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Metabolic reprogramming in cancer cells, consequences on pH and tumour progression: Integrated therapeutic perspectives with dietary lipids as adjuvant to anticancer treatment

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Abstract

While tumours arise from acquired mutations in oncogenes or tumour-suppressor genes, it is now clearly established that cancers are metabolic diseases, characterized by metabolic alterations in both tumour cells and non-tumour cells of the host organism (resulting in tumour cachexia and patients weakness). This review aims at delineating details by which metabolic alterations in cancer cells, characterized by mitochondrial bioenergetics deregulations and the preference for aerobic glycolysis, are critical parameters controlling the aggressive progression of tumours. In particular, metabolic alteration in cancer cells are coupled to the modulation of intracellular and extracellular pH, epithelial-to-mesenchymal transition and associated increased invasiveness, autophagy and the development of anticancer treatment resistance. Finally, based on mechanistic, pre-clinical and clinical studies, we wish to propose the adjuvant supplementation of dietary n-3 polyunsaturated fatty acids for a complementary global treatment of the cancer disease.

Key words (5):

Cancer cell metabolism, pH, cancer cell invasiveness, resistance to treatments, n-3 polyunsaturated fatty acids

Abbreviations:

α-SMA: α-smooth muscle actin; AMPK : AMP-activated protein kinase; bHLH : basic Helix-Loop-Helix; DHA : docosahexaenoic acid (22:6n-3); Drp1 :dynamin-related protein 1 ; ECM : extracellular matrix ; EMT : epithelial-to-mesenchymal transition ; EPA: eicosapentaenoic acid (20:5n-6); ERK1/2: extracellular signal-regulated kinase ; ^{18}FdG: 18-fluorodeoxyglucose; HIF : Hypoxia-Inducible Factor ; IFP : interstitial fluid pressure; LDH: lactate dehydrogenase; MAPK : mitogen-activated protein kinase; mCAT: mitochondrial catalase; MCT : monocarboxylate-H^+ co-transporter; MDR : multidrug resistance ; MMP : matrix metalloproteinases; n-3 PUFA: n-3 polyunsaturated fatty acid; Na_V: voltage-gated sodium channels; NHE : sodium-proton exchanger; NMU : N-nitroso-N-methylurea; OXPHOS : oxidative phosphorylation; PDH: pyruvate dehydrogenase; PET: positron emission tomography; P-gp: P-glycoprotein; PPAR : peroxisome proliferator activated receptor; PPI : proton pump inhibitor ; ROS : reactive oxygen species; TCA: tricarboxylic acid cycle; TMZ : temozolomide; UCP-2: mitochondrial uncoupling protein 2

1. Introduction

Cancer is a leading cause of death, and in 2012, it was estimated that 14.1 million new cancer cases and 8.2 million cancer deaths occurred in the world (1). These numbers are expected to increase rapidly in the next few years because of the growth and aging of populations, the changes in lifestyle and dietary behaviours, and/or the exposure to environmental conditions that are known or supposed to increase cancer risk. Cancer is often reduced as being a genetic disease. Indeed, the tumour mainly takes its origin in the occurrence of sporadic mutations leading to the amplification, or activation, of oncogenes (such as *SRC*, *AKT*) (2,3), the increased expression (or gain-of-function mutations) of proto-oncogenes (such a *RAS*, *MYC*, *WNT*) (4-6), or the acquisition of loss-of-function mutations in tumour-suppressor genes (such a *RB1*, *TP53*, *PTEN*, *APC*) (7-9). A small proportion of cancers, 5 to 15% of cases depending on cancer types, are hereditary due to the transmission of germline mutations in tumour-suppressor genes, such as the breast-ovarian hereditary cancers associated with mutations in *BRCA1/2* genes encoding for DNA repair enzymes (10). These initial mutations, along with the stochastic accumulation of multiple others, lead to the selection of some cancer cell clones, generating a heterogeneous primary tumour, characterised by six minimal parameters identified as being the hallmarks of cancer: a sustained signalling promoting proliferation, a replicative immortality, the independence towards growth suppressors, the resistance to cell death, the induction of tumour angiogenesis, and the activation of invasive and pro-metastatic properties (11).

Cancer progression

The growth of this primary tumour, and its progression towards an aggressive phenotype result from mutual interactions between cancer cells and their microenvironment in the host organism. This microenvironment comprises stromal, endothelial and immune cells, extracellular matrices, and also soluble factors, such as cytokines, growth factors, and can be submitted to fluctuations in ionic composition, nutrients availability and oxygen tension. At the cellular level, the acquisition of extensive migration and extracellular matrix invasion potencies by cancer cells are critical steps in cancer progression, in the metastatic cascade (12,13) and eventually in patient death (14). Over the last decade, important knowledge on cancer cell migration and invasiveness has emerged, leading to the classification of different types of migrations, individual *versus* collective, and different migrating cell phenotypes, mesenchymal *versus* amoeboid (15,16). These specific phenotypes are not absolutely strict, but versatile as a function of changes in the microenvironment. It is generally accepted, that the acquisition of pro-invasive capacities is associated with the epithelial-to-mesenchymal transition (EMT), which is a reversible phenotypical and functional programme, reminiscent of physiological mechanisms involved in embryonic development or tissue repairing (17). During the EMT, cancer cells of epithelial origin dedifferentiate, they lose their apico-basal polarity to the profit of a rear-to-front cell polarity, lose intercellular junctions (especially tight and adherens junctions), remodel their intracellular cytoskeleton and gain mobility and resistance to apoptosis (Figure 1). They also overexpress and secrete ECM-degrading proteases, and express mesenchymal markers (vimentin, N-cadherin, α-smooth muscle actin (α-SMA), *etc*.) (18). The EMT is proposed to favour the acquisition of stemness characteristics in cancer cells (19,20), to support their survival in the bloodstream and their extravasation in metastatic sites (21,22). Increasing evidence suggests that the EMT, and the reverse process called mesenchymal-to-epithelial transition (MET), may be better described

as a spectrum of intermediate states that might depend on the physical and chemical nature of the microenvironment. The initiation of the EMT programme is driven by several signalling pathways including those mediated by transforming growth factor β (TGF-β) (23), bone morphogenetic protein (BMP) (24) or integrin signalling (25), that stimulate EMT-inducing transcription factors (Snail1/2, Zeb1/2, Twist) which bind to the promoter region of critical genes such as those regulating cell-cell adhesions (26-29). In a primary tumour, the induction of EMT is generally visualized in areas of hypoxia (30). In fact, low oxygen tension induces transcriptional, metabolic and phenotypic changes that directly induce, or synergize with signalling pathways inducing EMT (see paragraph 2.1 – "Aerobic glycolysis, pH regulation and consequences on cancer cell invasiveness and resistance to treatments"), thus linking this phenotypical switch to metabolic parameters. In the mesenchymal mode of invasion, cancer cells harbour an elongated fibroblast-like morphology, with a rear-to-front lamellopodial cell polarity. Their motility is dependent on the interaction of integrins, at focal sites, with components of the substratum. In this mode of invasion, cancer cells self-generate a path through the participation of invadosomal structures that perform the proteolytic remodelling of the ECM by both membrane-associated and extracellularly-released soluble proteases, such as MMP2, MMP9 or cysteine cathepsins, which extracellular release and activation are promoted by extracellular acidification (31-34). In the "amoeboid" mode of invasion, cancer cells generally present a rounded morphology, but their shape changes in order to move into small gaps of the ECM, with no need to degrade it, and they display a high speed of migration due to strong actomyosin contractions (35). While different cancer cell types may preferentially engage into one mode or the other, the most aggressive cancer cells show high plasticity, with transitions called MAT, for mesenchymal-to-amoeboid transition, or AMT for amoeboid-to-mesenchymal transition (35). These transitions, orchestrated by RhoGTPases

family members (such as Rac1/2, RhoA/B, cdc42) (36-38), offer selective advantages and compensatory mechanisms to invading cancer cells, by counteracting and adapting to changes in the microenvironment (matrix composition and stiffness, accessibility to oxygen and nutrients), and are also proposed to abrogate the efficacy of some anticancer treatments (39-41). Recently, some reports describe a very aggressive hybrid mesenchymal-amoeboid phenotype in some cancer cell types (42,43). The selection of drug resistant cancer cells is another critical step in the progression of the disease leading to a decrease in the efficacy of chemotherapies. The multidrug resistance (MDR) phenotype is the result of a multicomponent process, among which is the over-expression of ATP-binding cassettes (ABC) transporters, such as the P-glycoprotein (P-gp, also known as *ABCB1* or *MDR1*), which are highly energy-demanding for their activity of anticancer drug efflux. Drug resistance is also characterized by the capacity of cancer cells to counteract the oxidative stress generated by some chemotherapeutic agents, through a modulated Redox status, or to resist to apoptotic inducers. Recent studies suggest that EMT is importantly involved in the selection of chemotherapy-resistant cancer cells (44,45).

