Phospholipids and cholesterol: Inducers of cancer multidrug resistance and therapeutic targets

Phospholipids and cholesterol: inducers and therapeutic targets in multidrug resistance

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Abstract

Lipids, in particular phospholipids and cholesterol, are the main components of plasma-membrane, where drug efflux ATP binding cassette (ABC) transporters are embedded. In this review, we will discuss how lipids strongly modulate the activity and expression of these transporters, contributing to the development of a multidrug resistant (MDR) phenotype. The mechanisms are pleiotropic. First, notwithstanding the high intra-and inter-tumor variability, MDR cells have an altered composition of plasma-membrane phospholipids and glycosphingolipids, enriched in very long saturated fatty acyl chains. This feature, together with the increased amount of cholesterol, reduces the fluidity of membranes, alters the spatial organization of membrane nano- and micro-domains, interacts with transmembrane helixes of ABC transporters, favoring the drugs' binding and release. Second, MDR cells show a peculiar composition of membranes of intracellular organelles, such as mitochondria and

endoplasmic reticulum. Particularly, they contain a lower amount of oxidizable fatty acids, resulting more resistant to oxidative stress and chemotherapy-induced apoptosis. Third, drug resistant cancer cells have a higher ratio of monosatured/polyunsatured fatty acids: this lipid signature reduces the production of reactive aldehydes with cytotoxic and pro-inflammatory activity and, together with the higher activity of anti-oxidant enzymes, limits the damages induced by lipid peroxidation. Finally, specific precursors of phospholipids and cholesterol, such as ceramides and isoprenoids, are highly produced within MDR cells: by acting as second messengers, they trigger multiple cascades that induce the transcription of efflux transporters and/or promote a metabolic rewiring supporting the MDR phenotype.

High-throughput lipidomics and computational biology technologies are a great help in analyzing the tumors lipid signature in a personalized manner and identifying novel biomarkers of resistance. Moreover, beyond inducing MDR, lipid metabolism offers a tremendous opportunity of reversing it, by using lipid analogs and repurposing lipid-targeting drugs (e.g. statins, aminobisphosphonates) that reprogram the lipid composition of resistant cells, turning it back to sensitive cells.

Keywords: phospholipids; cholesterol; isoprenoids; membrane fluidity; lipid peroxidation; drug resistance

1. Altered biosynthesis of phospholipids and cholesterol in cancer cells

More than 1,000 types of lipids have been characterized in living cells (Corradi et al., 2019), as circulating molecules or components of biological membranes. Cell membranes are made of three main classes of lipids: 1) glycerophospholipids (PLs), including phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI) and phosphatidylinositol phosphates (PIPs); 2) sphingolipids, including

sphingomyelins (SM) and glycosphingolipids; 3) sterols, i.e. mainly cholesterol (Chol) in mammalian cells. The lipids of the first two classes are strongly amphiphilic, bearing a polar head group and one or two fatty acyl chains with various degrees of saturation.

In cancer cells, many biochemical processes are altered, including fatty acid and lipid metabolism. Consequently, tumors' lipid profile often differ from normal tissues' profile (Zalba and Hagen, 2017; Islam and Manna, 2019). Notwithstanding the increasing number of lipidomic studies focused on cancer cells, differentiating the lipid signature of transformed and non-transformed cells is not an easy task. First, the lipid composition of membranes is strongly cancer-type dependent (Zalba and Hagen, 2017; Islam and Manna, 2019), meaning that there a high inter-tumor variability. Second, during progression, cancer cells acquire specific alterations in lipid biosynthesis: hence, the lipid composition of the same tumor may vary at different stages. The peculiar changes in lipid composition observed at advanced stages may identify cells potentially more aggressive and resistant to stressors, including chemotherapy. Indeed, the interaction with specific lipids is a critical factor affecting the activity of ATP binding cassette (ABC) transporters involved in drug efflux, such as P-glycoprotein (Pgp/ABCB1) which is encoded by the *mdr1* gene, multidrug resistance related protein 1 (MRP1/ABCC1), breast cancer resistance protein (BCRP/ABCG2) (Jung et al., 2015; Lee and Kolesnick, 2017).

In this review, we will analyze how changes in lipid metabolism and membrane composition may determine multidrug resistance (MDR), either in an ABC transporter-dependent or independent manner.

1.1. An altered phospholipid metabolism promotes drug resistance

The PL composition of plasma-membrane in sensitive and resistant cells can be analysed by lipidomic shotgun approaches. At the present, however, the studies on different tumors and drugs are limited. The main reason of this low number of experimental works is a technical limitation, because the amount of

biological material requested by lipidomic analyses often exceeds the amount that can be obtained from tumor specimens. In the attempt to overcome this limitation, lipidomic techniques have been applied to the extracellular vesicles (EVs), released by the tumor and recapitulating the lipid profile of the plasma-membrane of the original tissue. Using this approach, PC, PI, PG, lysoPC and lysoPI containing very long fatty acyl chains have emerged as the PLs most enriched in the EVs derived from gefitinib-resistant patients. Notably, this lipid signature discriminates sensitive from resistant patients (Jung et al., 2015).

Interestingly, more than single classes of PLs, the composition of their fatty acyl chains is the main discriminating factor between sensitive and resistant cells. Fatty acid composition of PLs depends on the acyltransferase enzymes, that – under this perspective – appear interesting druggable targets to improve the chemosensitivity. For instance, the abnormal synthesis of PC, promoted by the lipid droplets-associated enzyme lysoPC acyltransferase 2 (LPCAT2), induces resistance to oxaliplatin and 5-fluorouracil in colorectal cancer. The resistance mechanism is due to the increased resistance to apoptosis in response to endoplasmic reticulum (ER) stressors and to the higher resistance to the immunogenic cell death promoted by chemotherapy (Cotte et al., 2018). These data suggest that LPCAT2 activity likely alters the lipid composition of ER and plasma-membrane, determining a lower susceptibility to ER stress and to the recognition by the host immune system, respectively. The abundance of lipid droplets has been correlated with resistance to docetaxel also in progesterone-dependent breast cancers, because hydrophobic drugs such docetaxel are easily sequestered within droplets (Schlaepfer et al., 2012). Moreover, progesterone up-regulates the lipid droplet-associated enzyme stearoyl-coenzyme A desaturase-1 (SCD-1) that enriches plasma-membrane PLs of oleic acid. This enrichment has been correlated with the resistance to docetaxel (Schlaepfer et al., 2012), likely because it increases the membrane fluidity and alters the connections between plasma-membrane and cytoskeleton, allowing a better cell adaptation to cytoskeleton-targeting drugs such docetaxel.

Also the immediate precursors of PLs, i.e. lysophospholipids (lysoPL), mediate chemoresistance. In most cases they act through plasma-membrane independent mechanisms. A recent study demonstrated that lysoPC protects tumor cells from DNA damaging agents such as cisplatin. This mechanism is highly lipid-specific and drug-specific. Indeed, only lysoPC containing very long chain/long saturated fatty acyl chains induces chemoresistance. Second, cells are protected against DNA-damaging drugs, but retain their sensitivity to drugs acting with different mechanisms (Houthuijzen et al., 2014). This leads to hypothesize that lysoPC acts as a second messenger and an activator of the DNA repairing machinery, unveiling unsuspected biological effects of this PL precursor. Similarly, lysophosphatidate-1 (LPA-1), a precursor shared by most PLs, is a strong activator of the Nuclear factor erythroid 2-related factor 2 (Nrf2) that up-regulate several drug efflux transporters, such as MRP1, MRP2, MRP3 and BCRP, and several anti-oxidant enzymes (Venkatraman et al., 2015). In this way, LPA-1 activates at least two mechanisms promoting chemoresistance.

Not only PLs, but also their receptors, play a role in drug resistance. A recently-discovered family of PS receptors, including TYRO3, AXL and MERTK (globally known as TAM family), has been identified in several hematological and solid cancers with acquired or constitutive resistance to tyrosine kinase inhibitors (TKIs). The binding of PS activates multiple cross-talks between TAM receptors and receptor tyrosine kinases (RTKs), promoting pro-survival and oncogenic pathways, increasing cell survival and/or reducing apoptosis in response to TKIs (Graham et al., 2014). The production of chimeric TAM proteins allowed to identify MERTK as the predominant PS receptor determining chemo- and immune-resistance: indeed, the binding of PS exposed by apoptotic cells fosters MERTK-expressing cells to activate Akt-dependent pro-survival pathway and to up-regulate the immune checkpoint ligand programmed death-ligand 1 (PD-L1) (Kasikara et al., 2017). These experiments elegantly demonstrate the possible intratumor development of chemo-immune-resistant clones, able to

turn the pro-apoptotic messenger PS, exposed by dying cells, into a survival messenger after the activation of MERTK on nearby cells.

As far as sphingolipids are concerned, the metabolites that have been most strongly correlated with chemoresistance are ceramide (Cer), i.e. the precursor of SMs, shingosine (Sph), i.e. the precursor of Cer, and glucosylceramide (GC), a key component of glycosphingolipids.

Cer is the main backbone of sphingolipids. It is synthesized by at least six Cer synthases (CerS) that conjugate Sph with a fatty acid. Each CerS produces Cer with different fatty acyl chains, determining a broad spectrum of SMs that can cause either chemosensitivity or chemoresistance. (Brachtendorf et al., 2019). For instance, in head and neck squamous cell carcinoma, doxorubicin and cisplatin promote the ubiquitination of CerS1 (Sridevi et al., 2010). Since CerS1 mediates apoptosis if translocated to the outer mitochondrial membrane (OMM) where it synthesizes the lethal mitophagic derivative 18:Cer (Dany et al., 2016), the removal of CerS1 by the ubiquitin/proteasome system prevents the mitochondria-dependent cell death in response to chemotherapy. The chemosensitivity of colon cancer cells is determined by the ratio between CerS2 and CerS5, as well as by the status of *TP53*. In wild-type *TP53* cells, CerS5 prevails: the SMs produced by CerS5 induce resistance to oxaliplatin and 5-fluorouracil by promoting cytoprotective autophagy and increasing mitochondrial energy metabolism. In mutated-*TP53* cells, CerS2 is the predominant form and increases the production of pro-proliferative C24:0- and C24:1-Cer. Although the knock-down of CerS5 restores chemosensitivity in wild-type *TP53* cells, in mutated-*TP53* cells the knock-down of CerS2 does not (Brachtendorf et al., 2018). These results lead to hypothesize that, although important, the expression of specific CerS isoforms is not sufficient to determine *per se* drug sensitivity or resistance. The changes in CerS levels and isoforms likely give a partial contribution to the global lipid rearrangement and/or cooperate with other signaling pathways that are the real determinants of chemoresistance. As mentioned above, the *TP53* status is important in determining the chemoresistance of colon cancer cells mediated by CerS5 and CerS2

(Brachtendorf et al., 2018), but it is not the only factor. CerS2 promotes drug resistance by up-regulating the anti-apoptotic protein Bcl2L13 (Jensen et al., 2014) and increasing the activity of the vacuolar H^+-ATPase (V-H^+-ATPase) (Wang et al., 2017). The anti-apoptotic effect mediated by Bcl2L13 is enhanced by CerS2 heterodimerization with CerS6 (Jensen et al., 2014): since different tumor types exhibit different isoforms of CerS, the extent of chemoresistance mediated by Bcl2L13 is unequivocally tumor-dependent. The increased acidification of lysosomes is instead a well-known mechanism of MDR shared by different tumors, because it increases sequestration and inactivation of drugs that are weak bases, and favors the quick exocytosis of the sequestered drugs (Zhitomirsky and Assaraf, 2015; Zhitomirsky and Assaraf, 2016; Zhitomirsky et al., 2018).

