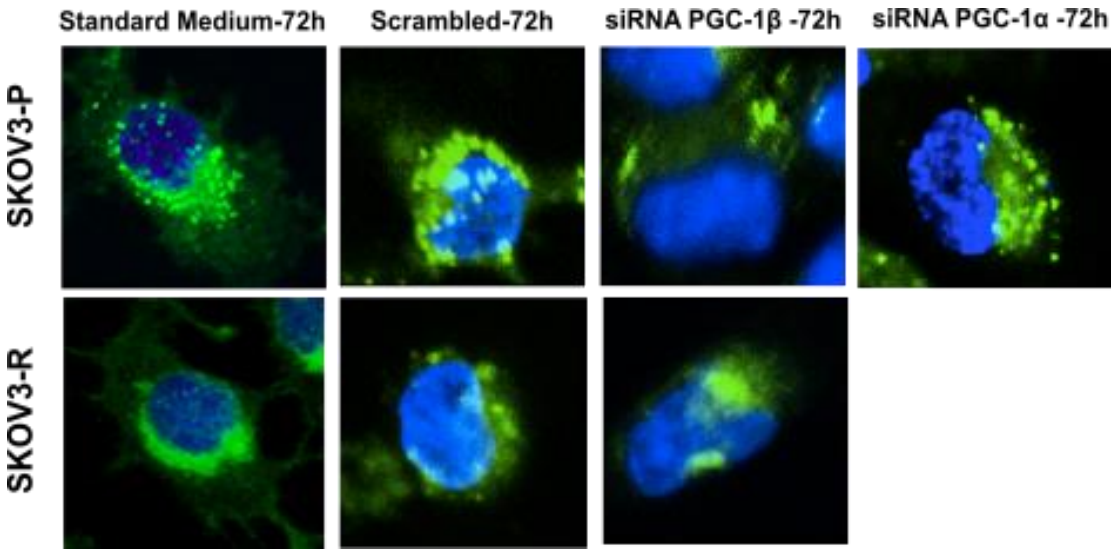


Fig. 25: Lipid droplets. *P- and R-cells cultured in standard medium: 11 mM glucose, 2 mM glutamine, 10% FBS. Scrambled siRNA represents negative control. BODIPY 493/503 staining for neutral lipids. Lipid droplets (green), DAPI: nuclei (blue). n=1 or 2*

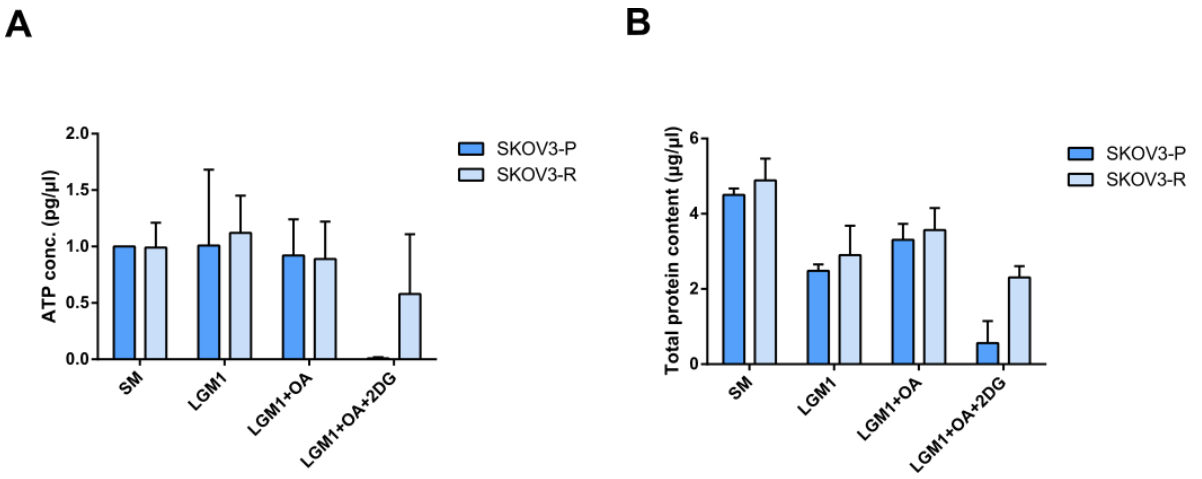


Fig. 26: ATP levels in different nutrient conditions. ***(A)*** *ATP levels;* ***(B)*** *Total protein content. P- and R-cells were cultured in the following nutrient conditions: SM: 11 mM glucose, 2 mM glutamine, 10% FBS; LGM1: 1 mM glucose, 2 mM glutamine, 0.1% FBS; OA: 100 µM oleic acid; 2DG: 10 mM 2-deoxy-D-glucose. Cells cultured in conditions for 72 h. Mean ATP conc. normalized to total protein content, and expressed relative to SKOV3-P in SM, STDEV, n=3. No significant differences detected.*

That R-cells expressed higher levels of CD36 than did P-cells (Fig.24A), suggests that PGC-1α is not involved in its regulation. Indeed, KD of PGC-1α or -β did not affect either CD36 expression (data not shown) or LD accumulation (Fig. 25). Although PGC-1α is well-known for activating the transcription of CD36 and other lipid metabolic enzymes (Cheng, et al., 2018), other regulators of CD36 expression exist, including the CCAAT/enhancer-binding proteins α and β (C/EBPα/β) and peroxisome proliferator receptor gamma (PPARγ) (Qiao et al., 2008; Supruniuk, Miklosz, & Chabowski, 2017).

Omental metastases in the KI cohort were found to express elevated levels of genes involved in lipid metabolism such as FA-transport, lipogenesis, LD formation, and lipolysis, without simultaneous increase in the expression of genes involved in FAO-related energy production (data not shown). It is plausible that there was no demand for increased ATP or that the FA-linked OCE was higher in omental metastases than in primary tumours, in which case evaluating the activity of respiratory complexes and other enzymes involved in energy production would be preferential. In omental metastases, the expression of ALDH1A correlated with the expression of lipid metabolic genes CD36, CPT1A (FA transport), ACADL (FAO), and ADIPOQ (see earlier Fig. 13F). Together, this data suggests an association between TIC-marker expression and elevated lipid uptake and storage, rather than elevated energy production, similar to the relationship observed between these processes in R-cells.

In summary, R-cells express no or very little PGC-1α. This loss and the peri-nuclear clustering of fragmented mitochondria typical in TICs (Shen et al., 2015) could be involved in cisplatin resistance. This change in mitochondrial organization/networking did not negatively impact mitochondrial respiration, but might instead be associated with enhanced FA metabolism evidenced by greater FA-linked OCE, increased expression of CD36, reduced LD accumulation in SM, and reduced sensitivity to 2DG. We showed that these metabolic characteristics occur independently of PGC-1. In HGSOC tumours, PGC-1 isoforms did not appear to play a role in CR and metastasis, and similar to P-and R-cells, PGC-1 expression did not associate with TIC-marker expression or lipid metabolism. However, TIC-marker expression associated with the expression of invasivity-markers, lipid metabolic enzymes, and metastasis.

The enhanced ability of TIC-like R-cells to metabolize FAs compared to P-cells, and the expression patterns of genes involved in lipid metabolism, invasivity and TIC-ness in omental metastases motivates additional evaluation of the effects of nutrient conditions that resemble the low glucose /FA-rich tumour microenvironment (TME) of the omentum. Additionally, PGC-1α expression is known to be sensitive to changes in nutrient availability and cellular stress (Bost & Kaminski, 2019), and PGC-1α levels could play a role in metastasis and chemoresistance (Gabrielson et al., 2014). We therefore studied the effect of different nutrient conditions, mimicking the low glucose/FA-rich TME of the omentum, on the expression of the PGC-1 isoforms as well as various metabolic and mitochondrial features.