Cancer as a metabolic disease

Cancer progression relies on bioenergetics parameters (46), and the metabolic reprogramming of cancer cells was added to the initial hallmarks of cancer (47). This reprogramming of the entire metabolism has consequences on protons dynamics in all cellular compartments (mitochondria, cytosol, lysosomes and autophagolysosomes), and extracellular compartments leading to a deregulated pH homeostasis, which, in adapted cancer cells, offers supplementary aggressive features to create a “perfect storm for cancer progression” (48). While the metabolic reprogramming is a general feature of tumours, it is not static and not

similarly applicable to all tumour cells. Furthermore, the nature of the metabolic reprogramming is highly dynamic, and might differ as a function of time and conditions (such as episodic deprivations of oxygen and nutrients, distance from blood vessels, stimulation by paracrine factors), as a function of cell types (cancer cells, cancer-associated fibroblasts, tumour-associated macrophages, endothelial cells, …) and populations (cancer cell clones). Therefore, the metabolic reprogramming should not only be considered as a simple and permanent state in a cancer cell, but should rather take into consideration tumour spatio-temporal heterogeneity (49). These versatile metabolic adaptations not only allow cancer cells to survive to the pressure of environmental conditions by providing energy demand required to support the maintenance of their high anabolic activity for uncontrolled proliferation, but also exacerbate their migrative, invasive activities and metastatic properties (50,51).

It should be mentionned that metabolic reprogramming does not solely concern cancer and non-cancer cells from the tumour (metabolic interplay between cancer and non-cancer cells from the tumour, and also between oxygenated and hypoxic tumour cells), but also operates, most probably through the participation of an inflammatory component, in distant non-cancer tissues from the host organism, such as in liver, adipose tissue and skeletal muscles, leading to an overall deregulation of the energetic balance (52-54). A severe state of tumour-induced metabolic reprogramming, known as cancer cachexia, is a complex syndrome characterized by the loss of adipose and muscle mass, anorexia, and a general weakening of patients, impeding their quality of life and preventing the application of an effective anti-cancer treatment (55,56).

Therefore, it is proposed that the cancer disease, considering not only the tumour and its aggressive progression but also the response of the host organism to the presence of a tumour, is a metabolic disease. This review, focused on cancer cells, is intended to highlight how

cancer cell metabolic reprogramming couples with completely deregulated protons homeostasis and dynamics, promoting some aspects of cancer cell aggressiveness and tumour progression, such as the gain in invasive potential, the development of metastases and the resistance to anticancer treatments.

2. Metabolic switches in cancer cells and consequences on cancer cell properties

Cancer cells in a tumour are selected under stringent environmental conditions of low oxygen levels, nutrients deprivation, acidic extracellular pH (pHe), exposure to anticancer treatments, and on their ability to fulfil intensive energetics needs required for their high proliferative, invasive and detoxifying activities. Under these stringent conditions, only cancer cells with an adaptative metabolism can survive, leading to the self-selection of the most aggressive cells.

2.1 - Hypoxia and the selection of cancer cells with high glycolytic activity

In rapidly growing primary tumours, areas located away from blood capillaries (distance generally estimated as being > 150 µm) reach the limit of oxygen diffusion and cancers cells in these regions are under temporary or sustained hypoxic conditions (57,58). The majority of cells, mostly relying on the oxygen-dependent mitochondrial oxidative phosphorylation (OXPHOS) to generate ATP, die in these conditions, in turn generating necrotic cores of tumours. In this scenario, only cells that are be able to cope with hypoxic conditions because of their metabolic preference for glycolysis, will survive. The principal cellular mechanism for the biological adaptation in response to hypoxia is the stabilisation of the Hypoxia-Inducible Factor (HIF), a transcription factor belonging to the basic Helix-Loop-Helix (bHLH) family. The HIF-1 is a heterodimeric factor composed of a stable HIF-1β subunit and

an unstable HIF-1α subunit, the half-life of which is controlled by the level of oxygen. Indeed, in normoxic conditions, HIF-1α is degraded by the successive action of oxygen-dependent prolyl hydroxylases (PHD) and Von Hippel-Lindau (VHL) ubiquitin ligases, while it is maintained under hypoxia (59). Stabilized HIF-1 factor inhibits mitochondrial biogenesis (60) and functionally cooperates with the MYC oncogene to promote the glycolytic metabolism (61). This includes the up-regulation of glucose transporters, particularly studied is the isoform 1 (GLUT1, gene *SLC2A1*), which allows an increase in glucose uptake, and the expression of glycolytic enzymes, such as hexokinases 1 and 2 (*HK1* and *HK2*), which are the primary enzymes needed for the entry of glucose in glycolysis, aldolase A (*ALDA*), which catalyses the reversible conversion of fructose-1,6-bisphosphate to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate, phosphoglycerate kinase 1 (*PGK1*), which catalyses the reversible transfer of a phosphate group from 1,3-bisphosphoglycerate to ADP producing 3-phosphoglycerate, pyruvate kinase (PK), which catalyses the transfer of a phosphate group from phosphoenolpyruvate to ADP, thus giving one pyruvate and one ATP, and lactate dehydrogenase A (LDHA), which catalyses the inter-conversion of pyruvate and L-lactate with concomitant inter-conversion of NADH,H^+ and NAD+ (62-64). An increasing number of studies also reports that hypoxia promotes the EMT in cancer cells (18). This can be mediated, not exclusively, through the HIF-1α-dependent induction of EMT-promoting genes such as TGF-β (100), ZEB2 (101), TWIST (102), and the loss of E-cadherin (103).

The resistance to hypoxia is therefore an important parameter leading to the selection of adapted cancer cells, and initial studies led to the observation that aggressive cancer cells display a highly increased glycolytic activity. Quite surprisingly, this metabolic pathway, in selected cancer cells, is still observed under normoxic conditions. This is contradictory to the "Pasteur effect", generally observed in normal cells, characterizing the fact that the presence

of oxygen results in the activation of OXPHOS and the relative inhibition of glycolysis, since OXPHOS is 18 times more efficient than glycolysis in generating ATP from one molecule of glucose. This particular metabolism was called "aerobic glycolysis", which might be confusing since glycolysis does not require oxygen. This is also often referred as being the "Warburg effect" (65,66). Since the initial description by Otto Warburg, the "aerobic glycolysis" was demonstrated in different cancer types (67) and the study of tumour metabolism became an important research field, for both basic and translational research. For example, the detection and imaging of tumours, displaying a high consumption of glucose, can be performed with the analogue ^{18}fluorodeoxyglucose (^{18}FdG) as a tracer in positron-emission tomography (PET) (68,69). While "aerobic glycolysis" might be a general mechanism associated with cancer progression, the hypothesis proposed by Otto Warburg that this might be the consequence of a defective mitochondrial activity (70) was not verified, and it is know that mitochondria are fully functional in cancer cells and contribute to cancer progression (51). In this context, it is proposed that the aerobic glycolysis is a supplementary and regulatory metabolic pathway fulfilling the high energy demand of cancer cells.