Sph and Cer can be phosphorylated by sphingosine kinase (SphK) 1 or 2, and by Cer kinase (CerK), producing Sph-1-phosphate (S1P) and Cer-1-phosphate (C1P), respectively (Mesev et al., 2017). By activating the membrane-bound enzyme cyclooxygenase-2 (COX-2), S1P and C1P stimulate the production of prostaglandin E2 (PGE2) that up-regulates the expression of Pgp/ABCB1, MRP1/ABCC1 and BCRP/ABCG2 (Brachtendorf et al., 2019). Cer and GC up-regulate Pgp expression also independently of COX-2 (Gouazé-Andersson et al., 2007), acting as second messengers promoting *mdr1* gene transcription. Also S1P determines drug resistance to gemcitabine in clear cell renal carcinoma by activating the Hypoxia inducible factor (HIF)-1α and HIF-2 transcription factors that promote tumor growth and alter tumor vascularization. In vivo validation studies using the S1P inhibitor FTY720, however, pointed out that the chemosensitization effects of the latter are independent on the restored vascularization (Gstalder et al., 2016). Although the chemosensitizing mechanism has not been investigated in depth, it is noteworthy that HIF-1α is a strongly transcriptional inducer of *mdr1* gene (Comerford et al., 2002), and FTY720 may prevent HIF-1α-induced up-regulation of Pgp. This may be the crucial chemosensitizing factor. The Sph/S1P ratio determines resistance to etoposide in retinoblastoma cells: in this tumor, sensitive and resistant cells increase the

synthesis of the pro-apoptotic mediator Sph, while only resistant cells simultaneously increase the levels of S1P that counteracts Sph-induced apoptosis (Kakkassery et al., 2019). Since etoposide is a substrate of Pgp and S1P up-regulates Pgp (Brachtendorf et al., 2019), we cannot exclude that a parallel mechanism of resistance is the increased expression of this efflux transporter induced by the high levels of S1P.

Multiple linkages between deregulated metabolism of Sph- and Cer-derivatives and ABC transporter expression exist. For instance S1P is both an inducer and a substrate MRP1 and BCRP (Ogretmen, 2017). Curiously, also chemotherapeutic drugs such as doxorubicin have the dual role of inducer and substrate of Pgp (Kopecka et al., 2015a), indirectly suggesting that S1P is as strong as chemotherapy in inducing drug resistance. If the intracellular S1P upregulates *mdr1* gene, the effluxed S1P binds to its G-protein coupled receptor, activating autocrine signaling pathways that increase cell proliferation and reduce apoptosis (Ogretmen, 2017), providing additional mechanisms of drug resistance. These mechanisms are particularly evident in hypoxic tumor bulk, where ShpK2 increases the synthesis of S1P, which is effluxed and autocrinally stimulates the p42/p44 mitogen-activated kinase (MAPK) pro-proliferative axis (Schnitzer et al., 2009), contributing to chemoresistance.

Similarly to S1P, the chemoresistance induced by GC is not mediated only by the up-regulation of Pgp. For instance, Pgp-positive/fludarabine-resistant clones of B-cells chronic lymphocytic leukemia (B-CLL) have an abnormal up-regulation of GCS synthase (GCS), also known as UDP-glucose ceramide glucosyltransferase (UGCG): this enzyme reduces the intracellular levels of the pro-apoptotic metabolite Cer, converting it into GC, that instead exerts pro-survival effects (Huang et al., 2018). This change in Cer/GC ratio contributes to the resistance against fludarabine. Notably, patients with unmutated *IGHV* chain, i.e. with the most aggressive and chemoresistant form of B-CLL, have reduced levels of Cer and increased levels of GC also before starting any treatment (Thurgood et al., 2019), suggesting that a high GC/Cer ratio may be an hallmark of constitutive resistance in this tumor. In

breast cancer cells resistant to doxorubicin, GCS up-regulation promotes chemoresistance by enriching plasma-membrane domains containing glycosphingolipids with GC. This change activates Akt- and ERK1/2-dependent pathways that up-regulate at the same time anti-apoptotic genes and *mdr1* gene (Wegner et al., 2018), providing multiple mechanisms of resistance.

1.2. An altered sterols metabolism supports drug resistance

Both the increased endogenous synthesis of Chol and the exposure to high circulating levels of Chol favor cancer progression. On the one hand, Chol is a critical structural component of lipid rafts, dynamic plasma-membrane domains rich of RTKs and MDR efflux transporters. On the other hand, as PLs, also Chol acts as a second messenger, able to activate oncogenic and stemness related pathways, such as mammalian target of rapamycin complex 1 (mTORC1)- and Hedgehog-dependent pathways (Ding et al., 2019).

Using *in vitro* models of acquired resistance to doxorubicin, Gene Ontology analysis has revealed that the Chol biosynthetic pathway, the so-called mevalonate (MVA) pathway, is generally up-regulated. Genes up-regulated belong to upper MVA pathway (e.g. 3-β-hydroxy-3-β-mehyl glutaryl coenzyme A synthase 1, *HMGCS1*; 3-β-hydroxy-3-β-mehyl glutaryl coenzyme A reductase, *HMGR*; isopentenyl diphosphate δ Isomerase 1, *IDI1*), committed to the synthesis of isoprenoids (geranyl pyrophosphate, GPP; farnesyl pyrophosphate, FFP; geranylgeranyl pyrophosphate, GGPP), and of lower MVA pathway (e.g. farnesyl-diphosphate farnesyltransferase 1, *FDT1*; squalene epoxidase, *SQLE*; 7-dehydrocholesterol reductase, *DHCR7*), committed to the synthesis of Chol (Greife et al., 2015). Indeed, both isoprenoids and Chol are elevated in MDR cells, as observed in colon cancer (Riganti et al., 2013), malignant pleural mesothelioma (Salaroglio et al., 2015), non-small cell lung cancer (Kopecka et al., 2015b), breast cancer (Kopecka et al., 2016). Tissue Cancer Genome Atlas (TCGA) analysis pointed out as the increased expression of genes involved in Chol biosynthesis is correlated with a lower patients survival (Kuzu et al., 2016), leading to hypothesize that this metabolic phenotype

mediates an accelerated tumor progression and/or a poor response to cancer treatments. In support of this hypothesis, a study on lung adenocarcinoma showed that patients with high hypercholesterolemia are prone to develop resistance to cisplatin, as a consequence of Chol-induced up-regulation of BCRP (Wu et al., 2015). Similarly, *in vitro* experiments demonstrated that Chol-LDL up-regulates Pgp and to a lesser extent MRP1, Chol-HDL decreases Pgp at high concentrations, while it produces opposite effects at low concentrations (Celestino et al., 2015). These results suggest that Chol is a transcriptional controller of several MDR efflux transporters. Beside the transcriptional effect, the amount of Chol regulates plasma-membrane fluidity, finely tuning the optimal conditions for the activity of ABC transporters, as detailed in Section 2.

Among the transcription factors controlling the expression of MVA pathway and Chol metabolism genes, the most active are the Sterol regulatory element-binding proteins (SREBPs) and the Liver X receptors (LXRs). Both families play a role in drug resistance, using either Chol metabolism-dependent or independent mechanisms. Intriguingly, in hypoxic conditions, where HIF-1α is active and up-regulates *mdr1* gene (Comerford et al., 2002), also SREBP1 is up-regulated (Furuta et al., 2008). Being with high levels of Pgp and high levels of Chol, hypoxic cells have at least two conditions favoring chemoresistance. Also ABCC2 is up-regulated by SREBPs, which act in combination with LXR in hepatoma HepG2 cells (Kobayashi et al., 2013).

LXRs up-regulate the transcription of Chol efflux proteins, such as ABCA1 and ABCG1 (Sharma and Agnihotri, 2019). The reduction in intracellular Chol produced by the activation of LXR should theoretically result in increased sensitivity to chemotherapy. However, this deduction is not always confirmed by the experimental data, because Chol and Chol-derived oxysterols are ligands of LXRs, triggering unexpected feedback mechanisms. For instance, Chol activates LXRβ in ovarian cancer cells, reducing the apoptosis mediated by cisplatin and at the same time up-regulating Pgp and BCRP expression. The mechanistic evidence that Chol and LXRβ trigger the resistance to cisplatin is proved

by the observation that cells depleted of Chol with β-methyl cyclodextrin (β-MCD) or silenced for LXRβ reacquire chemosensitivity (Kim et al., 2018). In non-small cell lung cancer, a quite complex scenario exists: indeed, if LXR physiological ligands have no effects on the efficacy of the EGFR inhibitor Gefitinib, LXR synthetic agonists reverse the resistance to this TKI (Hu et al., 2017; Liu et al., 2018). These findings suggest that LXRs may represent good druggable targets to reverse chemoresistance, but their targeting can have highly different and tumor-dependent impacts on chemosensitization.

Besides transcription factors controlling MVA pathway, other proteins controlling Chol metabolism are overexpressed in resistant cells. This is the case of the Progesterone receptor membrane component 1 (PGRMC1), an adaptor protein regulating Chol synthesis by modulating SREBP cleavage activating protein (SCAP). PGRM1 has been found overexpressed in doxorubicin-resistant MES-A/DxR uterine sarcoma cells compared to the sensitive counterpart, following the same trend of Pgp. Notably, PGCRM1 knockdown has synergistic effects with the Pgp inhibitor verapamil in restoring doxorubicin efficacy (Eugenie et al., 2015), suggesting that PGCRM1-driven mechanisms cooperate to maintain a high activity or expression of Pgp.