Oleic acid did not affect growth or PGC-1 levels and mitochondrial respiration

There were overall no differences in growth between P- and R-cells under different nutrient conditions (Fig. 27). Moreover, both cell lines were sensitive to low glucose and glutamine deprivation, and the addition of oleic acid (OA) did not restore growth (Fig. 27).

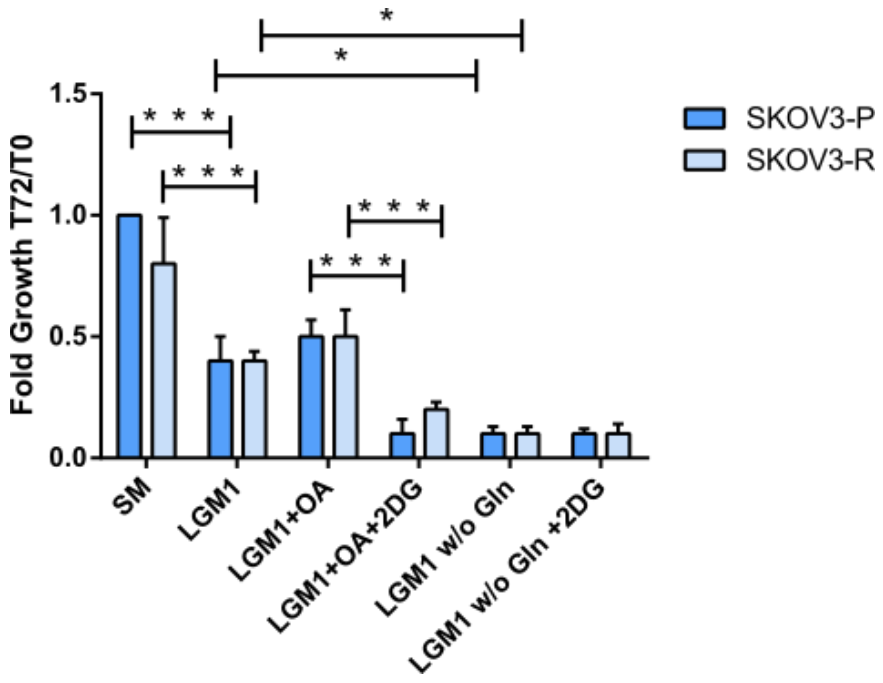


Fig. 27: Growth in different nutrient conditions. *P- and R-cells were cultured in the following nutrient conditions: SM: 11 mM glucose, 2 mM glutamine, 10% FBS; LGM1: 1 mM glucose, 2 mM glutamine, 0.1% FBS; OA: 100 µM oleic acid; 2DG: 10 mM 2-deoxy-D-glucose. (Growth was assessed after 72 h, as described in M&M. Mean fold growth ± STDEV are relative to SKOV3-P (n=3). *P<0.05 indicates significant differences.*

Of the PGC-1 isoforms, TFAM, and ATP5B in P-cells, only PGC-1α expression was affected by the different nutrient conditions, as it increased in low glucose/low FBS (LGM1) compared to SM, and the addition of OA did not further affect this (Fig. 28). The increase did not affect the expression of TFAM and ATP5B (Fig. 28) nor routine respiration and FA-linked OCE (Fig. 29). PGC-1α has several downstream effectors (Tan et al., 2016), and although PGC-1α is known as an indirect regulator of TFAM (Barshad et al., 2018) and ATP5B (D'Errico et al., 2011) expression, their upregulation under conditions of nutritional stress might not be necessary. Instead PGC-1α, which is known to be sensitive to nutrient changes could be upregulated in LGM1 and LGM1+OA to maintain survival through suppression of oxidative damage and the activation of autophagy (Tan et al., 2016). It is unlikely that PGC-1α is involved in the slight fragmentation of mitochondria observed in LGM1 (Fig. 30), since PGC-1α expression was also upregulated in LGM1+OA where mitochondria appear more fused (Fig. 30). As previously discussed, cancer related pathways such as MAPK or PI3K/AKT could play a role in changes in mitochondrial organization and networking observed here.

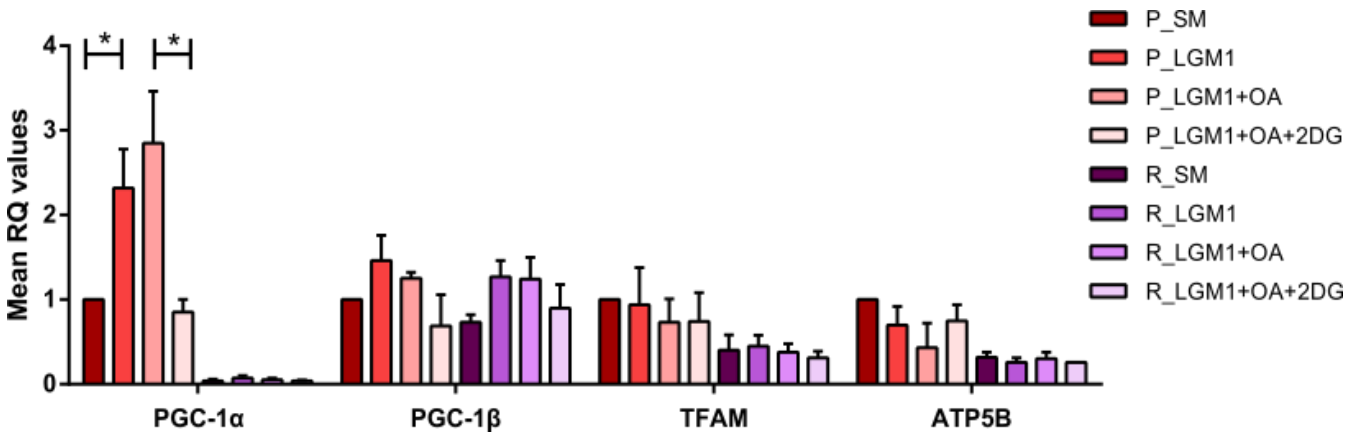


Fig. 28: PGC-1 isoforms, TFAM and ATP5B mRNA in different nutrient conditions. *P- and R-cells were cultured in the following nutrient conditions: SM: 11 mM glucose, 2 mM glutamine, 10% FBS; LGM1: 1 mM glucose, 2 mM glutamine, 0.1% FBS; OA: 100 µM oleic acid; 2DG: 10 mM 2-deoxy-D-glucose. Cells cultured in conditions for 72 h. Shown are mean RQ values ± STDEV relative to SKOV3-P (n=3 or n=2). *P-value < 0.05 indicates significant differences.*