It should be stated that within a tumour, there is a metabolic cooperation between cancer and non-cancer cells. As such, the understanding for a tumour metabolism with metabolic coupling between cancer cells and stromal cells from the tumour had been previously proposed by Lisanti and collaborators and was termed "The reverse Warburg effect" (71). In the tumour, cancer cells generate oxidative stress in neighbouring stromal fibroblasts or mesenchymal stem cells, thus leading to the onset of "aerobic glycolysis" in those non-cancer cells. In turn, the excess of lactate and pyruvate, the energy metabolites resulting from aerobic glycolysis from non-cancer cells, is transferred to adjacent cancer cells where they enter in the

tricarboxylic acid cycle (TCA), resulting in increased OXPHOS and efficient ATP production (71,72).

A metabolic cooperation has also been identified between cancer cells themselves and between cancer cells and non-cancer cells from the tumour microenvironment. In the case of ovarian cancers, adipocytes provide free fatty acids to cancer cells, after the hydrolysis of triacylglycerids (lipolysis) to fuel mitochondrial β-oxidation and promote tumour growth (73). In addition, a metabolic symbiosis between glycolytic and oxidative cancer cells was demonstrated through the exchange of lactate. In this model, lactate produced and excreted by glycolytic cancer cells, whether being in hypoxic conditions or not, is transported through monocarboxylate-H^+ co-transporters (MCT) in oxidative cancer cells. These later use LDHB to convert lactate into pyruvate and fuel the TCA cycle and promote tumour growth (74,75).

2.2 - Roles of mitochondrial bioenergetics in cancer progression

- Mitochondrial activity, cancer cell invasiveness and metastases -

Cancer cell migration and invasiveness are two critical aspects of the metastatic potential that may require a boost in energy and in this scenario, mitochondrial energy metabolism would be important. Therefore, several studies suggest that the regulation of the mitochondrial metabolic activity is an important determinant of the metastatic process. This has been elegantly shown *in vivo* by Tan and collaborators (76). In this study, the intravenous injection of tumour cells bearing no mitochondrial DNA (mtDNA), and therefore showing compromised mitochondrial energy metabolism, presented a delayed tumour growth in mice. Interestingly, metastastic cells were deriving from mtDNA-deficient carcinoma cells that obtained mtDNA from host murine cells, and exhibited full restoration of mitochondrial energy metabolism. These data indicate that “horizontal transfer” of mitochondria from host

cells to cancer cells in the tumour microenvironment play a role in metastases (76). Moreover, it has been shown that migratory/invasive cancer cells specifically favour mitochondrial oxygen consumption and have an increased ATP production (77). Invasive cancer cells use the transcription co-activator peroxisome proliferator-activated receptor gamma, co-activator 1 alpha *(PPARGC1A*, also known as PGC-1α) to enhance mitochondrial biogenesis and mitochondrial OXPHOS. Silencing of PGC-1α in cancer cells inhibited their invasive potential and attenuated metastasis (77). Correlatively, the increased expression of genes known to be involved in the mitochondrial energy metabolism, accompanied by a gain in mitochondrial biogenesis, was also observed in breast cancer cells originating from brain metastases (78). It has been shown that mitochondrial complex I activity was critical in defining the metastatic phenotype of breast cancer cells (79). Thus enhancing complex I activity inhibited metastasis, partly through the regulation of the tumour cell NAD^+/$NADH,H^+$ redox balance (79).

Mitochondria are high dynamic organelles with frequent fission and fusion that impact mitochondrial and cellular activities. It has been reported that hypoxia stimulated mitochondrial fusion and migration in metastatic breast cancer MDA-MB 231 cells, by increasing the expression of dynamin-related protein 1 (Drp1) (80). Moreover, inhibition of Drp1-dependent mitochondrial fission by Mdivi-1, an inhibitor of fission, or silencing Drp1 attenuated hypoxia-induced migration in MDA-MB 231 cells (80). Furthermore, it has been shown that EGF induced the translocation of its receptor EGFR to mitochondria that in turn induced mitochondrial fission, cellular redistribution of mitochondria to the lamellipodia, upregulated cellular ATP production, and enhanced cancer cell motility both *in vitro* and *in vivo* (81). In addition, the overexpression of Mitofusin 1, an actor of mitochondrial fusion, significantly alleviated the mitochondrial EGFR-mediated higher ATP production and cell

migration (81). Therefore, one could consider that the inhibition of mitochondrial fusion may be an important mechanism to prevent metastasis.

Mitochondria are major sites of reactive oxygen species (ROS) production, mostly occurring during the process of electron transfer through mitochondrial electron-transport chain enzymes at complex I and complex III (82,83). It has been shown that ROS induced by mitochondrial dysfunction enhances gastric cancer metastasis (84). By replacing the endogenous mtDNA of a poorly metastatic mouse tumour cell line, by the mtDNA derived from a highly metastatic mouse tumour cell line (cytoplasmic hybrid (cybrid) technology), it has been shown, both *in vitro* and *in vivo*, that mutations in mtDNA can enhance the metastatic potential of tumour cells by inducing defects in complex I, thus resulting in the increase of mitochondrial ROS production (85). Additional evidence implicating the role of the oxidative stress generated by mitochondrial activity in cancer metastasis comes from the study of Goh and colleagues (86). In their study, transgenic mice expressing a human mitochondrial catalase (mCAT) were crossed with MMTV-PyMT transgenic mice that spontaneously develop metastatic mammary cancer. Data showed that PyMT mice expressing mCAT had a lower incidence of metastasis compared to controlled PyMT mice not expressing mCAT. Moreover, PyMT tumour cells expressing mCAT had lower mitochondrial ROS levels than wild type tumour cells (86). A critical role for mitochondrial ROS in metastasis has also been reported by Porporato and collaborators (87). Indeed, they demonstrated that cancer cell migration, invasiveness and spontaneous metastasis in mice were promoted by the natural selection of two mitochondrial phenotypes leading to an enhanced mitochondrial ROS production: *i.e.* overload of the mitochondrial electron transport chain *vs.* partial mitochondrial electron transport chain inhibition. In addition, in this study, the specific scavenging of mitochondrial ROS with MitoTEMPO blocked tumour cell migration and

prevented spontaneous metastasis in both murine and human tumour models (87). In contrast, it has been shown that metastatic cancer cells can release mitochondrial ROS, induce oxidative stress and aerobic glycolysis in the non-cancer cells of the tumour microenvironment, which in turn generate L-lactate and ketone bodies that fuel mitochondrial energy metabolism in cancer cells, as a part of the reverse Warburg effect (88-90).

-Mitochondrial activity in the resistance to anticancer treatments-

Several studies have focused on the role of mitochondria and intracellular ATP in the development of anticancer drug resistance by cancer cells. For instance, this has been shown in CD44+/MyD88+ ovarian cancer stem cells that are resistant to apoptotic cell death induced by conventional chemotherapy agents. Indeed, in these extremely chemoresistant cells, the isoflavone derivative, NV-128, significantly depressed the mitochondrial function, as confirmed by a decrease in ATP synthesis, and by the increase in mitochondrial superoxide and hydrogen peroxide, and in turn activated cell death (91). On one hand, by comparing oxaliplatin-sensitive to –resistant cancer cells, it has been demonstrated that exogenous ATP delivery to sensitive cells partially blocked the cytotoxic effect of oxaliplatin (92). On the other hand, ATP depletion in resistant cells partially re-sensitized resistant cell lines to oxaliplatin, indicating the prominent role of intracellular ATP level in mediating the drug-resistant phenotype (92). Differently, by comparing several 5-fluorouracil (5-FU)-sensitive to –resistant colon cancer cells, the authors reported a down-regulation of mitochondrial ATP synthase subunits, a lower mitochondrial ATP synthase activity and a decreased intracellular ATP content in chemoresistant cells compared with parent cells (93). In addition to this, it was found that 5-FU sensitivity positively correlated with mitochondrial ATP synthase expression and activity. Finally, knocking-down the mitochondrial ATP synthase expression,