2. How the lipid profile of plasma-membrane impacts on drug resistance

2.1. Peculiarities of plasma-membrane lipid composition in drug resistant cells

As a barrier between the cell and its environment, plasma-membrane has an important function in sensing changes in the tumor microenvironment (TME). For this reason, plasma-membrane contains a wide range of different proteins and lipids, that determine distinct cellular reactions in response to extracellular stimuli and stressors such as chemotherapy. Since lipids are the major components of the

plasma-membrane, their qualitative and quantitative variations contribute to mediate these differential responses.

Recent lipidomic analyses, which identify more and more lipid species, have allowed the first qualitative and semi-quantitative comparisons of the plasma-membrane lipid composition between not-transformed cells and cancer cells (Brzozowski et al., 2018; Shen et al., 2017). The plasma-membrane lipid composition is cancer-type dependent (Hendrich and Michalak, 2003), making virtually impossible to build a general prediction linking the lipid composition and the response to specific drugs. For instance, the concentration of plasma-membrane PLs is increased in breast cancers compared to normal mammary tissues, as seen on 267 human specimens (Hilvo et al., 2011). This signature is associated with cancer progression and the enrichment in PLs has been proposed as a negative prognostic biomarker, potentially suggesting higher invasiveness and/or poor response to treatments. Similarly, the lipid profile (including 179 different PLs) was analyzed in malignant and non-malignant lung tissues from 162 non-small cell lung cancer patients: more than 90 PLs were differentially present in cancer cells. The increases of PE, PI and PC, and the decrease of PS relative concentrations were among the most prominent discriminating changes (Marien et al., 2015). This could be consistent with the fact that PS is a pro-apoptotic PL, and cancer cells maintain low its amount. By contrast, in 20 kidney cancer patients, PE 36:4 (16:0/20:4) was the most markedly down-regulated lipid, followed by the down-modulation of other 36:4 PLs, namely, PC 16:0/20:4 and PI 16:0/20:4 (Cífková et al., 2015). To make this scenario more complicated, it has been reported that PLs also change between primary and metastatic sites, e.g. between colorectal cancer primary tumor and liver metastases, and in different areas of the same tumor. For instance, while PS is a typical marker of apoptotic areas, PC was abundant in necrotic zones (Patterson et al., 2016). These differential changes between tumor and normal cells, or between different sites of the same tumors may mediate the cellular adaptation to the different environmental conditions that cancer cells encounter during their

progression, contributing to proliferation and invasion.

In addition to changes in the polar head groups, also the saturation of fatty acyl chains changes in cancer tissues, where mono-unsaturated PCs (e.g. PC 36:1) prevail over saturated forms, such as PC (36:0) and lysoPC (18:0) (Ide et al., 2013). Better than only focusing on single classes, a lipid signature, as defined by several classes of lipids containing fatty acids with specific degrees of saturation, may serve as predictive or prognostic biomarkers. For instance, the set of PG (34:0), SM (42:2 and 38:8), Cer (44:5), lysoPC (18:3 and 18:2), PE (O-36:3 and O-38:3) has emerged as a good discriminator between colorectal cancer patients and healthy volunteers (Shen et al., 2017). Similarly, a prognostic three-lipid signature (Cer 18:1,24:1, SMs 18:2,16:0, PC 16:0,16:0) is associated with shorter survival in patients with metastatic and castration-resistant prostate cancer (Lin et al., 2017).

Also the profile of sterols contained in plasma-membrane has been under scrutiny in cancer cells. The higher Chol relative concentration increases the membrane ordering and decreases the area-per-lipid compared to non-transformed cells (Jedlovszky and Mezei, 2003; Róg et al., 2009; Wu et al., 2014; Zalba and ten Hagen, 2017). These events lower membrane fluidity, affect the cell migration and the diffusion of metabolites and drugs across plasma-membrane. Sterols and sphingolipids are particularly abundant in lipid rafts (Cebecauer et al., 2018). The relative increase of Chol and sphingolipids within the rafts of MDR cancer cells affects the expression, recycling and biological activity of the proteins concentrated in these domains, including ABC transporters (Ye et al., 2019; Zalba and ten Hagen, 2017). This could be an additional lipid-related factor determining drug resistance.

2.2. The impact of plasma-membrane lipid composition on drug permeability

Lipid composition is critical in the delivery and intracellular accumulation of anti-cancer drugs, because - before reaching their biological target(s) - drugs have to cross many biological membranes. The intracellular content of the drugs is the net sum of passive diffusion and active transport, mediated

by membrane-associated influx and efflux transporters, such as solute carriers (SLCs) or ABC transporters (**Figure 1**). By modifying fluidity or other biophysical properties of membranes and membrane-associated proteins, the lipid composition strongly modulates the drug delivery across the plasma-membrane.

One mechanism of drug resistance is the decreased drug permeability, due to different mechanisms. First, PLs in cancer cells often contain very long fatty acyl chains that are uncommon in non-transformed tissues and may reduce the passive diffusion of hydrophilic and amphipathic drugs. Second, it has been demonstrates that intracellular pH alters the mechanical properties of plasma-membrane, making it less permeable to doxorubicin (Rauch, 2009). MDR cells have an increased number and activity of lysosomes and so far an altered intracellular pH (Zhitomirsky and Assaraf, 2016). It is known that this feature promotes the sequestration and subsequent exocytosis of weak bases as doxorubicin from MDR cells (Zhitomirsky and Assaraf, 2017; Zhitomirsky and Assaraf, 2015). Intriguingly, this phenotype also reduces the entry of doxorubicin across plasma-membrane. This redundancy of resistance mechanisms is typical of MDR cells.

The passive permeation of drugs roughly follows a 3-step process: membrane entry (i.e. insertion in the bilayer from the donor compartment), flip-flop from one leaflet of the bilayer to the other one, membrane exit (i.e. drug release into the acceptor compartment). This process is driven by the drug diffusion coefficient and the Gibbs energy profile along the perpendicular to the membrane. These values allow to build rational structure-property relationships and to establish good drug-candidates for passive permeation (Bochicchio et al., 2015; Comer et al., 2017; Dickson et al., 2017). In addition to the chemical feature of the drug, any modification in plasma-membrane fluidity and organization dramatically alters the diffusion coefficients or Gibbs energy profiles. For instance, an increase in the Chol relative concentration from 0% to 33% decreases the permeability of cisplatin by one order of

magnitude (Rivel et al., 2019). The loss of plasma-membrane asymmetry, a common effect in cancer cells, is a second factor decreasing the permeability of cisplatin in comparison to the membranes of normal cells (Rivel et al., 2019). The modification of lipid composition may increase the energetic barriers of drug influx: this event justifies the reduced entry of doxorubicin, an amphipatic drug, in the presence of membranes containing short chain sphingolipids (van Hell et al., 2014). Beyond acting on energy coefficients, the different ratio between PLs such as PC, PI and PE detected in MDR cells also modifies the relative concentrations of polar head groups (Zalba and ten Hagen, 2017). This implies a change in surface charges and steric hindrance, that constitute other reasons impairing the influx of specific drugs in chemoresistant cells.

2.3. The impact of plasma-membrane lipid composition on ABC transporters

Beside the reduced drug influx, MDR has been critically related to the increased drug efflux through ABC transporters. For this reason, FDA (Food and Drug Administration) and EMA (European Medicines Agency) have recommended the systematic pre-clinical assessment of the capacity of novel drugs to interact with specific ABC transporters, identified as critical determinants of drug efflux by the International Transporter Consortium (ITC) (Hillgren et al., 2013). ABC transporters are made of two nucleotide binding domains (NBDs) and several transmembrane domains (TMDs). Most ABC transporters have been described to function by the alternating-access mechanism, during which the protein goes from inward-facing open (IF-o) to outward-facing (OF) conformers (**Figure 1**), passing through various inward-facing close (IF-c) intermediate states. In these three geometries, the drug enters, is translocated and exits (Pan and Aller, 2018). This process is driven by ATP hydrolysis, triggered by the conformational modifications induced upon the substrate binding and by the interaction with the surrounding lipid bilayer (Immadisetty et al., 2019). The lipid environment surrounding the transporters is a crucial solubilizing matrix: by specific or non-specific interactions

(Corradi et al., 2019) lipids change the conformation of ABC transporters (Gustot et al., 2010), acting as allosteric modulators. The interactions between the annular lipids and the transmembrane helices may impact on the alternating access mechanism (Ward et al., 2012), as well as on the efficiency of the catalytic cycle. Indeed, it has been observed that lipids are particularly important in stabilizing the transporters in the low-energy and stable conformation. For instance, conversely to PLs containing short fatty acyl chains, PLs with long chains dynamically drive the transporters in a stabilized conformation, reducing the ATPase activity (Bao et al., 2013).

As mentioned above, the lipids surrounding the ABC transporters have been suggested to be organized into nano- or micro-domains, including lipid rafts, with a defined structural organization (Radeva et al., 2005). This hypothesis fully agrees with recent coarse-grained simulations made with lipid mixtures, showing a strong correlation between the extent of lateral diffusion of membrane proteins and the composition of a 50-100 annular lipid shell surrounding the protein (Barreto-Ojeda et al., 2018; Marrink et al., 2019). This annular belt constitutes a highly dynamic solvation shell for membrane transporters. The physical properties of this belt, including the fluidity or the curvature of fatty acyl chains, create non-specific lipid-protein interactions which transfer tensions from the membrane to the protein. This energy transfer impacts on the transporter conformation and consequently on its function. The huge number of lipids constituting human plasma-membranes limits the building of exhaustive in silico or in vitro membrane models to study the lipid-protein interactions. The modelling work is complicated by the fact that lipid bilayers are asymmetric, heterogeneous and dynamic, meaning that the lipid ratios can fluctuate from one plasma-membrane region to another one within the same cell. Moreover, the lipid composition is subjected to inter-individual variability, further increasing the complexity of studying clinically relevant membrane models. Another important point to be considered is that some ABC transporters can also translocate lipids (Bocer et al., 2012; Doerrler et al., 2004), thus modifying lipid composition and bilayer asymmetry. Multiple computational simulations supported by

high-throughput lipidomic analyses of the membrane domains surrounding ABC transporters may provide good theoretical models in the future. At the present, a few experimental data on a limited number of ABC transporters exists.