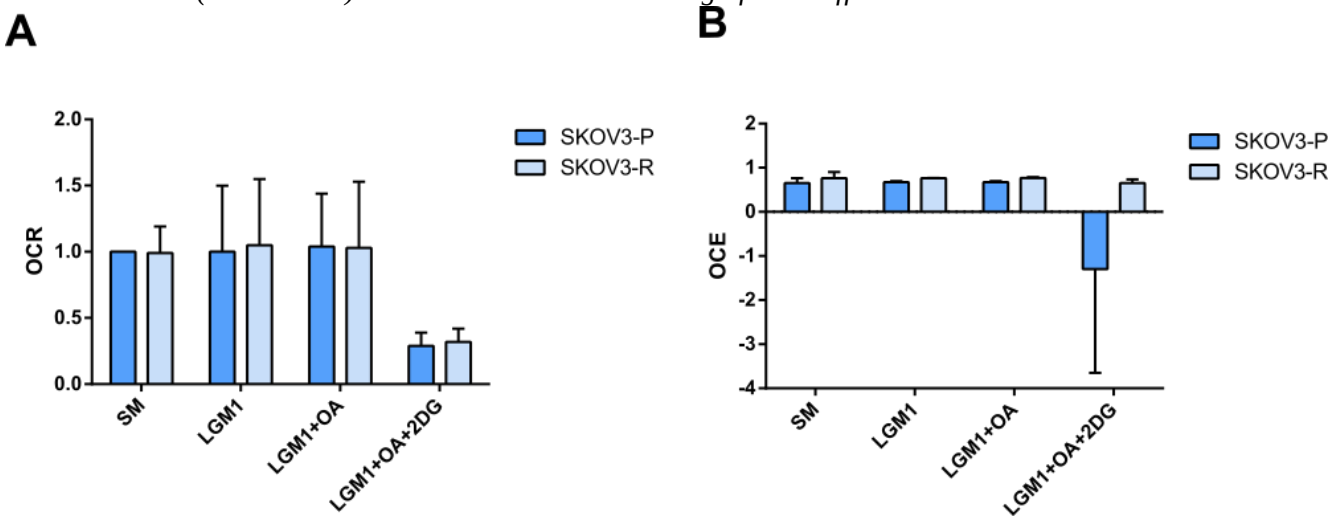


Fig. 29: Mitochondrial respiration in different nutrient conditions. (*A*) *Routine respiration and* **(*B*)** *FA-linked OCE in different nutrient conditions P- and R-cells were cultured in the following nutrient conditions: SM: 11 mM glucose, 2 mM glutamine, 10% FBS; LGM1: 1 mM glucose, 2 mM glutamine, 0.1% FBS; OA: 100 µM oleic acid; 2DG: 10 mM 2-deoxy-D-glucose. Cells cultured in conditions for 72 h. OCR: oxygen consumption rate; OCE: OXPHOS coupling efficiency, normalized to protein content and expressed relative to P_SM. (mean, STDEV, n=3). No significant differences detected.*

Oleic acid induced hyperfusion of mitochondria and LD accumulation in R cells

Given the increased ability of R-cells to metabolize FAs we evaluated mitochondrial organization/networking and LD accumulation in LGM1 with and without OA. In P-cells, LGM1 induced a slight fragmentation of mitochondrial networks whereas OA had very little effect (Fig. 30). Furthermore, LD accumulation in P-cells was similar in SM and LGM1 (Fig. 31). In R-cells, LGM1 had no effect on mitochondrial organization (Fig. 30) or LDs (Fig. 31), whereas the addition of OA to LGM1 resulted in the restoration of networks into long filaments spreading throughout the cell (Fig. 30), and in the formation of LDs (Fig. 31), thus resulting in similarity to P-cells in SM (Figs. 30 and 31).

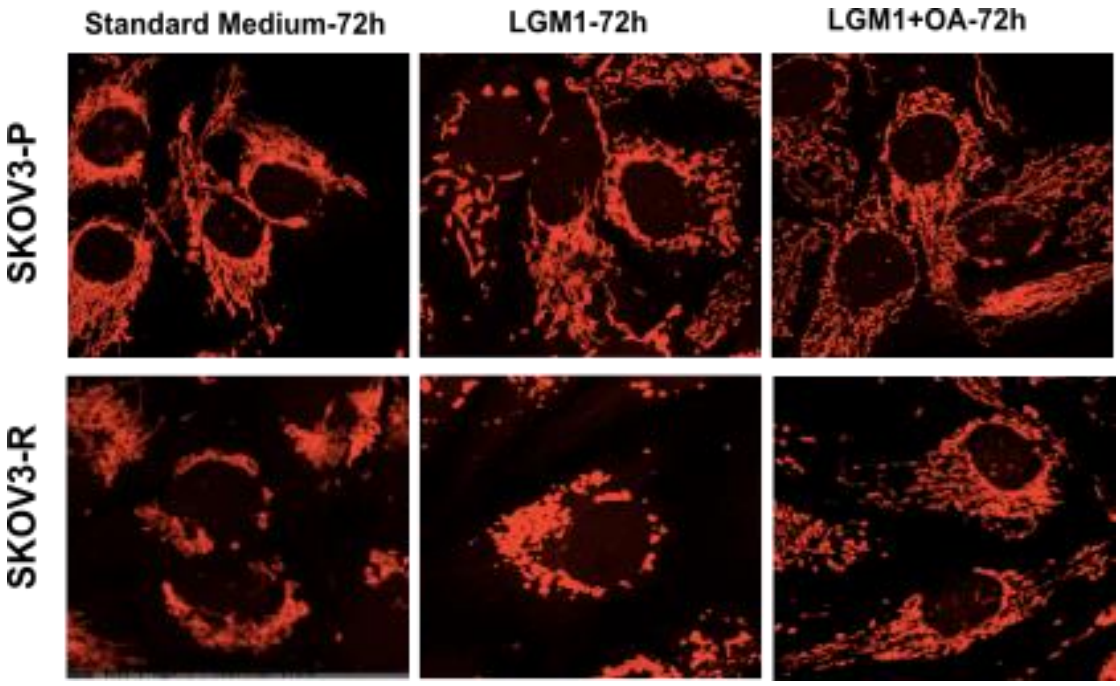


Fig. 30: Mitochondrial organization and networking in different nutrient conditions. *The following nutrient conditions were used: SM: 11 mM glucose, 2 mM glutamine, 10% FBS; LGM1: 1 mM glucose, 2 mM glutamine, 0.1% FBS; OA: 100 µM oleic acid; 2DG: 10 mM 2-deoxy-D-glucose. Cells cultured in conditions for 72 h. MitoTracker Red: mitochondria. n=2.*

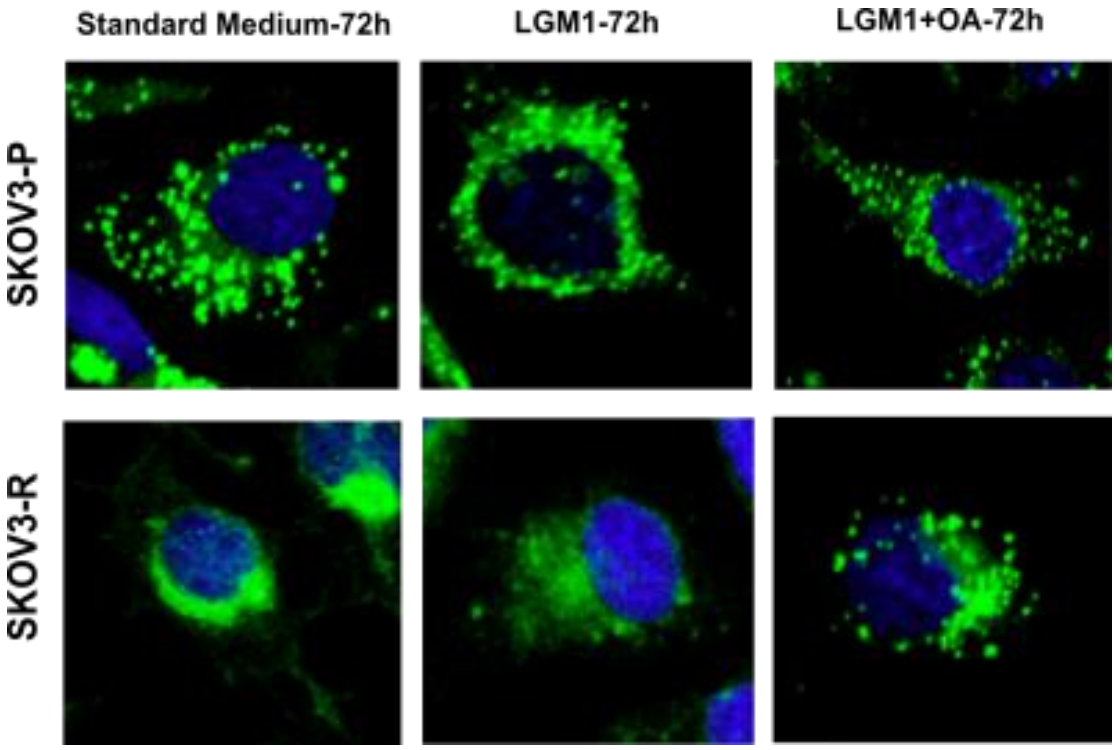


Fig. 31: Lipid droplets in different nutrient conditions. *The following nutrient conditions were used: SM: 11 mM glucose, 2 mM glutamine, 10% FBS; LGM1: 1 mM glucose, 2 mM glutamine, 0.1% FBS; OA: 100 µM oleic acid; 2DG: 10 mM 2-deoxy-D-glucose. Cells cultured in conditions for 72 h. Green: BODIPY 493/503 probe for lipid droplets; Blue: DAPI staining of nuclei n=2*