by using siRNA, induced a higher viability of sensitive cells under the treatment with different concentrations of 5-FU (93). One obstacle of chemotherapy efficacy is the overexpression of a superfamily of energy-dependent ATP binding cassette transporters that extrude anticancer drugs out of the cell. It has been suggested that the modulation of mitochondrial energy metabolism could reduce drug resistance by restoring the capacity to accumulate and retain drug in the cells. Thus, it has been showed that oligomycin could suppress the activity of the P-gp expressed at the plasma membrane, at an extent similar to the P-gp inhibitor verapamil, and increased accumulation of doxorubicin in doxorubicin-resistant HepG2 cells (94). Although ATP-dependent efflux pumps are generally considered to be localized at the plasma membrane, it has been showed that BCRP (breast cancer resistant protein), MRP1 (multidrug resistance protein 1) or P-gp could also be expressed in mitochondria from leukaemia, ovarian carcinoma, sarcoma and hepatocellular doxorubicin-resistant cancer cells (95-98). It has been described that BCRP and MRP1 pumps, expressed in the mitochondrial membrane, maintain the direction of transport similarly to that in the plasma membrane (inside towards outside). Therefore, it is proposed that mitochondrial ATP-dependent efflux pumps could be involved in the protection of mitochondria from damage due to anticancer drugs (Figure 2). Since functionally active mitochondrial pumps require ATP for their efflux activity, one could argue that mitochondrial energy production in chemoresistant cells participates to the ATP used for the functioning of mitochondrial efflux pumps.

It has been suggested that the toxicity level of drugs towards cancer cells was dependent on the mitochondrial mass. For example, the introduction of normal mitochondria from immortalized, untransformed MCF-12A cells in breast cancer cells MCF-7 increased their sensitivity to doxorubicin, abraxane, and carboplatin (99). In this context, it has been shown that the treatment of breast cancer cells with the thyroid hormone (T3) increased

mitochondrial mass, modulated the bioenergetics profile and enhanced doxorubicin-induced cell death (100). Such a link between mitochondrial mass and chemoresistance has also been found in oxygen-deficient conditions. Thus, it has been observed, in lung cancer cell lines, that hypoxia-induced resistance to cisplatin and doxorubicin were associated with a decrease in number and size of mitochondria (101).

Several studies have used dichloroacetate, a pyruvate dehydrogenase kinase inhibitor that activate mitochondrial OXPHOS, to target metabolism in cancer cells. Using this methodology, it has been suggested that the activation of mitochondrial OXPHOS was more effective than the suppression of glycolysis in overcoming sorafenib resistance in highly glycolytic hepatocellular carcinoma (HCC) cells (102). Indeed, although enhanced glycolysis was positively correlated with sorafenib resistance of HCC cells, dichloroacetate, by activating mitochondrial OXPHOS, markedly sensitised sorafenib-resistant cells to sorafenib-induced apoptosis. By contrast, the inhibition of glycolysis, by silencing the expression of hexokinase 2, was ineffective (102). In addition, it has been shown that combination of dichloroacetate and cisplatin exhibited a significant synergistic activity in reducing the viability of HeLa cells (103). Furthermore, it has been showed that the use of dichloroacetate, in combination with doxorubicin or cisplatin, reduced the cytotoxicity of these two drugs *in vitro*, on a range of human cancer cell lines (104). Even if the precise mechanisms still need to be clarified, these studies suggest that mitochondrial energy metabolism is involved in the chemoresistance of cancer cells and tumours (Figure 2).

Multiple anticancer drugs induce cell death through the generation of ROS, and even agents that have been characterized to generate cancer cell apoptosis through the interaction with DNA (such as cisplatin, bleomycin, doxorubicin, methotrexate, busulfan and fluorouracil) are known to generate mitochondrial ROS (105). The role of mitochondria in the interplay

between ROS production and chemoresistance has been studied by Alakhova and collaborators (106). In their study, they showed that part of the sensitizing effects of pluronic, a synthetic amphiphilic copolymer, in multidrug resistant cancer cells was associated with an increased production of mitochondrial ROS and the release of cytochrome c, induced by the impairment of the mitochondrial oxygen consumption (106). To test the hypothesis that chemoresistance arises from decreased production of ROS, mitochondrial function has been assessed in temozolomide (TMZ)-sensitive (U251) or -resistant (UTMZ) glioma cells and in TMZ-resistant glioblastoma xenografts (107). Firstly, data showed that mitochondrial coupling (an indicator of ATP synthesis efficiency) was higher, and consequently mitochondrial ROS production was significantly lower, in TMZ-resistant cells. Secondly, it has been found that mitochondrial DNA-depleted cells (U251ρ0), a model of non-functional mitochondria, became resistant to TMZ and had lower intracellular ROS levels after TMZ exposure compared with parental sensitive cells. Finally, restoration of ρ0 cells with mitochondria restored ROS production and sensitivity to TMZ (107). Taken together, these data indicate that chemoresistance to could be due to low mitochondrial ROS production. In another study, it has been showed that basal level of ROS production is lower or identical in chemoresistant cancer cells However, radiations increased levels of ROS in drug-sensitive cells and not, or even a slight reduction, in drug-resistant cells (108). Interestingly, this phenomenon was associated to a higher expression of the mitochondrial uncoupling protein 2 (UCP2) in drug-resistant cancer cells, suggesting that mitochondrial ROS production was lower (108). Although the exact role of UCP2 needs to be determined, various studies have shown that it could prevent mitochondrial oxidative stress generation by increasing the flow of protons into the mitochondrial matrix (increased proton leak), thus rendering electron flow through the mitochondrial respiratory complexes more elevated (109-111). By using drug-

sensitive HL-60 human acute promyelocytic leukemia cells and the drug-resistant HL-60/MX2 cells as models, it has been showed that genipin, by inhibiting UCP2, sensitized drug-resistant cells to cytotoxic agents (menadione, doxorubicin, and epirubicin) and that sensitization of drug-resistant cells was accompanied by increased levels of cellular ROS (112). In addition, the drug-induced increase in mitochondrial ROS was linked to genipin-mediated inhibition of mitochondrial proton leak (112). Pons and collaborators have recently conducted a study in which ROS production and sensitivity to cisplatin or tamoxifen were tested in two breast cell lines (MCF-7 and T47D) as a function of UCP2 expression and function (using siRNA and genipin, respectively) (113). Data showed that inhibition of UCP2 improved the efficacy of each drug to decrease cell viability, and increased the production of mitochondrial ROS in both cell lines (113). Similarly, a role for mitochondrial oxidative stress in chemoresistance was also identified in the case of the anticancer drug gemcitabine (GEM). It has been demonstrated that mitochondrial uncoupling, induced by the chemical uncoupler carbonilcyanide p-triflouromethoxyphenylhydrazone (FCCP), or by the over-expression of UCP2, strongly decreased GEM-induced mitochondrial ROS and protected cancer cells (pancreatic adenocarcinoma, non-small cell lung adenocarcinoma and bladder carcinoma) to GEM-induced apoptosis (114). In addition, GEM treatment and UCP2 inhibition (by genipin) strongly induced mitochondrial superoxide production and ROS-mediated apoptosis in cancer cells (114). A role for mitochondrial ROS in chemoresistance has also been demonstrated by Okon and collaborators (115). However, in this study, ROS levels were higher and catalase (antioxidant enzyme) levels were lower in gefitinib (a tyrosine kinase inhibitor)-resistant lung H1650G adenocarcinoma cell line than in the sensitive H1650 counterpart. This stress oxidative phenotype was associated with a lower mitochondrial oxygen consumption. In addition, when H1650 cells were grown over several weeks in the

presence of gefitinib (as carried out to generate gefitinib-resistant H1650 Clones) and the mitochondria-specific ROS scavenger MitoTEMPO, mitochondrial oxygen consumption was not decreased and ROS levels were not increased (115). This demonstrated a direct link between mitochondrial ROS production, mitochondrial dysfunction and resistance to gefitinib.