An enrichment in PC and Chol was observed in the annular belt surrounding Pgp (Fosso-Tande et al., 2017; Sharom, 2014). On the basis of these observations, plasma-membrane models containing Pgp-surrounding lipids have been built, by using POPC (1-palmitoyl-2-oleoyl-glycero-3-PC)/POPE (1-palmitoyl-2-oleoyl-glycero-3-PE)/POPS (1-palmitoyl-2-oleoyl-glycero-3-PS)/POPIP (1-palmitoyl-2-oleoyl-glycero-3-PIP)/SM/Chol (Enkavi et al., 2019) or POPE/POPC/POPS/SM/Chol, to mimic brain endothelial cell membrane (Domicevica et al., 2017) where Pgp is abundant. In these models, the electrostatic interactions drive the polar head of PS next to Pgp in the inner leaflet (Enkavi et al., 2019). Although it has not been clarified if this interaction changes conformation and activity of the transporter, this hypothesis is not unlikely because the charged head of PS may produce strong electrostatic forces, polar forces or even H-bonds. In a computational study, POPE induces the closure of the periplasm gate in the prototypical SAV1866 ABC transporter, in consequence of transient H-bonds with lysine 38, aspartate 42, threonine 276 and threonine 279, as well as electrostatic interactions with lysine 38 and aspartate 42. Conversely, POPC neither induces similar interactions nor affects the transporter's activity (Immadisetty et al., 2019). Not only the head groups, but also the length and saturation of fatty acyl chains participate in the effect of POPE, as 1,2-dioleoyl-sn-glycero-3-PE (DOPE) and 1,2-dipalmitoy-sn-glycero-3PE (DPPE) – i.e. PLs containing all unsaturated or all saturated fatty acyl chains - produce a lower closing than POPE with a mixed composition of acyl chains (Immadisetty et al., 2019).

A crevice between transmembrane helices 10-12 of Pgp favors the interaction of the protein with the surrounding Chol (Domicevica et al., 2017; Pan and Aller, 2018). Chol and PLs distribute

asymmetrically around Pgp: the outer leaflet is characterized by a ring of ordered Chol, while both PLs and Chol are present in the inner membrane (Alam et al., 2019). These lipid-protein interactions induce structural modifications, such as the kinking of the transmembrane helices 4 and 10 (Alam et al., 2019), that can modulate the catalytic cycle of Pgp.

A high amount of Chol is also detected in the plasma-membrane surrounding MRP4, where it induces dramatic conformational changes in the transporter with a potential modulation of its function (Chantemargue et al., 2018). Chol is also critical for the activity of BCRP, since the cell depletion of Chol decreases its activity: this is due to structural modifications such as alteration in H-bonds that impair the energy transduction between NBDs and TMDs (Ferreira et al., 2017; Telbisz et al., 2013).

Of note, some ABC transporters, such as Pgp, MRP1 and BCRP, can shift from lipid raft to non-lipid raft localization (Bacso et al., 2004; Storch et al, 2007; Klappe et al., 2009). Although the biological meaning of this shift is controversial, it affects the activity and the recycling of the transporters. For instance, the maintenance of high levels of Chol and sphingolipids within lipid rafts is critical to support the activity of Pgp here located. The resistance to doxorubicin has been correlated with the preferential localization of this drug in lipid rafts where doxorubicin interacts with Chol. This localization of doxorubicin, dictated by its chemical nature, increases the bioavailability of doxorubicin for Pgp, also concentrated within lipid rafts, reducing its intracellular concentration (Meyer dos Santos et al., 2007). In the HT29/DX subline, which expresses Pgp, MRP1 and BCRP, we found all these transporters associated with the lipid raft fractions. Of note, HT29/DX have a higher content of Chol and PLs containing saturated fatty acyl chains in lipid rafts than the sensitive counterpart. Disrupting such highly ordered geometry by supplementing cells with ω-3 fatty acids, e.g. docosahexaenoic acid (DHA) and eicosapentaenoic acid (EIPA), induces a shift of Pgp and MRP1 from lipid to non-lipid raft

domains, reducing their global activity, as demonstrated by the restoration of sensitivity to irinotecan (Gelsomino et al., 2013).

Beside binding to transmembrane helices, lipids can also bind specifically to the substrate chamber of ABC transporters, by electrostatic interactions and hydrophobic effects. Positively charged rings of arginine and lysine residues promote such lipid-protein interactions and drive substrates toward the transporter cavity (Marcoux et al., 2013). Studying the effects of lipids on drug binding and release from ABC transporters is a new field of investigation and is an additional way to understand how lipid environment modifies their conformational state and activity.

3. Membrane lipid composition of intracellular organelles sustains drug resistance

Plasma-membrane is continuously produced from intracellular organelles, starting from the lipid synthesis within the ER, their post-translational modification within Golgi apparatus and the fusion of intracellular vesicles to the plasma-membrane. Similarly, the opposite flux triggered by endocytosis, and fusion of endosomes with lysosomes, allows a redistribution of lipids derived from plasma-membranes within the intracellular compartments. Given the different lipid profile of plasma-membrane in chemoresistant and chemosensitive cells, this dynamic and bidirectional lipid flux should determine a differential composition of intracellular organelles.

Mitochondria metabolism and functions are widely altered in drug resistant cells, and these changes sustain the MDR phenotype (reviewed in Alexa-Stratulat et al., 2019). Beside many alterations in protein expression levels, differences in the lipid composition of mitochondrial membranes may cooperate to determine those features inducing chemoresistance.

In non-transformed cells, mitochondrial membranes are constituted of PC and PE for 70-80%, and of PI, PS ad SM for the remaining 1-5% (Horvath and Daum, 2013). Cardiolipin (CL) is a PL peculiarly found in mitochondria, which represents 20% of the inner membrane (IMM) and 5-10% of the outer membrane (OMM) (Suhaili et al., 2017). CL is essential for the correct IMM organization and stabilizes components of respiratory chain complexes (Pennington et al., 2019). CL can be easily oxidized by reactive oxygen species (ROS): this process triggers mitochondria-dependent apoptosis because oxidized CL is required for the release of cytochrome c into the cytosol (Pennington et al., 2019). The most striking difference between mitochondrial membrane and plasma-membrane is the content of Chol, that is much lower in mitochondria compared to plasma membrane (0.5-3%). Despite this fact, Chol plays an important role in regulating the physiological functions of mitochondria (Horvath and Daum, 2013).

Mitochondria of several cancers have altered mitochondrial PLs and Chol levels compared to normal tissue (Ribas et al., 2016). In particular they have different levels of CL and contain shorter and less unsaturated fatty acyl chains in PL (Kiebish et al., 2009). These changes affect energy production, apoptosis and fusion/fission ratio (Monteiro et al., 2013; Sassano et al., 2017). The differences, however, are highly tumor specific. For instance, lower levels of CL, and in particular of oxidized CL, are found in human hepatocellular carcinoma (HCC) compared to adjacent normal tissue (Zhong et al., 2017). These decreased levels make cells more resistant to the ROS-induced apoptosis (Huang et al., 2008). An increase in CL content was instead measured in human breast cancer MCF7 cells resistant to doxorubicin or cisplatin, compared to the sensitive counterpart (Todor et al., 2012). The presence of less oxidizable CL species may increase the resistance to apoptotic stimuli (Kagan et al., 2009), leading to hypothesize that the lower apoptosis induction by doxorubicin and cisplatin may be due to the prevalence of CL species more resistant to lipid peroxidation (e.g. with a higher saturated/unsaturated fatty acyls ratio).

An increased amount of Chol also alters the membrane fluidity and the metabolic activity of many complexes of electron transport chain of cancer cells (Ribas et al., 2016). The alterations in mitochondrial respiration increase the production of mitochondrial ROS but also reduces mitochondrial membrane permeabilization (MMP) (Elustondo et al., 2017; Sassano et al., 2017). Notwithstanding the higher Chol level within mitochondria membrane determines an increased levels of ROS, it also renders cells more resilient to apoptotic stimuli (Montero et al., 2008), determining chemoresistance. The constant and low production of ROS induced by the altered energetic mitochondrial metabolism can train the cells to survive in unfavorable conditions, triggering a mitochondria-dependent adaptation to stressors known as mitohormesis (Ristow, 2014). In support of this hypothesis, we recently found that keratinocytes undergoing a mitohormetic process induced by the constant production of mitochondrial ROS acquire radio- and chemoresistance (Tassone et al., 2017).

As anticipated above, mitochondrial Chol and CL prevent MMP, a key event in the apoptotic cell death. At the early stages of the apoptotic process, there is an increase of pro-apoptotic CL metabolite monolysoCL in sensitive cells (Crimi and Degli, 2011). By altering the mitochondrial membrane fluidity, Chol decreases the activation of pro-apoptotic Bax (Lucken-Ardjomande et al., 2008; Montero et al., 2008). Consistently, increasing mitochondrial Chol decreases the apoptotic response to stressors, while decreasing Chol levels increases chemosensitivity (Lucken-Ardjomande et al., 2008). This anti-apoptotic role of lipids contained within mitochondrial membrane could be exploited for new therapeutic options. For instance, edelfosine, an alkyl-lysoPL analog with anti-tumor properties, has been shown to promote the fusion of lipid rafts from plasma-membrane to mitochondria and to interact with the Chol-rich lipid rafts in mitochondrial membranes. Such interaction leads to mitochondrial disruption and apoptosis (Mollinedo et al., 2011). Similarly, the knockdown of steroidogenic acute regulatory protein (StAR), that regulates Chol import within mitochondria, is associated to chemosensitization of HCC cells and mimic the effects of statins (Montero et al., 2008). By contrast,

the inhibition of ABCA1 transporter that effluxes Chol on plasma-membrane, increases the Chol incorporated within the mitochondrial membrane and induces chemoresistance, by preventing apoptosis (Smith and Land, 2012).

The alteration of the membrane fluidity produced by Chol affects also the activity of specific mitochondrial transporters, including glutathione (GSH) carriers (Montero et al., 2011). In normal cells, an enrichment in Chol results in a depletion of mitochondrial GSH, favoring a pro-oxidant environment in this organelle. Conversely, cancer cells maintain unaltered the mitochondrial GSH, notwithstanding the higher Chol levels and are protected from ROS damages (Ribas et al., 2014). Cells undergoing a mitohormetic processes have a constitutive high level of anti-oxidant pathways, such as the pentose phosphate pathway (PPP) (Tassone et al., 2017). This metabolic rewiring may explain the high GSH levels in resistant cancer cells, despite the presence of high levels of mitochondrial Chol and ROS.