As ATP levels were not affected by fusion or fragmentation of mitochondria, no connection could be established between bioenergetics and mitochondrial dynamics as typically described in the literature, i.e., that fused networks of mitochondria are believed to be more energized and vice versa (Liesa & Shirihai, 2013). Furthermore, the accumulation of LDs observed in response to the addition of OA to LGM1 is likely unrelated to FAO or cell growth since FAO-linked OCE (Fig. 29) and cell growth (Fig. 27) were unaffected under the same nutrient conditions. Instead, the storage of lipids might indicate the activation of survival mechanisms to protect against oxidative stress, nutrient deprivation, lipid overload or autophagy under conditions of stress (Petan et al., 2018).

Additionally, R-cells expressed significantly less CD36 in LGM1 than in SM (Fig. 32). As fatty acid synthase (FASN), the key enzyme in *de novo* synthesis of lipids, was not differentially expressed (data not shown), the unrestored CD36 expression in response to OA (Fig. 32) might indicate that LD formation depends on increased expression and activity of another FA transporter, e.g. FABP4 (Nieman et al., 2011) (Ladanyi et al., 2018).

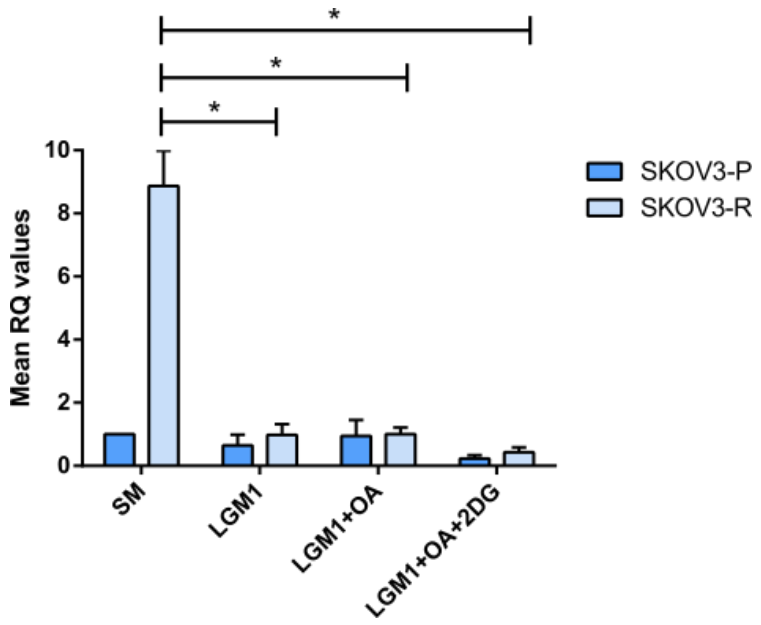


Fig. 32: mRNA expression of CD36 in different nutrient conditions. *P- and R-cells were cultured for 72 h in the following nutrient conditions: SM: 11 mM glucose, 2 mM glutamine, 10% FBS; LGM1: 1 mM glucose, 2 mM glutamine, 0.1% FBS; OA: 100 µM oleic acid; 2DG: 10 mM 2-deoxy-D-glucose. Mean RQ values expressed relative to SKOV3-P, STDEV, n=3). *P-value < 0.05 indicates significant differences.*

In omental metastases, compared to primary ovarian tumours, an upregulation of lipid metabolic genes involved in FA transport (CD36, FABP4), LD formation (CIDEA) and lipogenesis (GPD1) and enrichment of pathways and processes related to cell growth and proliferation was observed, with no change in the expression of genes related to FA-linked energy production.

In summary, cell growth in both cell lines was equally sensitive to low glucose and glutamine deprivation, and was unaffected by OA under standard conditions (SM) as well as in low glucose/low FBS medium (LGM1). In P-cells, PGC-1α expression was increased in LGM1, and coincided with slight fragmentation of mitochondria, albeit with no effect on mitochondrial respiration and FA-linked OXPHOS coupling efficiency, the addition of OA had no further effect. In R-cells, addition of OA to LGM1 had no effect on mitochondrial routine respiration or OCE, but resulted in the formation of long filamentous networks of fused mitochondria and the accumulation of LDs. Furthermore, CD36 expression was significantly reduced in response to low glucose. This is possibly due to very low levels of FAs in the culture medium. Although addition of OA could not restore CD36 expression, it led to the formation of LDs, suggesting that FABP4 might be the preferred port of uptake, in line with the observed upregulation of FABP4 in omental metastases.

GENERAL DISCUSSION

This is to our knowledge the first study with a direct comparison of gene expression patterns in primary, omental, sigmoid colon and diaphragm HGSOC tumours. It is also unique in its focus on metabolic processes in tumour progression. Differential gene expression analysis of primary tumours and omental metastases from the KI-cohort depicts omental metastases as proliferative tumours with greater lipid metabolic and invasive/metastatic capabilities. This is likely supported by increased uptake and metabolism of FAs and stimulation by inflammatory cytokines, both of which are prevalent in an adipose-rich TME such as the omentum. In line with this data, Nieman et al. reported rapid tumour growth and enhanced invasion and metastasis as a result of increased FA uptake, beta-oxidation, and stimulation by inflammatory cytokines released by omental adipocytes (Nieman et al., 2011). It remains unknown if upregulated FAO-derived energy production plays a role in omental metastases, since mitochondria-associated genes in the KI-cohort, particularly those related to energy production, were not differentially expressed. We suggest that the activity of enzymes involved in FA-linked energy production would be a better measure of bioenergetics than gene expression.

Gene expression in omental metastases in the KI-cohort indicated enhanced lipogenesis and LD formation. Lipid droplets play an important role in preventing the toxic effects of excess lipids such as autophagy and lipid peroxidation by sequestering lipids, from extracellular or endogenous sources, into inert lipid molecules such as triglycerides (Petan et al., 2018). This acts to support processes that contribute to tumour progression including cell proliferation and growth (Cruz et al., 2020). Indeed, cell survival and proliferation pathways were enriched in omental metastases from the KI-cohort. Additionally, FAs and lipids originating from the breakdown of LDs may also serve as signaling molecules by interacting with TFs including PPARs or, when converted to bioactive lipid mediators, may act as paracrine or autocrine messengers, thus influencing inflammatory signaling, metabolism, proliferation, and metastasis (Petan et al., 2018). This is in line with the enrichment of several signaling pathways in omental metastases, including lipolysis, proliferation, motility, and PPAR and inflammatory signaling pathways, and describes a multifaceted role for increased LD accumulation in OVCA tumour progression.