Interestingly, recent studies pointed out the role of mitochondrial dynamics in chemoresistance. Thus, it was showed that piceatannol, a natural metabolite of the stilbene resveratrol, was a potent enhancer of cisplatin sensitivity in different human ovarian cancer cell lines (sensitive or not to the drug) (116). Interestingly, this effect was associated, among other things, with an increase in mitochondrial fission. Indeed, the number of cells showing signs of apoptosis and fragmented mitochondria were increased by the co-treatment of piceatannol with cisplatin, while they were lower when mDivi-1, an inhibitor of fission, was added (116). Differently, the study conducted by Santin and collaborators suggested that the increased fusion of mitochondria, to the detriment of fission, may be the mechanism responsible for drug resistance. In B50 neuroblastoma rat cells, an expected apoptosis was induced after a 48h-long exposure to cisplatin, but under a long-term exposure to the drug, the elongation of mitochondria was associated with a reduction of the apoptosis rate (117).

2.3. pH regulation as a consequence of metabolic reprogramming, effect on cancer progression and resistance to anticancer treatments

One consequence of this complete metabolic reprogramming, and high activity is the increased production of acid equivalents that are exported in the extracellular space leading to an acidic pHe (Figure 2). While there might be some metabolic heterogeneity within the tumour (118), there is a good correlation between glucose consumption in tumours, as

monitored by PET using ^{18}FdG-Glucose uptake, and acidic regions as monitored using pH-sensitive fluorescent probes or Magnetic Resonance Imaging (119,120). This would involve the glycolytic metabolism in the production of protons. However, the production of lactate from glucose, by the glycolysis does not produce any proton. Therefore, the majority of the production of acid equivalents associated with the glycolytic activity might be produced at the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) step, or even consecutive to the correlative high energetic demand, leading to the hydrolysis of ATP to ADP. While some authors argue for the dissociation of lactic acid into lactate and H^+ (pKa 3.9), the conversion reaction of pyruvate into lactate by the LDHA consumes protons. Because LDHA- and glycolysis-deficient tumours are still producing extracellular acidification, it is proposed that the glycolysis is not the only source of H^+ in cancer cells (121-123). Additional sources of protons could come from the CO_2 produced during the pentose phosphate pathway in the cytosol and the tricarboxylic acid (TCA) cycle in mitochondria (Figure 2).

In any case, excessively produced H^+ are extruded in order to maintain the intracellular pH (pHi) and permit cancer cells to survive (124-126). This is allowed by overexpression of proton-extruding plasma membrane transporters, such as the electroneutral Na^+/H^+ exchanger type 1 (NHE1), MCT monocarboxylate-H^+ co-transporters (particularly the MCT-4 isoform, which is mediated by hypoxia through HIF-1α), bicarbonate transporters (such as the electroneutral or electrogenic Na^+/HCO_3^- co-transporters NBCs, electrogenic Na^+-dependent Cl^-/HCO_3^- exchangers NDCBE) and proton pumps (such as the electrogenic vacuolar-type H^+-ATPase V-ATPase), and for inducible transmembrane carbonic anhydrase isoforms 9 and 12 (CA-9 and CA-12) (127-129). *In vitro*, the expression and activity of these acid-extruding transporters were demonstrated to control the 3-dimensional growth of human breast cancer cells spheroids (130). These numerous plasma membrane proteins are essential for

maintaining pHi and their activities result in the decrease of pHe. It is interesting to point out that some of these are active transporters (ATPases) hydrolysing high amounts of ATP to export protons produced by the glycolytic energetic pathway, and allow cancer cell survival. This could appear as being an aberrant situation, if we did not consider the fact that this extrusion of H^+ in the extracellular space also provides selective advantages to cancer cells by conferring them with enhanced proliferative (131), migratory and invasive activities (32,132-134), while reducing the anticancer immune response (124,135).

While there might be some differences depending on the considered tissue, pHi is generally comprised between 6.9 and 7.2, and is slightly more acidic than pHe, which is comprised between 7.2 and 7.4 under physiological conditions (125,136). It is also noticeable that the activities of H^+ transporters and pumps cited above, not only maintain pHi to values compatible with cell survival, but further enhance the extrusion of protons, thus reversing the proton gradient in cancer cells with a pHi > 7.2 and a pHe < 7.0 (48). Far from being deleterious to cancer cells, this extracellular acidification in solid tumours (57) triggers the apoptotic cell death of non-cancer cells (137). In cancer cells, the acidic pHe further participates to genomic instability by reducing DNA repair and promoting the acquisition of further mutations (138).

-pH regulation, cancer cell invasiveness and metastases -

Among all pH regulators, NHE1 was functionally associated with cancer cell survival, migration and metastatic progression (32,125). NHE1 uses the inwardly directed electrochemical Na^+ gradient to exchange H^+ for Na^+ ions with a stoichiometry of 1:1 (139). This plasma membrane exchanger is ubiquitously expressed. It is quiescent at physiological pHi ~7.2, but activates rapidly upon intracellular acidification (140) and is therefore involved

in the protection of normal cells upon intracellular acidification bursts (128) while acidifying the extracellular environment. Its activity is tightly regulated by multiple hormonal and mitogenic pathways (141). In cancer cells, NHE1 is both overexpressed and overactivated (142), and is involved in cancer cell motility and matrix degradation (33,143). The increased NHE1 expression and activity are considered to constitute an unfavourable prognostic factor, such as in the case of ovarian cancers (144).

NHE1 overexpression in cancer cells is also suggested to be a part of the EMT. Indeed, in prostate cancer cells, the expression of NHE1 is strongly correlated with the expression of Zeb1, a crucial transcription factor activating the mesenchymal gene expression program (145). Furthermore, Zeb1 was found to bind to the promoter of NHE1 gene (*SLC9A1*) and support its expression. NHE1 up-regulation was even proposed to drive carcinogenesis (142). Importantly, it was demonstrated that low levels of intracellular ROS, which confer cells with resistance capacities to death stimuli, could up-regulate the expression of NHE1 during carcinogenesis (146).

NHE1 was subsequently involved in the trafficking of lysosomes to the plasma membrane and the secretion of proteases involved in cancer cell invasion (145). Of interest is the recent publication from D. Lagadic-Gossmann's group showing that the genotoxic and carcinogenic agent, benzo[a]pyrene, a polycyclic aromatic hydrocarbon pollutant from exhaust fumes and cigarette smoke, found in food and drinking water, also promotes metabolic reprogramming in hepatic cancer cells and EMT through NHE1 regulation (147). Benzo[a]pyrene treatment was responsible for a reduction of mitochondrial respiration, and correlative increases in glucose oxidation, lactate release and extracellular acidification, which recapitulated a switch towards an aerobic glycolysis. These changes were prevented by the use of the NHE1 inhibitor cariporide (147).

NHE1 is known to support the ECM degradative activity that is associated with the mesenchymal phenotype. Indeed, it has a predominant role in extracellular acidification, the activity of invadopodia and in the invasiveness of cancer cells (33,125,134,148) (Figure 1). NHE1 has also been identified as a key regulator of oriented actin polymerisation for invadopodia initiation. This occurs both through scaffolding properties, as it interacts with actin-binding proteins of the ERM (ezrin, radixin and moesin) family, and through its activity, as the resulting intracellular alkalinisation allows the release of the actin-severing factor cofilin and disrupts its inhibitory interaction with cortactin (125,149,150). Furthermore, the activation of focal adhesion kinase (FAK), which is an important parameter of cancer cell migration and survival, was found to be sensitive to pHi. Alkaline pHi was demonstrated to induce FAK conformational changes allowing its autophosphorylation on tyrosine residue 397. This was prevented by knocking-down NHE1 expression and resulted in the impairment of focal adhesions and cell spreading (151).

It is worth noticing that, in cancer cells, not only the expression is increased, but most importantly the activity of NHE1 is enhanced, despite the relatively alkalinized pHi of cancer cells (125), a condition for which it would be inactive in normal cells. Signalling pathways that are often overactivated in cancer cells are also known to upregulate the activity of NHE1. This is the case for some growth factor (141), extracellular signal-regulated kinase (ERK1/2) (152,153), or B-Raf kinase (154) signalling pathways that would enhance the extrusion of H^+ through NHE1 activity in alkaline pHi. Therefore it seems that several signalling pathways, such as mitogenic pathways, up-regulate the expression of several proton extruders, including NHE1, thus responsible for intracellular alkalinisation, but also sustain their activity in alkaline pHi.