In other intracellular compartments, sphingolipids are the main lipids most frequently associated with the MDR phenotype. Cer is produced in response to stress conditions and chemotherapy in sensitive cells, where its accumulation promotes cell death (Morad and Cabot, 2013; Praharaj et al., 2019). CerS is localized mainly in the ER, but they have been found also in mitochondria-associated membranes (MAM) (Szymański et al., 2017), the compartment connecting mitochondria and ER, regulating the Ca^{2+} exchange and allowing the correct function of mitochondria (Herrera-Cruz and Simmen, 2017). ER and MAM contain lipid rafts rich in Chol and sphingolipids (Herrera-Cruz and Simmen, 2017), together with the proteins involved in lipid metabolism, such as CerS (Brachtendorf et al., 2019). Recent studies have correlated the distribution of Cer-derivatives between organelles with drug sensitivity or resistance. For example, in sensitive cancer cells, chemotherapy promotes the translocation of CerS1, which generates C18-ceramide, to the mitochondria, increases C18-ceramide

levels in the OMM, and triggers a Cer-dependent mitophagy. In drug-resistant cancer cells, chemotherapeutic drugs induce instead the ubiquitination and proteasomal degradation of CerS1 (Dany et al., 2016). Also Cer6 isoform is localized in the ER and it is overexpressed in several tumor types, including acute lymphoblastic leukemia, where it has been related to drug-resistance (Verlekar, 2018).

Overall, if specific lipid signatures of mitochondria, ER or MAM have been correlated with the response to chemotherapy, to the best of our knowledge no data exists on the lipid profile in lysosomes of sensitive and resistant cells, leaving the field open to a plethora of new investigations that may identify new biomarkers and druggable targets in MDR cells.

4. A different balance between lipid peroxidation and anti-oxidant defenses characterizes drug resistant cells

The composition of fatty acyl chains of the different lipid class is important for the sensitivity to oxidative stress, induced by different stressors including chemotherapy. Oxidative stress is a state of disturbed redox balance and is elicited by ROS and other electrophilic species. The sources of ROS damaging lipids, proteins and DNA (Guéraud et al., 2010) in the cellular environment are multiple. For instance the electron leakage occurs physiologically in the electron transport chain generates ROS(Esterbauer et al., 1991; Suski et al, 2018). A constant chronic inflammation, often observed within TME, increase ROS as well (El-Kenawi and Ruffell, 2017).

Lipids are among the first molecules to be affected by ROS. The sensitivity of lipids to oxidative stress depends on their fatty acid moiety. In general, fatty acids are grouped based on the number of double bonds in saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs), with zero, one or more double bonds. Double bonds in MUFAs and PUFAs show a prevalent *cis* conformation, which produces bends and limits their rigid packing (Plumb et al., 1993).

This feature lowers the melting temperature, increases the fluidity of membrane and affects the functions of membrane-associated proteins, regulating the proteins' lateral diffusion and the lipid-protein interactions (Plumb et al., 1993). Since double bonds make fatty acyl chains more susceptible to oxidative stress, lower levels of PUFAs in the membrane render the latter less prone to ROS damages. In support of this hypothesis, in breast cancer cell lines with increasing malignancy, PUFA content is progressively decreasing (Rodrigues et al., 2019), suggesting that the most aggressive and resistant cells may be protected by ROS thanks to their PUFA-low composition. It should be noted that most of the research on PUFAs in cancer is not focused on the endogenous composition of membranes, but on the beneficial effects of diet on the ω-3/ω-6 PUFA ratio in preventing specific cancers, such as colorectal cancer. Some preclinical studies reported indeed a direct toxicity of both ω-3 and ω-6 PUFAs in cancer cell lines, with a higher sensitivity showed by MDR cells (Gelsomino et al., 2013; Liu et al., 2010; Plumb et al., 1993; Scheim, 2009). The cytotoxicity of PUFAs can be due in part to their role in the production of inflammatory mediators. For instance, arachidonic acid is a ω-6 PUFA, which can undergo enzymatic oxidation forming prostaglandins, thromboxanes and leukotrienes. These molecules can drive pro- or anti-tumorigenic processes. A second important mechanism of toxicity is the generation of lipid peroxidation (LPO) products, following the ROS damages on PUFAs.

The degradation of PUFAs starts at the carbon atom farthest from carboxylic group (i.e. ω1 or n1) (Aglago et al., 2019; Chiu et al., 2012; Corsinovi et al., 2011; Stratakis et al., 2018). LPO cascade has three phases: initiation, propagation and termination (Zarkovic et al., 2013). The process is initiated by the reaction of single electrophilic species with double bonds and the formation of a carbon-centred lipid radical. In the following step, this lipid radical reacts with dioxygen and a lipoperoxyl radical is formed. The propagation occurs as the lipoperoxyl radical can react with a neighboring lipid, forming a new carbon-centred lipid radical and a lipid hydroperoxide. The latter are highly unstable and are

degraded to reactive aldehydes, leading to LPO termination (Esterbauer et al., 1991; Gasparovic et al, 2013; Guéraud et al., 2010).

Unlike ROS, which are extremely reactive molecules with a limited diffusion range because of such high reactivity, aldehydes are more stable and are therefore called "second messengers of free radicals" (Zarkovic et al., 2013). Although some ROS are stable enough to diffuse, such as H_2O_2 or $HOCl^-$, they act locally, without eliciting any systemic response, differently from reactive aldehydes (Gasparovic et al., 2017a). The type of aldehydes produced is determined by the original PUFAs which underwent LPO. Among these aldehydes, the most investigated are 4-hydroxy-2-nonenal (HNE), the end product of ω6-PUFAs; acrolein, a product of several different chemical reactions beyond LPO; malondialdehyde (MDA), often used as a marker of LPO (Gasparovic et al., 2017b; Guéraud et al., 2010). Numerous other reactive aldehydes, with similar biological activities to HNE, such as 4-oxo-2-nonenal (ONE) or 4-hydroxy-2-hexenal (HHE), are produced by LPO and are intensively investigated (Gęgotek et al., 2016; Yang et al., 2019).

4.1 Biological effects of lipid peroxidation in cancer cells

The aldehydes produced during LPO may have a different spectrum of toxicity, due to their intrinsic reactivity, coupled with a higher stability than ROS and lipid hydroperoxydes (Guéraud et al., 2010; Vistoli et al., 2013). Consequently, several types of chemical reactions between aldehydes, proteins and lipids occur. For instance, HNE binds to PE, changing the membrane properties (Jovanović et al., 2015; Vazdar et al., 2017) and influencing the properties of membrane-associated proteins, such as H^+ and Na^+ transporters (Jovanović et al., 2019). Such alterations induce a plethora of pH- and sodium-dependent cellular processes. Yet, these chemical interactions with plasma-membrane lipids are poorly investigated, especially in the context of (cancer) cell biology.

In contrast to lipids, the biological effects of the interactions of aldehydes with proteins are well investigated. Reactive aldehydes bind to cysteine, lysine, histidine and arginine moieties, causing

structural modifications and changing proteins' activity (Milkovic et al., 2015; Zarkovic et al., 1993). The bound with the cysteine is reversible and is the mainly known mechanism of reactive aldehyde bioactivity (Castro et al., 2017). By targeting specific proteins, reactive aldehydes act as signalling molecules activating EGFR-dependent pathways, several isoforms of PKC, PI3K/Akt axis, c-Jun and TP53 (Gasparovic et al., 2017a). As a result, reactive aldehydes promote cancer growth and metastasis. Moreover, LPO can subtract substrates - i.e. PUFAs – for the production of prostaglandins, thromboxanes and leukotrienes, altering the spectrum of inflammatory and anti-tumor mediators produced within TME.

HNE is one of the most investigated reactive aldehyde and was among the first ones described to exert a pro-proliferative effect in HeLa cells (Zarkovic et al., 2013). Since then, our knowledge on HNE and its role in cancer growth is expanding. HNE regulates proliferation, differentiation and apoptosis (Gasparovic et al., 2017a; Shoeb et al. 2014; Sovic et al. 2001), by modulating proteins involved in NF-κB, PI3K/Akt, p21 and mTORC-dependent pathways (Kovalchuk et al., 2018; Laurora et al., 2005; Timucin and Basaga, 2016; Zhang and Forman, 2017). The effects of HNE, however, are not uniform, since it can act both as an activator or an inhibitor of the target proteins, depending on the cell type and the HNE concentration (Zhang and Forman, 2017). Similarly to HNE, ONE also bounds to cysteine and histidine moieties of proteins, such as pyruvate kinase isoform M2 (PKM2). Both HNE and ONE cause conformational changes in PKM2: ONE is more potent and at a high concentrations it induces cross-linking reaction within PKM2, decreasing its activity in RKO cancer cells (Camarillo et al., 2017). In neuroblastoma cells under stressing conditions, i.e. after glucose and glutamine deprivation, HNE and ONE have different effects on cell viability and activity of matrix metalloproteinases (MMPs): HNE is less cytotoxic and it strongly reduces MMP in glucose-free medium, not in glutamine free-one, while ONE produces strong cytotoxic effects in both glucose- and glutamine-deprived media without significant differences (Zimmermann et al., 2017). These differences can be explained by the

fact that ONE is a stronger electrophile than HNE, and can interact with DNA and proteins at higher rates than HNE. Interestingly, while the amount of HNE is highly variable, depending on the tumor type (Marquez-Quiñones et al., 2010), the production of ONE decreases during tumor progression (Sakuma et al., 2009). This is not surprising, since progression is a biological process that selects resistant cells, able to neutralize dangerous LPO species, such as ONE. Another reactive aldehyde, HHE, has similar effect to HNE on the activation of TNF-α production, the modulation of redox signalling pathways and the deprivation of GSH (Ishikado et al., 2013; Long et al., 2008; Muzio et al., 2016). Overall, HNE, HHE and ONE sensitize cancer cells to apoptosis, by down-regulating the pro-apoptotic Bcl-2 protein in HT29 cells, or by activating the anti-apoptotic Bax protein in HepG2 cells (Timucin and Basaga, 2016).

Also MDA, the most stable aldehyde (Gasparovic et al., 2013), shares similar properties with other reactive aldehydes, covalently binding DNA, lipids and proteins. In addition, MDA bind to different epitopes (Busch and Binder, 2016), which – after the MDA covalent binding – are recognized better by the immune cells. This leads to hypothesize that MDA-protein adduct may serve as endogenous immune-sensitizers.