We show, for the first time, that compared to primary ovarian tumours, omental and diaphragm metastases, but not sigmoid colon metastases, share a similar upregulation of

three lipid metabolic genes, namely GPD1, CIDEA and FABP4. The diaphragm and sigmoid colon, unlike the omentum, are not adipose-rich tissues. Although the peritoneal folds, or mesentery, of the sigmoid colon help to store fat in addition to their conduit function, they do not form part of the sigmoid colon. By contrast, the diaphragm is a major drainage site for lipid and immune-cell rich lymph called chyle from the small intestine (Abu-Hijleh et al., 1995) (Bibby and Maskell 2019) (Nagai et al., 2015). In this way tumours of the diaphragm may benefit from the lipid-rich TME created by lymphatic drainage, while sigmoid colon tumours do not.

It should be noted that the origin of metastases, i.e., their phylogenesis, remains largely unknown. This is in part due to a lack of studies investigating the development of metastases and its interactions in more physiological environmental settings, and a shortage of studies examining the genomic characteristics of the different clones that make up metastases (Allgayer et al., 2020). Two proposed modes of tumour evolution include the linear and parallel progression models (Caswell & Swanton, 2017). In linear tumour progression (Fig. 33) the primary tumour develops multiple subclonal populations before tumour cells disseminate and form a metastasis. In contrast, in parallel progression (Fig. 33) tumour cells metastasize at an early stage and the primary tumour and metastases progress in parallel, with multiple subclonal populations (Caswell & Swanton, 2017). In a recent study, the majority of OVCA metastases were shown to arise through linear progression from primary tumour cells, resulting in reduced heterogeneity and increased sharing of mutations between primary tumours and metastases (Masoodi et al., 2020). However, in the same study some patients did demonstrate parallel progression in which only one driver mutation, in this case TP53, was present in the disseminating clone and was needed for ectopic survival, while the majority of somatic mutations occurred in metastases in order to adapt to the new microenvironment (Masoodi et al., 2020). Although primary tumours and metastases from the UNIBO cohort shared TP53 mutations, we are, without further mutational analyses, not able to discern their mode of progression. However, given the adipose-rich nature of the omental and diaphragm metastatic sites and the similarity in the expression levels of lipid metabolic genes in these metastases, a parallel progression model could be possible.

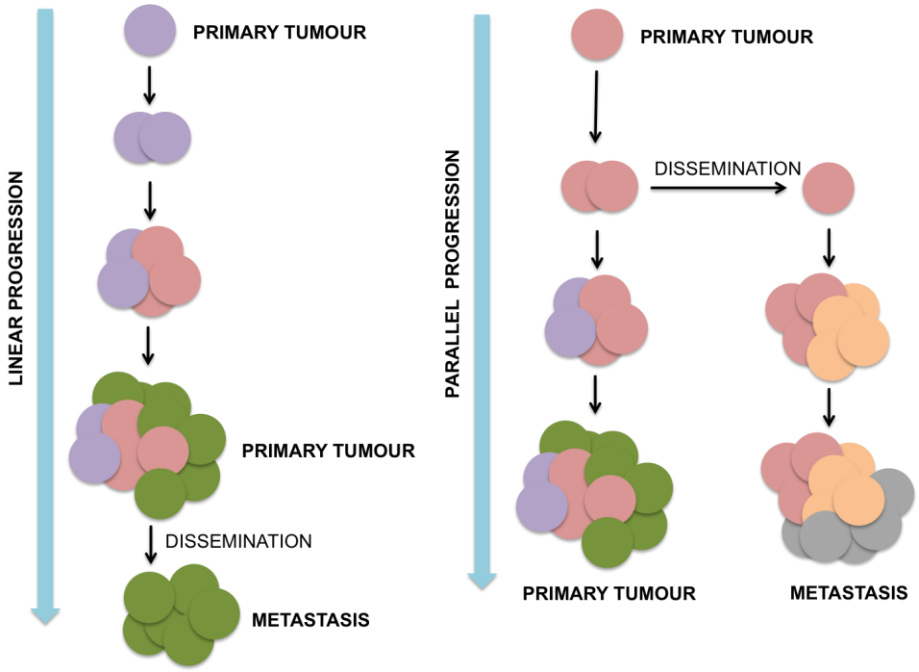


Fig. 33: Linear versus Parallel progression models of tumour progression adapted from Caswell and Swanton 2017

We suggest that lipids derived from the TME play a role in regulating the expression of GDP1, CIDEA, and FABP4. Indeed, numerous studies report the promotion of tumour growth and invasion/metastasis by stromal adipocytes in primary BRCA and OVCA metastases (Balaban et al., 2017)(Huang et al., 2017) (Fujisaki et al., 2015) (Ladanyi et al., 2018) (Nieman et al., 2011) (Wang et al., 2017) (Wu et al., 2019). The influence of the TME on gene expression is further substantiated by higher expression of genes associated with inflammatory cytokines and related receptors, as well as the enrichment of inflammatory signaling pathways in omental metastases in the KI-cohort. The upregulation of adipocytokines LEP and ADIPOQ and its correlation with lipid metabolic genes in omental metastases is in line with the known roles of leptin and adiponectin in lipid metabolism, and the relationship between their upregulation and tumour progression in omental metastases (Ladanyi et al., 2018) (Slomian et al. 2019) (Wang et al., 2017). The release of inflammatory cytokines from adipocytes also promotes invasion and metastasis (Duong et al., 2017) (Wu et al., 2019), and cancer cells may adopt this secretory profile to perpetuate the beneficial effects of an inflammatory TME. We propose that extracellular FAs as well as specific inflammatory factors released from stromal adipocytes influence gene expression in omental tumours. It should be noted that interactions in the TME are not the only drivers of metabolic reprogramming in cancer cells. Oncogenic mutations are also known to be associated with

metabolic reprogramming in cancer (DeBerardinis & Chandel, 2016), and interactions in the TME are likely influenced by oncogene-driven metabolic reprogramming to promote tumour progression (Min & Lee, 2018). Similarity in oncogene-drivers of metabolic reprogramming could therefore also affect the expression of genes in metastases in both KI- and UNIBO-cohorts. However, we have not compared oncogene mutations between the different tumours.

We established a gene category, LMID, with prognostic value in a Pan-cancer dataset of primary ovarian tumours. Three lipid metabolic genes identified from the LMID category, FABP4, GPD1, and CIDEA, correlated with better surival in omental metastases in the KI-cohort, and also in primary BRCA tumours (METABRIC dataset). As primary BRCA tumours also reside in an adipose-rich TME, this once again illustrates a role for the TME in regulating gene expression and its prognostic value. Our results suggest either a tumour suppressive role for FABP4, GPD1, and CIDEA or a better response to treatment. Indeed, GPD1 has been described as a tumour suppressor in primary BRCA tumours, and high expression of this gene correlated with better survival (Zhou et al., 2017). Moreover, overexpression of GPD1 via viral transfection, independently and when combined with metformin treatment, demonstrated anti-proliferative effects *in-vivo* and in several cancer cell lines, including OVCA cell line SKOV3 (Xie et al., 2020). In primary ovarian tumours from patients diagnosed with HGSOC, high expression of FABP4 correlated with poor survival (Gharpure et al., 2018). In the same study, tamoxifen (selective estrogen receptor modulator) treatment of OVCA cells significantly downregulated FABP4 expression, FA uptake, and migration potential. Furthermore, Nieman et al., 2011 demonstrated that inhibition of FABP4 significantly reduced tumour weight *in vivo*, implicating a role for FABP4 in tumour cell proliferation and growth. The relationship between CIDEA expression and patient outcomes has not previously been investigated in any cancer type. CIDEA localizes in the mitochondria, nucleus, ER, and LDs, and its functions, including the activation of apoptosis and LD formation and the inhibition of beta-oxidation and lipolysis, depends on its localization (Yonezawa, Kurata, Kimura, & Inoko, 2011). How CIDEA might contribute to better OS is unclear, since it appears to have opposing functions. On the one hand, ectopic overexpression of CIDEA, typically expressed in adipocytes, in glioblastoma has been shown to trigger apoptosis via the upregulation of PPARγ (Chatterjee et al., 2015). On the other hand, oleate-induced LD formation and increased interaction of CIDEA with LDs has been shown to reduce fat-specific protein 27 (FSP27)-mediated apoptosis, through direct interaction with the pro-apoptotic site of FSP27, in several non-adipocyte cell lines (Liu et