Further to the regulations previously mentioned, it was demonstrated that voltage-gated sodium channels (Na_V), overexpressed in carcinoma cells (155-157), and known to participate to the mesenchymal invasion of cancer cells, further enhance NHE1 activity in cancer cells. Na_V channels were initially proposed to be characteristics of excitable cells, such as skeletal and cardiac muscle cells, while their expression is generally repressed in non-excitable cells, such as epithelial cells. Na_V activity, through a transient entry of sodium charges, leads to the depolarization of the membrane responsible for the initiation and propagation of action potentials (158-163). More recently, Na_V were found to be expressed in carcinoma, such as in prostate, breast, lung, colon, cervix, ovary cancers, while they are not expressed in non-cancer tissues (164). It was proposed that their expression and activity are associated with cancer progression (155,156,165), metastases development and patients' death (166,167). In highly aggressive human breast cancer cells, the activity of the $Na_V1.5$ pore-forming subunit participated to the acquisition of a mesenchymal phenotype by controlling Src kinase activity and the phosphorylation of the actin-nucleation promoting factor cortactin (168). Furthermore, $Na_V1.5$ enhanced extracellular matrix (ECM) degradation and cancer cell invasiveness in 2 and 3-dimension matrices, by increasing the activity of extracellular cysteine cathepsins B and S (168-170) which have a maximal activity in acidic conditions (171) (Figure 1). While the activity of Na_V is transient (generating rapidly-inactivating, inward currents), $Na_V1.5$ was proposed to promote cancer cell invasiveness through a persistent window current at the membrane potential of cancer cells. This current, through a not yet described signalling pathway, was responsible for the allosteric activation of NHE1, rendering it more sensitive to intracellular H^+, thus increasing its H^+ efflux activity and the activation of extracellular cathepsins (168,172). While it is demonstrated that $Na_V1.5$ activity supports mesenchymal invasiveness of breast cancer cells, *i.e.* invasion that require ECM

proteolysis, the participation of Na_V channels to the EMT have not been fully characterized. A recent study from Nelson and collaborators indicated that the down-regulation of $Na_V1.5$ expression, using specific shRNA, resulted in an increase in MDA-MB-231 cell circularity and favoured a more rounded epithelial-like phenotype (173), thus corroborating previous findings (168,174). However, this was not associated with any alteration in E-cadherin, N-cadherin, vimentin, slug or snail expression. In contrast, the protein level of CD44, known to promote invasiveness and metastasis of breast cancer cells, was significantly reduced (173).

Further supporting the critical role of Na_V-NHE1 complexes in cancer progression, the $Na_V1.7$ pore-forming subunit was recently identified to promote gastric cancer cell invasiveness through the metastasis-associated in colon cancer-1 (MACC1)-dependent up-regulation of NHE1 (175). Na_V channels in cancer cells are critical for the metastatic colonization of organs, and their inhibitors that are clinically used for the treatment of other pathologies were demonstrated to be powerful pharmacological tools to inhibit cancer cell invasiveness and prevent metastatic colonization (166,174,176,177).

While NHE1 activity is electroneutral, some of the other pH regulators cited above are electrogenic and their activity might regulate the membrane potential (Em) of cancer cells at the same time they control H^+ homeostasis, thus also controlling the activity of voltage-sensitive ion channels and/or the driving force for some ions. Furthermore, several ion channels are directly gated by extracellular H^+, such as acid-sensing ion channels (ASIC) (178), while some other are regulated by acidic pH such as TRPV1 (179,180) and TRPA1 (181,182). These ion channels have recently been proposed to be involved in cancer progression through the promotion of lymphatic metastasis (183), and to be responsible for pain associated with cancer (184-186).

The acidic pHe has also been demonstrated to participate to the acquisition, by cancer cells, of aggressive features that are characteristics of the EMT. Indeed, the acidic pHe induces the loss of β-catenin from adherens junction in hepatocarcinoma cells through the activation of Src kinase (187), and the degradation of E-cadherin (188). The acidic pHe also participate to the acquisition of a mesenchymal, fibroblast-like, elongated cell phenotype, that is involved in an increased migratory activity (133,189) and an ECM-degradative function that participate to the metastatic evasion/invasion of tissues (32). While some of these aspects may be associated with pH by itself, some others were directly correlated with the activity of specific pH regulators. The acidification of the tumour microenvironment, is implicated in a positive feedback loop further enhancing the mesenchymal phenotype and the degradation of the extracellular matrix. Zeb1 promotes the NHE1-dependent anterograde lysosome trafficking to the plasma membrane, supporting ECM degradation and invasion by cancer cells (145). With an acidic pHe, the number of lysosomes decreased, owing to their displacement to the cell periphery and their exocytosis. This was shown to increase the secretion of degradative enzymes, but also the formation of filopodial structures, which together contributed to cancer cell invasiveness (34). The acidic pHe also indirectly promotes EMT in cancer cells. In a recent study, it has been shown that mesenchymal stem cells grown in low pHe conditions favour the growth and progression of melanoma through a release of TGF-β and the acquisition of an EMT program in melanoma cells (190).

Lastly, the acidic environment of tumours also controls the metabolic activity of cancer cells. In the tumour, cancer cells are exposed to intermittent hypoxic episodes between which oxygen becomes sufficient again to allow mitochondrial respiration, while the acidic microenvironment is maintained. This acidic microenvironment also interferes with the metabolism of oxygenated cancer cells, in proximity of blood vessels, in the tumour. The two

recent studies of Corbet and collaborators demonstrate that cancer cells subjected to an acidic environment (pH 6.5) have a preferred glutamine reductive metabolism (191), and fatty acid oxidation to provide acetyl-coA to the TCA cycle (192). The consequences of this rewiring of metabolism are the preferred consumption of fatty acids to provide energy, but also the reduction of ROS production due to the hyperacetylation of mitochondrial complex I, which together support cancer cell proliferation and tumour growth (192).

-pH regulation and resistance to anticancer treatments-

Major mechanisms contributing to chemo-resistance are the down-regulation of cell death mechanisms, but also the limitation of anticancer drug diffusion mainly due to the poor vascular perfusion of tumours, and the overexpression or activity of P-gp mediating the ATP-dependent efflux of drugs. The characteristics of tumour microenvironment, *i.e.* hypoxia, low nutrients concentration, high lactate concentration, and acidic pHe, which are consequences of the abnormal vascularization of tumours and the glycolytic metabolism of some tumour cells, are critical factors contributing to drug resistance.

The acidic pHe of the tumour microenvironment plays an important role in limiting drug diffusion. This particular phenomenon, called “ion trapping”, occurs when the plasma membrane permeability of a drug is different for its ionized or non-ionized form (193,194). The ratio of ionized *versus* non-ionized form of a drug is pH-dependent. At acidic pH, molecules that are weak bases are ionized into protonated, positively charged drugs with limited diffusion through the plasma membrane. Common chemotherapeutic drugs are weak bases such as anthracyclines, anthraquinones and vinca alkaloids. As such, both *in vitro* and *in vivo* studies evidenced that an acidic pHe prevents the distribution of these weak bases inside cancer cells, thus leading to a decreased efficacy in treatment (195-198) (Figure 2). On

the opposite, drugs that behave as weak acids are permeable in their non-ionized form at acidic pH, are deprotonated and become negatively charged when they reach the slightly alkaline intracellular milieu. Therefore, they are trapped intracellularly and gain in efficacy. This is the case of chlorambucil, cyclophosphamide (alkylating agents) and 5-FU (antimetabolic), which all have an enhanced uptake in acidic pHe conditions (197,199,200). As a result, the experimental acidification of the extra-tumoral pH *in vivo*, by -0.2 pH unit, using glucose administration, led to an increased efficiency of the weak acid chlorambucil, while it inhibited the tumour growth-reduction effect induced by the weak base doxorubicin (196). On the contrary, the cytotoxic action of the alkylating agent melphalan, which is indicated for advanced breast and ovarian cancer and myeloma, and which can be assimilated as an acid drug, is more effective at acidic pH under hypoxia (201,202). This consideration was taken into account for the treatment of confined advanced metastatic melanoma, by using the isolated limb infusion of melphalan under non-oxygenated conditions to reduce pHe (203). Taken together, these studies clearly highlight the contribution of tumour metabolism to produce acidity and its relevance to cancer treatment.