4.2. The role of lipid peroxidation and antioxidant enzymes in drug resistance

LPO is both a cause and a consequence of metabolic changes occurring during cancer progression and MDR development. Metabolic changes are not drastic, but rather they act cumulatively, as adaptive responses to stressors, supporting cancer cell growth in unfavourable conditions and drug resistance (Alexa-Stratulat et al., 2019) (**Figure 2**). Since MDR cells have an altered lipid metabolism, they inevitably change the SFAs:MUFAs:PUFAs ratio (Gelsomino et al., 2013; Rodrigues et al., 2019; Zalba and ten Hagen, 2017). These changes alter the spectrum of LPO products generated within MDR cells and able to exert cytotoxic or pro-inflammatory effects. The reduction in LPO-toxic species can

contribute to increase the growth of clones with significant resistance to stressors, including chemotherapy.

Reactive aldehydes are buffered by the same antioxidant and detoxifying systems that neutralize ROS. Cell antioxidant systems can be divided into two groups: small molecules acting non-specifically and enzymatic cascades acting toward specific targets. The main intracellular small molecules are vitamins C and E for hydrophilic and lipophilic compartments, respectively, as well as glutathione (GSH), ubiquinone, urea. Among these antioxidants, vitamin E is the most involved in the protection of MUFAs and PUFAs from LPO, as it is located mostly within the membranes (Zingg et al., 2019). On the other hand, GSH is the main scavenger for HNE. Indeed, the HNE detoxification starts with its conjugation to GSH (Svrivastava et al., 1998). GS-HNE is either exported from the cell by RLIP76 protein (Singhal et al., 2016), or is inactivated by aldose reductase at the expense of intracellular NADPH. PPP enzymes, GSH-regenerating enzymes such as glutathione reductase (GR), glutathione peroxidase (GPx), cytosolic and mitochondrial superoxide dismutases (SOD) are more active in MDR cells than in sensitive ones (Alexa-Stratulat et al., 2019). These features limit the extent of unbuffered HNE in MDR cells, limiting the cytotoxic effects exerted by the reactive species.

Aldehyde dehydrogenases (ALDH) are another family of HNE scavenger (Mol et al., 2017). ALDH2, which has the highest efficiency to oxidize HNE (Yoval-Sánchez and Rodríguez-Zavala, 2012), is also one key marker of cancer stem cells, which are responsible for recurrence and therapy resistance in most tumors (Liu et al., 2014; Mele et al., 2018). Notably, mitochondrial ALDH protects cells from doxorubicin toxicity (Ge et al., 2016). Hence, highly expressing-ALDH cells are typical examples of cancer cell subpopulations with high clonogenicity potential and multiple mechanisms of resistance, since they are more protected by the damages induced by chemotherapy and ROS.

Non-toxic concentrations of HNE activate the Nrf2 transcription factor, which is in turn responsible for the transcription of numerous antioxidant enzymes (Milkovic et al., 2017), MRP1-3 and BCRP

(Venkatraman et al., 2015; Salaroglio et al., 2017). In this perspective, HNE can be regarded as a "trainer" towards the acquisition of resistance. Similarly to what occurs in cells producing low amount of ROS (Tassone et al., 2017), we may speculate that HNE favors the expansion of cells with increased resistance to oxidative stress and increased drug efflux transporters, two features at the basis of MDR phenotype.

Since MDR is related to a higher resistance to oxidative stress (Alexa-Stratulat et al., 2019), it is not surprising that compounds with both antioxidant and transporters-blocking activities have been preliminarly investigated (Cindric et al., 2010). The dual targeting characterizing these compounds may open new possibilities of treatments against MDR tumors.

5. Isoprenoids as drivers of drug resistance

An increasing number of evidences demonstrate that in cancer cells of different origin, such as B-CLL (Rigoni et al., 2015), multiple myeloma (Clendening et al., 2010), breast (Sethunath et al., 2019), pancreatic (Carrer et al., 2019) and prostate (Ashida et al., 2017) cancers, MVA pathway is more active and contributes to drug resistance by changing the intracellular concentration of isoprenoids such as FPP and GGPP. The pivotal role of isoprenoids is demonstrated by the apoptosis elicited by the HMGCR inhibitor simvastatin in different cancer cells, e.g. glioblastoma (GB), neuroblastoma, non-small cell lung cancer. Notably, the addition of Chol did not rescue apoptosis (Alizadeh et al., 2017), showing that the statin induced apoptosis is a MVA-dependent, but Chol-independent process.

The mechanisms involved in the dysregulation of MVA pathway include mutations in *TP53* that activates SREBP transcription factors, mutations in key MVA pathway enzymes such as HMGCR and FPP synthase (FPPS), constitutive activation of SCAP and HIF-1α, increased activity of Akt, decreased activity of AMP kinase (AMPK) (Batahie et al., 2017). FPP and GGPP are used as substrates for protein prenylation, a post-translational modification which anchors proteins to the internal leaflet of

cell plasma-membrane. Prenylation is catalyzed by farnesyl-transferase (FT) and geranylgeranyl-transferase (GGT) that covalently attach farnesyl or geranylgeranyl units to the cysteine residue in the CAAX motif at the C-terminal of proteins (Wennerberg et al., 2005). When prenylated and bound to GTP, the GTPases translocate to the membrane and undergo a conformational change, engaging down-stream transducers that control cytoskeletal organization, cell survival and proliferation, transformation, and vesicular trafficking. Monomeric GTPases such of Ras, Rho/Rac/Cdc42 and Rab families are the isoprenylated proteins most involved in these processes (Wennerberg et al., 2005). Mutated, constitutively active Ras proteins (such as K-Ras, N-Ras, and H-Ras) are found in 15 to 30% of human tumors, with percentages of 90% in pancreatic cancers, 50% in colon and thyroid cancers, 30% in lung cancers, 25% in melanomas (Hobbs et al., 2016). Similarly, Rho proteins (RhoA, Cdc42, Rac1) are overexpressed in different human tumors including breast, lung, and testicular carcinomas (Cardama et al., 2017).

Different tumors have different sensitivity to MVA inhibitors, depending on the rate of their MVA flux. GB, the most common tumor of the central nervous system, is highly dependent on the MVA pathway for survival and an abnormally active MVA flux has been found in GB cells compared with their normal counterparts (Villa et al., 2016). Such up-regulation of the MVA pathway has been associated with low survival (Kambach et al., 2017), leading to hypothesize that MVA pathway mediates tumor progression, recurrence and/or drug resistance. GB easily acquires resistance to treatments because of the presence of cancer stem cells within tumor bulk. Interestingly, FPPS, that promotes the synthesis of FPP, is important to maintain GB stemness and drug resistance. Indeed the aminobisphosphonates zoledronate and alendronate, two FPPS inhibitors, significantly decrease the formation of GB spheres (Kim et al., 2018), a feature that is associated with reduced stemness and drug resistance. Similarly, a high rate of MVA pathway is responsible for MDR phenotype in colorectal cancer and malignant pleural mesothelioma cells, where doxorubicin-resistance is reversed by

zoledronate (Riganti et al., 2013). A high activity of ERK1/2, a downstream effector of Ras, determines chemoresistance in malignant pleural mesothelioma (Shukla et al., 2014), with unknown mechanisms. Noteworthy, ERK1/2 phosphorylates and stabilizes HIF in colorectal, breast, non-small cell lung cancer, malignant pleural mesothelioma, B-CLL (Kopecka et al., 2016; Kopecka et al., 2015b; Riganti et al., 2013; Rigoni et al., 2015; Salaroglio et al., 2015). HIF-1α up-regulates Pgp at transcriptional level (Comerford et al., 2002), HIF-2 activates the transcription of BCRP in breast and ovarian cancers (He et al., 2019; Xiang et al., 2012).

The constitutive activation of Ras/ERK1/2 is frequent also in myeloid leukemia cells, where it contributes to up-regulate ABC transporters and resistance to imatinib, a substrate of Pgp (Dharmapuri et al., 2015). The linkage between MVA pathway and resistance in hematologic malignancies has been confirmed by the efficacy of FT inhibitors (FTIs) and statins in killing malignant hematopoietic cells and/or increasing their susceptibility to cytotoxic drugs (Gritsman et al., 2014).

Beside Ras, also RhoA/RhoA kinase is a parallel axis contributing to the phosphorylation and activation of HIF-1α. Therefore, a reduced activity of Ras/RhoA achieved with zoledronate, as well as a targeted inhibition of ERK1/2 or RhoA kinase, all reverse the resistance to doxorubicin mediated by Pgp (Riganti et al., 2013; Rigoni et al., 2015).

In parallel to the up-regulation of MDR efflux transporters, an active Ras/ERK1/2/HIF-1α axis increases the transcription and activity of glycolytic enzymes, the glucose flux through glycolysis and tricarboxylic acid cycle, and the ability of producing ATP via both anaerobic and aerobic glycolysis. Moreover, isoprenoids produced in the MVA are incorporated into the hydrophobic tail of ubiquinone, the electron shuttle between Complex I and Complex III of electron transport chain. A high MVA activity has been correlated with a high synthesis of ubiquinone, and with a consequent high rate of oxidative phosphorylation. This process further increases the synthesis of ATP (Kopecka et al., 2015b; Kopecka et al., 2016). Overall, these metabolic events provide cells with higher amounts of ATP that

fuels ABC transporters, supporting the active efflux of different chemotherapeutic drugs. Indeed, the pharmacological inhibition of the MVA pathway with zoledronate or the silencing of FPPS restore the sensitivity in vitro and in vivo not only to substrates of Pgp and BCRP such as doxorubicin (Kopecka et al., 2016), but to substrates of multiple ABC transporters (Kopecka et al, 2015b). These data indicate that targeting MVA is a "broad spectrum" chemosensitizing approach (**Figure 3**).

Notably, in MDR cells of malignant pleural mesothelioma (Salaroglio et al., 2015) and breast cancer (Kopecka et al., 2016), Ras/ERK1/2 axis also activates the transcription factor STAT3. Among the genes up-regulated by STAT3 in MDR cells, there is indoleamine 1,2-dioxygenase (*IDO*). IDO catabolizes tryptophan, an essential aminoacid for effector T-lymphocytes, and produces the immune-suppressive metabolite kynurenine. The inhibition of Ras/ERK1/2/STAT3 axis by zoledronate, beside increasing chemosensitivity, reduces the proliferation of immune-suppressive T-regulatory (Treg) cells and re-instates a correct proliferation of effector $CD3^+CD8^+$ T-infiltrating lymphocytes (TILs) (Salaroglio et al., 2015; Kopecka et al., 2015b). Overall, this strategy turns a chemoresistant, immune-suppressive and pro-tumor milieu into a chemosensitive, immune-active and anti-tumor environment.