al., 2009). Moreover, in the present study both CIDEA and FSP27 were upregulated in omental metastases, while PPARγ was not. However, the association of CIDEA and FSP27 with LDs and the interaction between these two proteins remains unknown. Since omental metastases in the KI cohort were enriched for cell proliferation/growth and survival pathways, and FABP4, GPD1, and CIDEA expression correlated with the expression of 7 genes with documented roles in cell proliferation; we propose that residual cancer cells from these tumours following resection responded better to adjuvant chemotherapeutic targeting of highly proliferative cancer cells, thereby contributing to better survival in OVCA patients. When combined with standard cytotoxic chemotherapy, and/or other anti-cancer drugs, stimulation of FABP4, GPD1, and CIDEA expression in cancer cells could improve outcomes in patients with tumours expressing low levels of these genes.

In omental metastases in the KI-cohort, ALDH1A expression correlated with expression of lipid metabolic genes CD36, CPT1A, ACADL, and ADIPOQ, as well as APOD, PODN, DUSP and FOS genes related to cell proliferation. This is in line with reports where ALDH1A expression and TIC-properties such as sphere formation were reduced by suppression of lipid metabolism via inhibition of lipid metabolic genes ACC, SCD-1, and PPARγ (Corominas-Faja et al., 2014) (Li et al., 2017) (Wang et al., 2013). Furthermore, Chefetz et al., 2020 found that ALDH1A does not have a role as an inducer of mitochondrial UCPs in programmed cell necrosis in $CD133^{+}$ OVCA cells. Uncoupling proteins are mostly known for their regulation of lipid metabolism particularly in adipocytes (Bouillaud et al., 2016) and are frequently upregulated in cancer (Robbins & Zhao, 2011). Inhibition of ALDH1A stimulated UCP1 and 3 expression, reduced ATP levels, and induced necroptosis (Chefetz et al., 2020). Whether ALDH1A expression induces lipid metabolism or the other way around is yet to be determined.

In vitro, i.e., in the absence of an adipose TME, metabolic reprogramming in R-cells involved upregulated CD36 expression, unaffected ATP levels following the inhibition of glycolysis, and LD accumulation. Although PGC-1α has a known role as regulator of FA oxidation (Bost & Kaminski, 2019), the lack of PGC-1α in R-cells indicates other modes of regulation. Moreover, no relationship could be established between PGC-1α and lipid metabolic gene expression in omental metastases. Greater FA-linked OCE/ATP producing capabilities were observed in R-cells despite the low PGC-1α, TFAM and ATP5B levels,

suggesting a need for increased energy production that involves the upregulation of activity rather than expression of proteins related to FA-linked ATP production.

Similar to omental metastases, R-cells showed high ALDH1A and low TFAM levels, and that ALDH1A expression may be independent of PGC-1α. In addition, KD of PGC-1α in P-cells did not affect ALDH1A expression. Supporting this, no relationship between ALDH1A and PGC-1α expression was found in omental metastases. It is possible that upregulated NF-κB signaling in omental metastases could play a role in ALDH1A regulation, since a positive feedback loop between NF-κB and lipid metabolism that maintains CSC properties has been shown to involve ALDH1A (Li et al., 2017). In the same study, increased unsaturated FAs via ALDH1A and retinoic signaling were shown to promote NF-κB signaling and upregulation of lipid desaturases and maintenance of CSC properties (Li et al., 2017). Although the relationship between ALDH1A and lipid metabolism is not yet well understood, our findings support that ALDH1A expression associates with upregulation of lipid metabolism and related energy metabolism. Indeed, several reports indicate that upregulated ALDH1A is involved in the supportive role that lipid metabolism and OXPHOS have in maintaining TIC-properties (Chen et al., 2016) (Wang et al., 2017) (Yi et al., 2018).

Contrary to expectations, no associations were detected between PGC-1, TIC-markers, and metabolic/mitochondria-related genes, nor between the expression of these genes and survival or cisplatin resistance. However, a role for PGC-1α, but not PGC-1β, in cisplatin sensitivity was identified *in-vitro,* in that its loss was associated with resistance. The fact that no relationships could be established between PGC-1β expression, lipid metabolism and mitochondrial organization/networking speaks to its role in maintaining basic mitochondrial function (Villena, 2015). This could also offer an explanation for the high ratio of the β to α isoforms in both R-cells and in omental metastases compared to P-cells and primary tumours, respectively.

The effects of loss of PGC-1α appear to be unrelated to its known regulatory functions of FA metabolism and mitochondrial OXPHOS (Cheng et al., 2018), as evidenced by the fact that KD of PGC-1 in P-cells did not affect CD36 expression or FA-linked OCE and LD accumulation. However, the peri-nuclear clustering and fragmentation of mitochondria induced by KD of PGC-1α suggests that the role of PGC-1α loss in cisplatin resistance could involve mitochondrial organization/networking. In fact, peri-nuclear clustering of mitochondria has been proposed as a protective mechanism through ROS-induced

upregulation of nuclear transcriptional activity in conditions of cellular stress (Al-Mehdi et al., 2012) such as repeated cisplatin treatment. In addition, heat shock has been shown to induce peri-nuclear mitochondrial clustering, resulting in increased ROS in the nucleus that activates the heat shock response known to protect against cellular stressors such as exposure to DNA damaging reagents (Agarwal & Ganesh, 2020). Furthermore, the role of ROS in chromatin remodeling that influences transcriptional activation is well described (Kreuz & Fischle, 2016) (Rahman et al., 2004) (Sundar et al., 2013). We did not observe differences in the ROS and antioxidant levels between P- and R-cells. However, we evaluated total cellular ROS, and could therefore have missed an upregulation of ROS in the peri-nuclear area. Reintroduction of PGC-1α in R-cells could offer some insight. Fluorescent labeling of ROS and live cell imaging could be used to evaluate ROS levels and its intracellular localization following cisplatin treatment with or without nocodazole, a disrupter of microtubule dynamics. Additional comparison of gene expression involved in mediating cellular protection in response to ROS-induced transcription could indicate if this mechanism plays a role.

Altogether, we suggest that co-localization with mitochondria involving the loss of PGC-1α creates an oxidant-rich nuclear domain that offers protection against cisplatin-induced cellular stress (Fig. 34). This regulatory effect of PGC-1α on mitochondrial organization/networking likely occurs in a glucose-independent manner since PGC-1α expression did not play a role in mitochondrial organization/networking in low glucose with or without OA.