In addition, this dysregulation of the pH homeostasis occurring in cancer cells influences drug distribution within the different intracellular compartments. Weak base anticancer drugs in the intracellular medium can be sequestered in acidic organelles (lysosomes, endosomes), thus limiting their availability to exert cytotoxic effects. Osteosarcoma cells exposed to an pHe of 6.5, have been shown to present a higher population of lysosomes being more acidic, and this was associated with a reduction in the cytotoxic effect of the anthracyclin doxorubicin, because of a reduced nuclear localization (198). This intracellular trapping of anticancer drugs is exacerbated in multidrug resistant breast cancer cells since they have more acidic

endosomes and lysosomes compared to sensitive cells. As a result, this provokes an accumulation of basic drugs in these acidic compartments and a reduced cytotoxicity (204).

Recent studies report that the reduced efficacy of anticancer drugs in acidic pH is also related with P-gp-dependent efflux. While the expression of the P-gp does not seem to be modified under acidic environment, its drug efflux activity is enhanced (205,206) (Figure 2). It was proposed by the authors that the acute exposure to an acidic pHe (6.6) induces a reduction of intracellular Ca^{2+} concentration, which increases the transport rate of P-gp. While the molecular details of the control of the intracellular Ca^{2+} homeostasis was not described, a comparable effect was found with the inhibition of protein kinase C (PKC). This effect was potentiated by hypoxia, which is a critical parameter of the tumour microenvironment (205,206). Furthermore it has been proposed that alterations of cell membrane mechanical properties induced by pHi in cancer cells may also participate to the control of doxorubicin diffusion inside the cells and to the acquisition of the resistance phenotype (207).

Therefore, the reverse pH gradient that is characteristic of tumours has important consequences for the efficacy of the anticancer treatment, and development of resistant tumours. This reality should be taken into account for the development of more effective anticancer treatments, leading to new therapies targeting the abnormal pH of tumours, alone or in combination with conventional treatments. This concept has been entitled “the buffer therapy” (194). Mathematical models argued that the best buffer to neutralize pHe in the tumour microenvironment should have a pKa of 7 (208). Even if the pKa of bicarbonate (pKa 6) is not ideal, this is for the moment the best known buffer suitable for systemic administration. The use of bicarbonate gave the first evidence that systemic buffering in mice can reduce pHe of tumours (209). Bicarbonate had no effect on primary tumour growth but improved the effect of doxorubicin to reduce tumour volume (209). Furthermore, in a model

of mammary tumour, bicarbonate administration in the drinking water of mice elevated pHe in the tumour and importantly decreased spontaneous metastasis (210). The evaluation of the buffering capacity of food suggests that a new strategy based on a controlled nutritional intake may reduce pHe in the tumour microenvironment and may be effective to improve the efficacy of chemotherapy (211).

In addition, it has been proposed that interfering with pHe and reversing the acidic pH of organelles, using proton pump inhibitors (PPI), would avoid the accumulation of drugs in acidic compartments and would increase their distribution in the cytosol and in the nucleus of cancer cells. Targeting the lysosomal pH using omeprazole, a PPI that is generally used to treat excess stomach acid production and symptoms of gastroesophageal reflux, was demonstrated to improve the cytotoxic effect of doxorubicin, and to decrease tumour volume in a mouse model of osteosarcoma (198). In breast cancer, the other PPI lansoprazole enhanced the therapeutic effect of doxorubicin by both improving its distribution and its cytotoxic activity (212).

2.4. Autophagy and resistance to anticancer treatments

Autophagy is not specific to tumours, but is definitely an important metabolic pathway that allows cancer cells to degrade their own compartments and components, such as proteins, nucleotides or lipids (in that case called lipophagy), used as energetic substrates to fulfil high anabolic demands required to sustain the enhanced cell proliferation rate and survival under stressful conditions (ischemic episodes, exposure to anticancer treatments) (213) (214-217). Recent studies also suggest that autophagy may be a key inducer of EMT in cancer cells (218,219), further delineating the relationship between metabolism and the acquisition of aggressive parameters, and a potential role in resistance to anticancer treaments. The non-

selective form of autophagy is called macroautophagy (later called autophagy), whereas mitophagy, lipophagy or pexophagy are related to the specific recycling of mitochondria, lipid droplets and peroxisomes, respectively. The chaperone-mediated autophagy is an additional form of autophagy which lacks the formation of an isolation membrane and directly addresses cytosolic proteins containing a consensus pentapeptide for their degradation in lysosomes (220). During the autophagic process, the autophagosome, a double membrane vesicle, is formed and allows for the isolation of non-specific or specific cellular materials to be degraded. In this process, the ATG proteins, encoded by the autophagy-related genes (ATG), are responsible for the conjugation of phosphatidylethanolamine (PE) to LC3 (microtubule-associated protein 1 light chain 3), thus resulting in its incorporation into autoeagosome membranes. This is a critical step in autophagosome elongation and maturation, and as such, the presence of LC3–PE, also referred as LC3-II, is often used as a marker of autophagy. The autophagosome then fuses with the lysosomal compartment that provides enzymes. The internal acidification of lysosomes by the V-ATPase is essential for the degradation activity of these proteolytic enzymes. Autophagy can be initiated during nutrients starvation, when the intracellular ATP/ADP ratio becomes low, leading to the activation of the AMPK and the inhibition of the mTOR signalling pathway (221,222), to allow the recycling of amino acids and free fatty acids. This defines the metabolic function of autophagy sustaining cell viability when energetic supplies are reduced, such as in the ischemic regions of tumours. Moreover, autophagy can also assume a protective role against stress conditions, such as those that generate ROS. This could be the case under some anticancer therapies, such as radiation therapy or the exposure to chemotherapeutic agents generating oxidative stress, such as anthracyclines (doxorubicin, epirubicin, daunorubicin), alkylating agents, platinum coordination complexes (cisplatin, carboplatin, oxaliplatin), epipodophyllotoxins (etoposide,

teniposide), and the camptothecins (topotecan, irinotecan) (Figure 2). In those cases, autophagy allows for the degradation of dysfunctional organelles and aggregated proteins. Besides these roles of autophagy in response to stressful conditions, the maintenance of a normal autophagic flux is also needed for the physiological function of multiple organs in the whole body, as it is the case in brain, muscle and liver (217,223).

The role of autophagy in tumours is complex and it is now admitted that it could have a dual role in tumour progression (224,225). Studies, in which autophagy genes were knocked-out in mice, evidenced the protective role of autophagy against tumour initiation mainly by inducing oxidative stress (226,227). However, in advanced tumours stages, it has been demonstrated that cancer cells rely on autophagy for their survival, which is favourable for tumour growth and progression (224,228,229). Besides, the stressful conditions of the tumour microenvironment (hypoxia, low nutrients availability, presence of ROS) are known to activate autophagy. In a recent study, we have shown that lactate, which is elevated in tumours because of the elevated glycolytic metabolism, is associated with autophagy (230). In this study, we demonstrate that autophagy is not only activated by nutrient deprivation, but also by lactate. In cancer cells, lactate oxidation by the lactate dehydrogenase B (LDHB) participates to lysosomal acidification to promote autophagy, which is favourable for cancer cell survival and tumour growth. LDHB was found to be in close proximity with the V-ATPase, expressed at the lysosome. It is therefore proposed that the H^+ released by the LDHB, during the conversion of lactate to pyruvate, are transferred to the lysosomal compartment through the activity of the V-ATPase (230).