Other proteins of Ras family involved in drug resistance are Rab GTPases. Rabs control vesicular transport, regulating the vesicle docking and fusion (Guadagno and Progida, 2019). Therefore, they can control the trafficking and recycling of Pgp. It has been shown in MDR cells that the overexpression of Rab4 increases their drug sensitivity, by favoring the shift of Pgp from plasma-membrane into cytosolic endosomal compartments, where it is sequestered by Rab4 (Ferrándiz-Huertas et al., 2011). Similarly, the overexpression of Rab6c in breast cancer cells increases the intracellular localization of Pgp and the intracellular accumulation of anticancer drugs that are Pgp substrates (Shan et al., 2000). Rab members, however, do not act all in the same direction. Indeed, the cell transfection with the dominant negative Rab5 protein increases the intracellular localization of Pgp and the content of daunorubicin (Fu et al., 2007). These studies highlight the importance of Rab proteins in controlling

Pgp-mediated drug response, but also the difficulty in exploiting Rab as potential chemosensitizing tool, since different Rab members produce opposite effects on Pgp localization. Considering the high inter-patient and intra-tumor variability, only the exact knowledge of the prevailing Rab isoform in a specific tumor may provide useful tips on the most effective Rab-targeting strategy in each case.

As anticipated above, also RhoA contributes to the MDR phenotype by up-regulating Pgp. In general, Rho family members and interactors are overexpressed in chemoresistant cells (Goto et al., 2006), suggesting their putative involvement in the MDR phenotype. Their role, however, is quite controversial. Interestingly, the Rho-GDP dissociation inhibitor 2 (RhoGDI2), that reduces Rho activity by preventing its dissociation from GDP, up-regulates Pgp in gastric cancer cells and increases the resistance to 5-fluorouracle. In this case, however, the effects on Pgp are not mediated by the activation of Rho, but by the activation of Rac1, as demonstrated by the down-regulation of Pgp in Rac1-silenced cells (Zheng et al., 2015). By contrast, the inhibition of RhoA reduces the expression of Pgp and MRP1 in irinotecan-resistant colorectal cancer cells (Ruihua et al., 2016), in line with previous observations (Riganti et al., 2013). RhoA not only controls the expression but also the activity of ABC transporters via nitric oxide (NO). Indeed, RhoA silencing increases NF-kB activity and NF-kB-driven expression of inducible NO synthase (iNOS). NO nitrates critical tyrosines on Pgp and MRP3; following these covalent modifications the ATPase catalytic efficiency of the transporters are strongly reduced (Doublier et al., 2008).

Notably, isoprenoids metabolism and monomeric G-proteins are also mediators of the TME-driven chemoresistance. For instance B-CLL cells are protected by apoptotic stimuli and chemotherapy by the presence of bone marrow stromal cells (BMSC) (Coscia et al., 2011; Rigoni et al., 2015). By releasing soluble factors not yet identified, BMSC activate the synthesis of FPP and GGPP, and the downstream Ras/ERK1/2 and RhoA/RhoA kinase axes, leading to the activation of HIF-1α and to the transcription of *mdr1* gene (Rigoni et al., 2015). This cascade makes B-CLL surrounded by BMSC, i.e. growing in

experimental conditions resembling their physiological environment, resistant to a large spectrum of standard chemotherapeutics and targeted therapies. Targeting MVA pathway using aminobisphosphonates, reverses, at least ex vivo, such resistance and may be considered as a feasible clinical approach.

6. Reversing drug resistance by modulating lipid profile: towards new approaches?

By affecting drug passive diffusion, permeability organization of nano- and micro-domains, functions of ABC transporters and SLCs (Escriba, 2006), the modulation of plasma-membrane lipid composition is a potent theoretical tool in reversing MDR. (Hegedüs et al., 2015). This aim can be pursued using different approaches. Synthetic lipid analogues, for instance, have been proposed to facilitate the influx of doxorubicin, through the formation of transient channels that improve drug bioavailability (Ward et al., 2012). Alky-lysoPL and alkyl-PC modulate plasma-membrane permeability and fluidity, decrease proliferation and induce apoptosis. Thanks to their effects on both plasma-membrane properties and cell proliferation/apoptosis, they can be considered as adjuvant agents of chemotherapy. Edelfosine is a typical alkyl-lysoPL exhibiting these properties (Mollinedo et al., 2011). Although it has gastrointestinal toxicity, hemolytic potential and metabolic instability, other derivatives as perifosine reached further development stages, entering phase 2 clinical trials (Bernardes and Fialho, 2018). Another alky-lysoPL, ethyl-phosphate 10-(octyloxy) decyl-2-(trimethylammonium), also induces apoptosis in leukemia cells by disrupting the organization of membrane nano-domains (Bernardes and Fialho, 2018). Beside lipid analogues, membrane fluidity can be modulated by azurin, an anticancer protein of bacterial origin. Azurin increases the efficacy of paclitaxel and doxorubicin, likely acting as a plasma-membrane lipid modifier and increasing the drugs permeability (Bernardes and Fialho, 2018). Also Chol-targeting agents, including bio-compatible chelators (filipin, β-MCD), modulators of systemic Chol (epigallocatechin gallate, emodin) induce lipid raft disruption, modifying the

localization and functions of raft-associated proteins (Bernardes and Fialho, 2018; Zalba and Hausen, 2017). This is of particular interest for Pgp, MRP1 and BCRP, that are present within lipid rafts (Ferreira et al., 2015; Gelsomino et al., 2013; Klappe et al., 2009) and are activated by the high Chol content in the surrounding membrane (Meyer dos Santos et al., 2007). Other abundant components of lipid rafts are glycosphingolipids, also functional to support the activity of ABC proteins (Hinrichs et al., 2005): they may provide additional targets to overcome the drug resistance mediated by efflux transporters.

Exploiting the different lipid composition between sensitive and resistant cells, detergent-based reagents have been proposed as new agents able to selectively kill MDR cells, that display a higher sensitivity – the so-called collateral sensitivity (CS) – to these agents than sensitive cells (Pluchino et al., 2012).

The limitations of all the above-mentioned approaches rely on their low specificity. Indeed, although some differences in lipid membrane composition have been identified for specific lipids, other classes are present in both MDR tumors and non-transformed tissues. Hence, detergents and synthetic lipid analogues may alter the membrane fluidity of normal cells, impairing several physiological functions and inducing heavy side toxicities. Although effective in vitro, the feasibility and safety of plasma-membrane targeting approach is still elusive in preclinical and clinical applications.

By their nature liposomes or nanoparticles are preferentially accumulated within tumor mass for the enhanced permeability and retention (EPR) effect. Such delivery is even more effective with functionalized liposomes or nanoparticles that allow a higher delivery within the tumors (Golombek et al., 2018). Exploiting these features, liposomes and solid lipid nanoparticles (SLNs) are potentially useful tools to deliver specific lipids with a certain selectivity to tumor cells. We demonstrated that liposomal doxorubicin is superior to free doxorubicin in delivery the drug within Pgp-expressing cells, because the lipids contained in the liposomal shell redistribute themselves within the plasma-membrane

and the lipid rafts, altering their ordered structure. This event reduces the catalytic efficacy of Pgp (Riganti et al., 2011). Moreover, beside altering the surrounding environment, specific liposomal lipids directly interfere with drug efflux transporters: for instance, PEGylated distearoyl-PE, contained in liposomal shelves, is a potent allosteric inhibitor of Pgp, able to bind the glycine at position 185 and impair the Pgp catalytic cycle, increasing doxorubicin retention and cytotoxicity (Kopecka et al., 2014). Synthetic, pH-activated and thermosensitive lipopeptides, containing a leucine zipper and a 6-carbon alkyl chain, are other tools able to insert themselves within plasma-membrane and release their cargo only at the intratumor pH and upon heating the tumor mass (Wang et al., 2018). These hybrids can deliver chemotherapeutic drugs, with high efficacy and selectivity within the tumor.

These are just a few examples demonstrating the efficacy of liposomes or appropriate nanoparticles as donors of both lipids and chemotherapeutic drugs. Although these strategies are innovative and promising in vitro, a robust validation in preclinical models is mandatory to evaluate their efficacy and safety.

An alternative approach is exploiting lipid-targeting drugs already used for other indications. ω-3 fatty acids are dietary supplements with beneficial effects on cardiovascular system. Several in vitro works demonstrated that they exert cytotoxic and chemosensitizing effects against MDR cells (Gelsomino et al., 2013; Granci et al., 2013). Of note, ω-3 fatty acids have already been administered to oncological patients, where they have been well tolerated and have improved the efficacy of anthracyclines in patients with breast cancer (Bougnoux et al., 2009), and the efficacy of cisplatin plus vinorelbine in patients with non-small cell lung cancer (Murphy et al., 2011). Anthracyclines, cisplatin and vinorelbine are substrates of Pgp or MRP1, whose activity is reduced by ω-3 fatty acids (Gelsomino et al., 2013). Although the molecular bases of the chemosensitizing effects exerted by ω-3 fatty acids have not been investigated in depth in patients, it is likely that the inhibition of the MDR efflux transporters plays a key role.

The most widely used drugs employed to lower Chol are statins, inhibitors of HMGCR enzyme, that have various effects on incidence, recurrence and mortality for breast, prostate, lung and colorectal, cancers. The diverse effects are due in some cases to the different types - i.e. hydrophilic or lipophilic – of statins (Beckwitt et al., 2018). In breast cancer, lipophilic statins (but not the hydrophilic ones) have reduced the risk of recurrence, indirectly suggesting that they may increase the efficacy of chemotherapy. The effects are Chol-independent, but rely on the reduction of isoprenylated monomeric G-proteins or on the reduction of inflammatory cytokines. By contrast, hydrophilic statins have shown benefits in liver and prostate cancers, possibly due to the abundance of membrane importers of statins in these two tumors (Beckwitt et al., 2018). In some tumors, the chemosensitizing effect of statin is an off-target effect: for instance, simvastatin sensitizes GB cells to temozolomide-dependent apoptosis in a MVA-independent way, but by interfering with autophagolysosome functions (Shojaei et al., 2019). Although clinical trials using statins as adjuvant agents have been designed (https://clinicaltrials.gov), the use of these drugs is highly controversial as it has produced contradictory results and in some cases toxic effects (Beckwitt et al., 2018). These conflicting results in vivo can be explained by the great differences in half-life, clearance and metabolism of each statin, and by the lack of selectivity for tumor tissue.