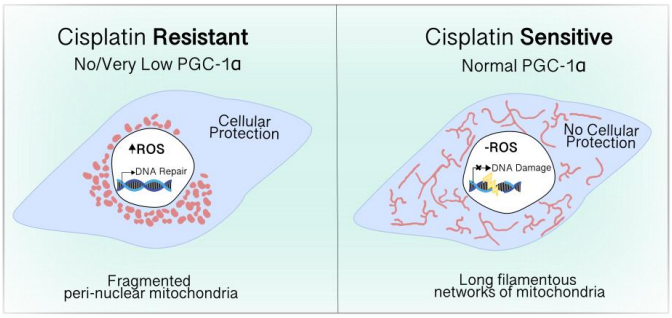


Fig. 34: Hypothetical model of cellular protection versus no protection through mitochondrial organization/networking. *In cisplatin-resistant cells expressing no or very low levels of PGC-1α, mitochondria become fragmented and organize peri-nuclearly. This induces or supports reactive oxygen species (ROS) production in the nucleus activating transcription of proteins that protect the cell against the damaging effects of cisplatin treatment.*

PGC-1α expression varies greatly between tumour and cell types, and can have both positive and negative effects on cancer cell survival (Bost & Kaminski, 2019) (Mastropasqua et al., 2018). However, no studies have evaluated PGC-1α expression or related mitochondrial functions, biogenesis and organization/networking under conditions of low glucose/FBS with or without FAs, i.e., mimicking more physiological conditions. To increase PGC-1α activity, several signaling pathways, including p38-MAPK, AKT and AMPK, stimulate binding of transcription factors to the PGC-1α promoter, and induce post-translational modifications (Fernandez-Marcos and Auwerx 2011) (Luo et al., 2019). Although increased PGC-1α activation typically correlates with increased mito-biogenesis, OXPHOS and FA oxidation, (Bost & Kaminski, 2019), it has also been shown to support tumour growth and cancer cell survival during periods of nutritional stress, for example by increasing glutamine flux through the TCA cycle (McGuirk et al., 2013). Here, PGC-1α expression was upregulated in P-cells cultured in low glucose medium with and without OA. We thus suggest that that this upregulation sustains survival rather than stimulates energy production, whereas in R-cells, where PGC-1α expression was unaffected by the different nutrient conditions, other survival pathways must be present, possibly such as those enriched in omental metastases, e.g., PI3K/AKT which when activated stimulates cancer cell proliferation, survival, and motility (Yang et al., 2019).

Although the addition of OA to low-glucose medium had no effect on mitochondrial routine respiration, FA-linked OCE, or ATP production, it stimulated the formation of long filamentous networks of mitochondria, which could be described as hyperfused, and the accumulation of LDs. This disputes the common assumption that filamentous networks of fused mitochondria are energized, and instead implies that mitochondrial dynamics cannot consistently be tied to a specific bioenergetic state (Silva Ramos, Larsson, & Mourier, 2016). Mitochondrial dynamics involves a constant cycling between fission and fusion, and responds to the nutrient environment by regulating nutrient utilization and energy production accordingly. Studies indicate that mitochondria in a nutrient-rich environment take on a fragmented architecture, whereas mitochondria exposed to starvation tend to stay in their fused state for longer (Gomes et al., 2011) (Molina et al., 2009). In fact, hyperfusion of mitochondria is considered a transient protective response to cellular stress, and protects against apoptosis and autophagy (Shutt & McBride, 2013) (Wai & Langer, 2016). Furthermore, upregulation of MFN1 has been shown to stimulate mitochondrial trafficking to the cytoskeleton to facilitate cancer cell migration away from unfavourable conditions to

support cell survival (Caino et al., 2015). Therefore, the fused and the fragmented states of mitochondria under standard nutrient conditions in P- and R-cells, respectively, suggest that these cells differ metabolically, and that R-cells are more efficient than P-cells at utilizing the available substrates. This is reflected in the metabolic flexibility and enhanced FA metabolic capabilities of R-cells in standard conditions. The accumulation of LDs is known to protect against cellular stress by preventing the oxidation of FAs that would contribute even more to cellular stress during conditions of starvation (Petan et al., 2018). We propose that in our *in vitro* model, the accumulation of LDs and hyperfusion of mitochondria reflect protective as well as energizing responses to the combination of OA and low glucose. This is line with emerging notions that cancer cells can run different metabolic programs in parallel to broaden their adaptability to changes in the microenvironment (Li & Wang, 2020).

CONCLUDING REMARKS & FUTURE PERSPECTIVES

Metabolic reprogramming has long since been established as a hallmark of cancer with high glucose consumption and metabolism at its centre. However, research on the role of lipid metabolism and mitochondrial organization and networking in tumour progression is still in its infancy. This thesis not only provides novel insights into the understanding of cellular metabolism as it relates to tumour progression in omental HGSOC metastases and TIC-like OVCA cells, but has also generated numerous compelling hypotheses for further investigation.

Through the identification of relationships between gene expression and enrichment pathways we could characterize omental metastases as proliferative and invasive tumours with increased lipid metabolic capabilities. Fatty acids are known to influence cancer cell gene expression and might underpin the upregulation of lipid metabolic genes observed in omental metastases. Moreover, we established a category of genes, LMID, with prognostic value in primary ovarian tumours, and detected three novel candidate genes, FABP4, GPD1, and CIDEA, with prognostic value in omental metastases. The relationship between high expression of these 3 genes in the omental metastases and better survival was also found in primary BRCA tumours, with similar TMEs. This suggests that the prognostic value of FABP4, GPD1, and CIDEA expression involves interaction between cancer cells and other cells or factors in an adipose-rich TME. Combining standard adjuvant chemotherapy, and/or

other anti-cancer drugs, with some manner of FABP4, GPD1, and CIDEA overexpression in omental metastases expressing low levels of these genes might improve treatment outcomes in HGSOC patients. Indeed, the application of targeted gene editing techniques, such as CRISPR-CAS9, has showed promising results for the treatment of numerous cancers *in-vivo*, including ovarian cancer (Akram et al., 2020) (Rosenblum et al., 2020).

In-vitro, metabolic reprogramming in the TIC-like R-cells, which similar to omental metastases, was found to involve enhanced lipid metabolic capabilities, including increased FA-linked ATP production potential despite loss of PGC-1α, and lower expression of TFAM and ATP5B. This highlights the importance of enzyme activity over gene expression when evaluating bioenergetics, wherefore, to further evaluate bioenergetics, assessing enzyme activities is necessary. We also propose that loss of PGC-1α could be involved in cisplatin resistance possibly through peri-nuclear clustering of mitochondria, but not in upregulation of TIC-marker ALDH1A. PGC-1β on the other hand appears to uphold its known role in maintaining basic mitochondrial function.

In light of the collective evidence, lipid metabolism relating to the TMEs could be of importance in understanding tumour progression and predicting overall survival in patients. However, the relationship between TIC-marker expression and metabolic reprogramming, in particular lipid metabolism, remains unknown. The relationships we established between the expression of ALDH1A and lipid metabolic and cell proliferation genes in tumours, as well as the upregulation of lipid metabolic capabilities in R-cells certainly point towards a role for ALDH1A in the regulation of lipid metabolism. KD of ALDH1A and evaluation of the expression and activity of specific lipid metabolic and mitochondrial genes and proteins respectively could offer more insight into the role of ALDH1A in metabolic reprogramming in OVCA cells.

The role of mitochondrial dynamics in tumour progression in more physiologically relevant conditions requires further investigation. Future *in-vitro* studies could thus include culturing cancer cells in different nutrient conditions containing e.g. adipocytokines, FAs, or other inflammatory factors and evaluating the tumourigenic and metastatic potential as well as the expression and activity of genes and proteins involved in lipid metabolism, FAO-linked ATP production, and mitochondrial dynamics/transport. High resolution respirometry could be used to assess FA-linked ATP producing capabilities, the activity of other related proteins

could be determined using specific enzyme activity assays, and fluorescently labeled mitochondrial dynamics and transport proteins could be used to assess their localization and movement during live cell imaging.

The new insights into metabolic reprogramming, a well-established hallmark of cancer, produced in this thesis not only expand the understanding of the roles of lipid metabolism and mitochondrial dynamics in ovarian cancer progression, but have also generated hypotheses and ideas for the exploration of the underlying mechanisms involved.

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APPENDIX I - LIST OF ABBREVIATIONS

2DHG:	D-2-hydroxyglutarate
ACADL:	Long-chain acyl-dehydrogenase
ACAT:	Acyl coenzyme A cholesterol acyltransferase
ACC:	Acetyl-CoA carboxylase
ADP:	Adenosine diphosphate
ALDH1A:	Aldehyde dehydrogenase 1 A
AMPK:	5' AMP-activated protein kinase
ATF2:	Activating transcription factor 2
ATGL:	Adipose triglyceride lipase
ATP:	Adenosine triphosphate
ATP5B:	ATP synthase F1 subunit beta
BMI:	Body Mass Index
BP:	Biological processes
BRAF:	V-raf murine sarcoma viral oncogene homolog B1
BRCA:	Breast cancer
C/EBPα/β:	CCAAT/enhancer-binding proteins alpha and beta
CA-125:	Cancer antigen 125
CAMKIV:	Calmodulin dependent protein kinase IV
CC:	Cellular compartments
CD113:	Cluster of differentiation 113
CD117:	Cluster of differentiation 117
CD133:	Cluster of differentiation 133
CD24:	Cluster of differentiation 24
CD36:	Cluster of differentiation 36
CD44:	Cluster of differentiation 44
CD87:	Cluster of differentiation 87
CE:	Cholesteryl ester
CFD:	Complement factor D
CIDEA:	Cell death inducing DFFA-like effector A
CIDEC:	Cell death inducing DFFA-like effector C
COX7B:	Cytochrome c oxidase subunit 7B
CPT1/2:	Carnitine palmitoyltransferase 1/2

CPT1A: Carnitine palmitoyltransferase 1 A

CPT1B: Carnitine palmitoyltransferase 1 B

CR: Clinical resistance

CSC: Cancer stem cell

DCIS: Ductal carcinoma *in-situ*

DEG: Differentially Expressed Genes

DHAP: Dihydroxyacetone phosphate

DRP1: Dynamin related protein 1

EMT: Epithelial-mesenchymal transition

EOC: Epithelial ovarian cancer

ERBB2: Also known as HER2 or Human epidermal growth factor receptor 2

ERRα/β: Estrogen-related receptor alpha/beta

ESA: Epithelial surface antigen

ETC: Electron transport chain

ETFP: Electron-transferring-flavoprotein dehydrogenase

FA: Fatty acid

FABP1: Fatty acid binding protein 1

FABP4: Fatty acid binding protein 4

FABP7: Fatty acid binding protein 7

FACS: Fluorescence-activated cell sorting

FADH2: Flavin adenine dinucleotide

FAO: Fatty acid beta-oxidation

FASN: Fatty acid synthase

FASTKD3: FAST kinase domain-containing protein 3

FCFc: Cytochrome c control factor

FDA: U.S. Food and Drug Administration

FFA: Free fatty acid

FH: Fumarate hydratase

FIGO: International Federation of Gynaecology and Obstetrics

FIS1: Fission protein 1

FOXO-1: Forkhead Box O1

FOXO3: Forkhead Box O3

FPKM: Fragments per kilobase of transcript sequence per millions base pairs sequenced

FSP27: Fat specific protein 27

FXR: Farnesoid X receptor

G-3-P: Glycerol-3-phosphate

GAC: Glutaminase C

GBM: Glioblastoma

GCN5: General control non-depressible 5

GLS: Glutaminase

GLUT4: Glucose transporter 4

GO: Gene Ontology

GPD1: Glycerol-3-phosphate dehydrogenase 1

H&E: Hematoxylin and Eosin

H+: Proton

HCC: Hepatocellular carcinoma

HGSOC: High-grade serous ovarian carcinoma

HIF-1α: Hypoxia inducible factor 1 alpha

HKII: Hexokinase II

HSL: Hormone sensitive lipase

IDH: Isocitrate dehydrogenase

IL-6: Interleukin-6

IL-8: Interleukin-8

IL-8R: Interleukin 8 receptor

JAK: Janus Kinases

KD: Knock-down

KEGG: Kyoto Encyclopedia of Genes and Genomes

KGA: Kidney-type glutaminase

KRAS: Kirsten rat sarcoma

LD: Lipid droplet

LDHA: Lactate dehydrogenase A

LEP: Leptin

LGM1: Low glucose and low FBS medium

LPL: Low-density lipoproteins

LXRα/β: Liver X receptor alpha/beta

MAGL: Monoacylglycerol lipase

MALT: Mucosa-associated lymphoid tissue

MEF2:	Myocyte enhancer factor-2
MF:	Molecular functions
MFF:	Mitochondrial fission factor
MFN1/2:	Mitofusin 1/2
MIRO1/2:	Rho GTPases 1/2
Mito:	Mitochondrial
MMP19:	Matrix metalloproteinase 19
MMP2:	Matrix metalloproteinase 2
mtDNA:	Mitochondrial DNA
mTOR:	Mammalian target of rapamycin
MYC:	Myelocytomatosis oncogene
NAD+:	Nicotinamide adenine dinucleotide
NADH:	Adenine dinucleotide
NFκB:	Nuclear Factor Kappa B
NIH:	National Institutes of Health
NPC:	Nasopharyngeal carcinoma
NRF1/2:	Nuclear respiratory factor 1/2
OA:	Oleic acid
OCE:	OXPHOS coupling efficiency
OCT:	Optimal cutting temperature compound
OCT4:	Octamer-binding transcription factor 4
OPA1:	Optic atrophy 1
OS:	Overall survival
OVCA:	Ovarian cancer
OXPHOS:	Oxidative phosphorylation
p-AMPK:	phosphorylated 5' AMP-activated protein kinase
p38-MAPK:	p38-mitogen-activated protein kinases
PARP:	Poly ADP-Ribose polymerase
PCR:	Polymerase chain reaction
PDHC:	Pyruvate dehydrogenase complex
PDK4:	Pyruvate dehydrogenase kinase 4
PFI:	Platinum-free interval
PFS:	Progression free survival
PFS:	Progression free survival

PGC-1α/β:	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha/beta
PI3K-AKT:	Phosphoinositide 3-kinase-protein kinase B
PKA:	Protein kinase A
PKM2:	Pyruvate kinase isoform M2
PLIN1:	Perilipin 1
PLIN4:	Perilipin 4
PPARα:	Peroxisome proliferator-activated receptor alpha
PPARγ:	Peroxisome proliferator-activated receptor gamma
PPP:	Pentose phosphate pathwayb
PRMT1:	Protein arginine methyl transferase 1
PTEN:	Phosphatase and tensin homolog
RAS-MAPK:	Ras-mitogen activated protein kinase
ROS:	Reactive oxygen species
RTK:	Receptor tyrosine kinase
SCD-1:	Stearoyl-CoA desaturase-1
SDH:	Succinate dehydrogenase
SERBP:	Sterol regulatory element-binding protein
siRNA:	Short-interfering RNA
SIRT1:	Sirtuin 1
SM:	Standard Medium
SOX2:	SRY (sex determining region Y)-box 2
SREBPF1/2:	Sterol regulatory element-binding transcription factor ½
STAT1:	Signal transducer and activator of transcription 1
STAT3:	Signal transducer and activator of transcription 3
TAG:	Triacylglycerol
TCA:	Tricarboxylic acid
TF:	Transcription factor
TFAM:	Mitochondrial transcription factor A
TFB2M:	Transcription factor B2 mitochondrial
TFG-β:	Transforming growth factor beta
TIC:	Tumour-initiating cell
TIDC:	Tumour-infiltrating dendritic cells
TIMP-1:	Tissue inhibitor of metalloproteinases
TLR4:	Toll-like receptor 4

TME: Tumour microenvironment

TP53: Tumour protein 53

TRβ: Thyroid hormone receptor beta

UCP1: Uncoupling protein 1

UCP2: Uncoupling protein 2

uPAR: Urokinase receptor

WHO: World Health Organization

Wnt/β: Wingless and Int-1 beta