According to the results obtained in vitro and in preclinical models, targeting MVA pathway with aminobisphosphonates, FTIs or GGTIs is a feasible anti-proliferative and chemosensitizing strategy (Bertolio et al., 2019; Kuzu et al., 2016). These inhibitors are more directed on isoprenoids and therefore reduce more than statins the Ras/Rho-downstream signaling. For instance, zoledronate synergizes and reverses the resistance to Everolimus in preclinical models of osteosarcoma, thanks to its ability of reducing the isoprenylation of Ras (Moriceau et al., 2010). As for statins, mixed results have been obtained in clinical trials using zoledronate in patients with breast cancer (Costa and Ferreira, 2017). A limitation of the aminobisphosphonate is that they are rapidly taken up by the bone,

where they reach millimolar concentrations, and have a plasmatic half-life of less than 1 h. To overcome this issue, self-assembled tumor-targeting nanoparticles carrying zoledronate have been synthesized: they successfully reverse chemotherapy in preclinical models of breast and non-small cell lung cancer cells (Kopecka, 2015b; Kopecka, 2016). Safety and cost/efficacy studies, however, have not been performed. Therefore nothing can be inferred in patients at the present.

FTIs, GGTIs and inhibitors of GGPP synthase have been the object of intensive studies, since they are even more specific than aminobisphosphonates in reducing the prenylation process, and have a more favorable pharmacokinetic profile (Haney et al., 2017; Waller et al., 2019). Although effective, they suffer from the same lack of tumor specificity that characterizes statins and aminobisphosphonates. For this reason, their use in preclinical and clinical settings has shown heavy side-effects. Also for this type of compounds a more vectorised approach, e.g. the use of tumor-targeting nanocarriers, may improve the therapeutic efficacy and limit the toxicity on non-transformed tissues.

In summary, by analyzing the lipid profile of plasma-membrane and intracellular compartments, as well as the peculiarity of lipid metabolism in MDR cells, it is possible to detect a plethora of unsuspected biomarkers and potentially druggable targets that can improve the efficacy of chemotherapy. The high number of different lipid species, the high inter- and intra-tumor variations in lipid composition make difficult to draw chemosensitizing strategies effective in each tumor. However, the deepest technological advances in the fields of high-throughput lipidomics and computational biology could rapidly overcome these limitations, leading to the identification of more accurate biomarkers and to the development of effective, safe and personalized strategies targeting lipids for the treatment of MDR tumors.

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Conflict of interest

None.

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Figures and legends

Figure 1

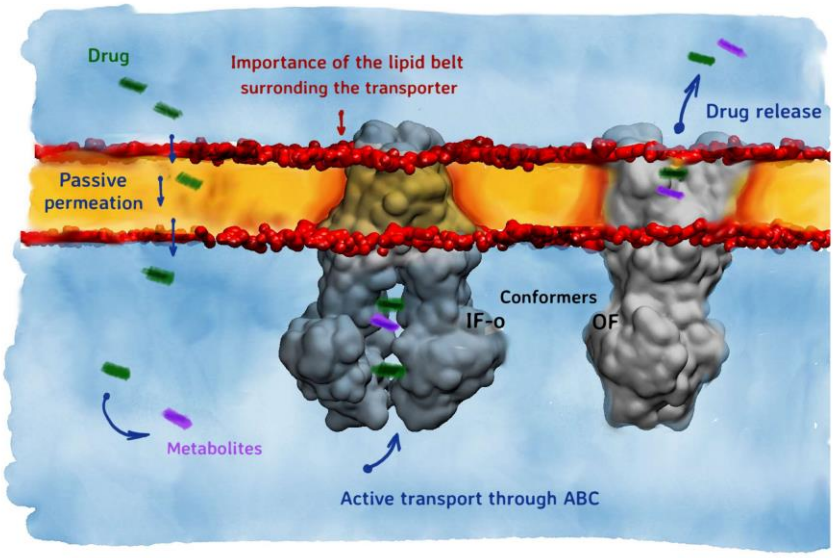

Figure 1. Role of plasma-membrane lipids in drug efflux

Schematic view of drug transport through membranes, occurring via passive permeation and active efflux though ATP biding cassette (ABC) transporters. Passive permeation is strongly influenced by the relative concentrations of phospholipids and cholesterol. ABC transporters dynamically change their conformational states along drug transport, from inward-facing open (IF-o) to outward-facing (OF) conformers. The lipid belt surrounding the ABC transporters modulate drug binding, ATPase catalytic cycle and drug release by interacting with specific helices of ABC transporters, favoring or impairing this conformation change.

Figure 2

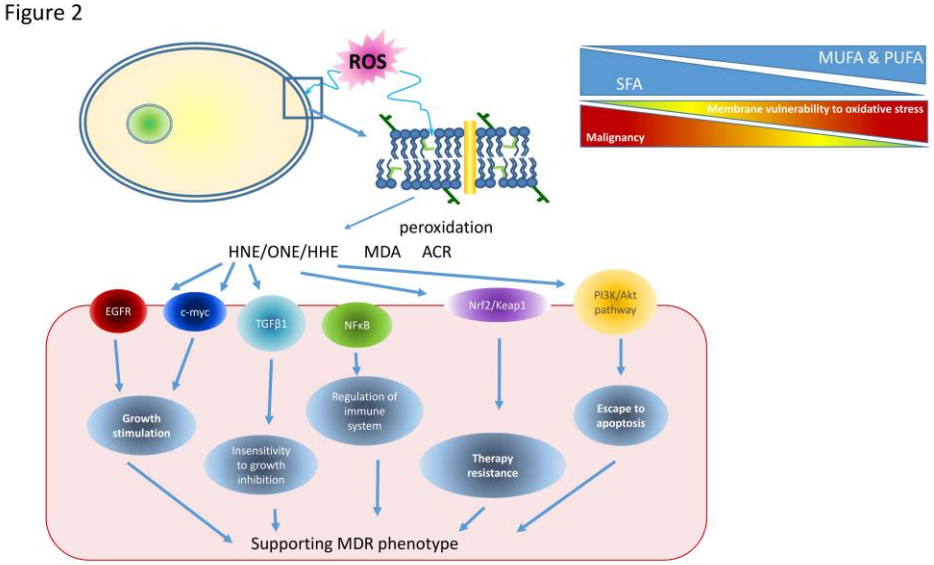

Figure 2. Lipid peroxidation supports MDR phenotype

Polyunsaturated fatty acids (PUFAs) in the membrane are susceptible to the oxidation by reactive oxygen species (ROS), which trigger the lipid peroxidation (LPO) cascade, generating a plethora of reactive aldehydes as end products. These aldehydes can modify cellular proteins, stimulating or inhibiting multiple transduction pathways. 4-hydroxy-2-nonenal (HNE), 4-oxo-2-nonenal (ONE) and 4-hydroxy-2-hexenal (HHE) stimulate several pro-survival and anti-apoptotic pathways. For instance they activate EGFR-dependent signaling and c-myc transcriptional program, favoring tumor growth; TGFβ production, which makes cells insensitive to growth inhibition; NF-κB activity, that controls cell survival, inflammation and immune reactions; PI3K/Akt pathway that allows the escape from apoptosis; Nrf2/Keap1 pathway that increases the expression of antioxidant enzymes and ABC transporters. The cumulative effects of reactive aldehydes on these pathways lead to increased resistance to oxidative stress and chemotherapy. Moreover, although reactive aldehyde support MDR phenotype, with the increase in malignant potential, cancer cells decrease PUFAs content, removing substrates potentially damaged by ROS that can results in lethal reactions. This change in the lipid profile further increases the resistance.

Figure 3

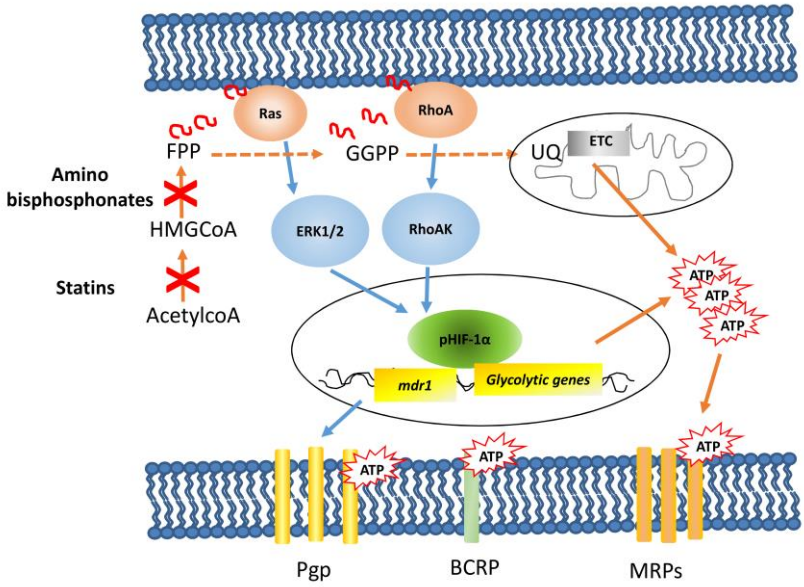

Figure 3. **An active isoprenoid synthesis induce the MDR phenotype**

Drug resistant cells have a high flux through the mevalonate pathway (MVA) that converts acetyl-coenzyme A (acetylCoA) into 3-β-hydroxy-3-β-mehyl glutaryl coenzyme A (HMGCoA) and produces isoprenoid moieties, such as farnesylpyrohosphate (FPP), geranylgeranyl pyrophosphate (GGPP) and the hydrophobic tail of ubiquinone (UQ). Farnesylation and geranylgeranylation determine the activation of Ras and RhoA monomeric proteins that engage their respective downs-stream transducers, extracellular regulated kinase 1/2 (ERK1/2) and RhoA kinase (RhoAK), that in turn phosphorylate and activate the Hypoxia inducible-1α (HIF-1α) transcription factor. HIF-1α up-regulate *mdr1* and glycolytic genes, increasing the amount of P-glycoprotein (Pgp) and the amount of intracellular ATP produced by anaerobic glycolysis. In parallel, ubiquinone, by favoring the activity of the mitochondrial electron transport chain (ETC), further supplies ATP. As a results, the activity of several drug efflux transporters – Pgp, breast cancer resistance protein (BCRP), multidrug resistance related proteins (MRPs) – is increased. This complex signaling is druggable by statins and aminobisphohonates, i.e. lipid-targeting drugs that can be repurposed as potential chemosensitizing agents.