The ways by which autophagy is regulated by nutrients availability and by the acidic pHe in the tumour microenvironment are still poorly understood. Besides its role in lysosomal acidification, the V-ATPase could have an important role in the regulation of autophagy and

subsequent cell growth. Recent evidence shows that pHi and lysosomal V-ATPase link glucose metabolism with cell growth. In yeast, the V-ATPase has been considered as being a sensor of the cytosolic pH, in response to glucose availability (231,232). In aerobic conditions, a correlation was found between the concentration of extracellular glucose and a high pHi, and this promoted the assembly and the activation of V-ATPase. V-ATPase interacted with the GTPase Arf1 and Gtr1, leading to the activation of TORC1 and Ras to promote cell growth (233). Similarly, the "sensing" of amino acid availability was proposed to be dependent on the V-ATPase and to the Rag GTPase (234,235), but seemed independent on cytosolic pH (233). It has been proposed that this mechanism for pHi sensing of glucose metabolism could be conserved in mammals (234) since V-ATPase also interacts with Rag GTPase (mammalian homologue for Gtr1 and Gtr2 in yeast), which promotes mTORC1 activity (235,236). Although the direct involvement of cytosolic pH in the regulation of autophagy is still lacking in mammals, it has been demonstrated that acidic pHe, leading to a decrease in pHi, inhibits mTORC1, a master negative regulator of autophagy (237). While the mechanistic link is still elusive, one could speculate that the deregulated pH gradient found in cancer cells could regulate cancer cell proliferation through the inhibition of mTORC1 and autophagy.

Autophagy has been described as a process activated during cancer treatment by both radiotherapy and chemotherapy, and was identified as being responsible for tumour resistance (238). The existence of an acidic pHe has also been linked with resistance to treatment through the participation of autophagy. In human melanoma and breast cancer cells, both acute and chronic acidosis activate autophagy, probably through PI3K/Akt/mTOR inhibition (239,240). In this context, the activation of autophagy is an adaptative mechanism to acidosis and promotes cancer cell survival. In addition, autophagic markers have been detected in the

outer region of tumours corresponding to hypoxic regions that are probably acidic (240). One contradictory study exists where acidic pH blocks autophagy in non-cancer breast epithelial cells (MCF-10A) and less aggressive breast cancer cells (MCF-7) as compared to MDA-MB-231) (241), suggesting a more complex regulation of autophagy by pHe.

These studies highlighting the influence of the extracellular microenvironment on the regulation of cancer cell survival and autophagy led to the proposition that controlling pHe of tumours might inhibit autophagy and increase anticancer treatment efficacy. In this direction, it has been shown that the use of bicarbonate, as a systemic buffer, decreased the expression of the autophagic marker LC3 in breast tumours (240). Another strategy used to inhibit autophagy through pH modulation was to disrupt lysosomal pH, by repurposing the weak base chloroquine, approved by the Food and Drug Administration (USA) as an antimalarial drug, and its derivatives. In preclinical models, chloroquine effectively reduced autophagy and improved anticancer therapy using alkylating drugs (242). This permitted chloroquine and hydroxychloroquine to be considered for clinical trials for anticancer treatments. The first results of these trials documented safety and pharmacokinetic data of their use, and were very promising (243). However, these weak base compounds present the disadvantage of having a limited diffusion in the acidic extracellular microenvironment of tumours. For this reason, the efficacy of chloroquine to block autophagy was reduced at acidic pH *in vitro* and in the hypoxic region of tumours *in vivo* (244). To counteract these limitations, the autophagy inhibitor salinomycin was proposed to be a possible substitute to chloroquine (245). However, the effects of salinomycin on autophagy seemed to be very complex, and were opposite depending on the dose used (246). Furthermore, these drugs are not specific and act as global pH modulators. As such, the development of specific inhibitors of lysosomal acidification is needed.

In the context of cancer treatment, while the overexpression of autophagy markers in cancer cells and tumours is commonly accepted, the biological consequences of autophagy remain controversial. Several studies have shown that radiation therapy could induce autophagy in cancer cells (247-249), which was supposedly due to a decreased phosphorylation of mTOR (250) and to the involvement of the integrin-associated protein CD47 (251). However, conflicting results were initially obtained, and it is not clear whether autophagy induction in this context is leading to cancer cell death, or their survival. Two different and opposing hypotheses were formulated. The first one was to consider autophagy as a cell death mechanism ("autophagic cell death") induced by radiations. In this hypothesis, the induction of autophagy would be beneficial to kill cancer cells, and it should be enhanced. However, the term "autophagic cell death" has been considered as being a misnomer, and to be in fact cell death associated with markers of autophagy, rather than being a specific type of cell death (252,253). The second hypothesis, which recently gained more insights, is to consider autophagy as a survival mechanism involved in cancer cell resistance to radiotherapy, and in that case, its inhibition would be beneficial to cancer treatment. This hypothesis is supported by the demonstration that, in cells deficient for autophagy, radiations induced the accumulation of p62, which inhibited the recruitment of DNA-repairing factors such as UIMC1/RAP80, BRCA1 and RAD51 to double strand break sites, leading to impaired DSB repair and decreased cell survival (254). Clinical trials with hydroxychloroquine and radiation therapy gave promising results (255). Few studies investigate the role of acidic pHe involvement for radiation therapy but it has been evidenced that at acidic pH, radiations induced less apoptosis (256). It has also been proposed to use PPI to overcome the autophagy induced by chemotherapies. This idea has brought some hopeful results with omeprazole, which was effective in re-establishing chemosensitivity of resistant melanoma cells to

cisplatine (257), and with pantoprazole, also enhancing the efficacy of docetaxel on the growth of human tumour xenografts by inhibiting autophagy (258). At this point, it is probably worth mentioning that the apparent contradictions on the biological significance of autophagy induction during anticancer treatment might also differ as a function of the level of induction and its persistence in time. Furthermore, autophagy is also important for anticancer or permissive immune response. It has been proposed that activating autophagy could improve an anticancer immune response and radiation therapy (259).

3. The perspectives of using dietary lipids for an integrated strategy in the treatment of cancers

The molecular mechanisms of the cancer hallmarks mentioned above are/can be targeted by specific drugs interfering with deregulated pH in cancer cells, such as the use of pH buffers (210), or drugs inhibiting H^+ transporters (260-262) or their key regulators (174), alone or in combination with conventional chemotherapeutics. This strategy seems to be very promising, at least at the basic research level and in pre-clinical models, but needs to be further studied in clinical trials. Another tempting strategy would be to identify molecules that have the capacity to target more than one of these mechanisms without displaying adverse side effect. Dietary lipids might be of interest to do so. Indeed, cancers are diseases for which the development and progression are also under the dependence of environmental factors that present the benefit of being modifiable. This is the case of nutritional factors. Some dietary compounds have been recognized to influence both the apparition and the survival to different carcinoma.

The adipose tissue plays an essential function in the storage of lipids, such as fatty acids and sterols, and it is a qualitative biomarker of past dietary fatty acids intake. Being in close

proximity to multiple epithelial tissues, such as the adipose tissue in the mammary gland, the periprostatic adipose tissue, or the mesenteric adipose tissue close to the colorectal tracts, the adipose tissue has been shown to importantly control primary tumour progression and may influence clinical markers of tumour aggressiveness (Figure 3).

Lipids, such as polyunsaturated fatty acids (PUFA, such as n-6 or n-3 PUFA) and sterols have been proposed to regulate, either positively or negatively, tumour development and disease progression by modulating cellular properties in both cancer and non-cancer cells of the tumour microenvironment. Studies performed on breast cancer have already demonstrated the importance of adipose tissue and epithelial tissue fatty acid composition, from triacylglycerids and phospholipids, respectively, on breast tumour development. Among dietary lipids, long chain n-3 PUFA, from marine fatty fishes, have been identified from epidemiological studies for their potential to prevent the appearance of breast cancer (263,264). From *in vivo* and *in vitro* studies, n-3 PUFA have been reported to have multiple anti-tumour effects and their dietary consumption was associated with a lower risk of cancers, such as breast or colorectal cancers (264-266). Also, they have been shown to reduce chronic inflammation associated with these cancer types (263,264,267). Even though n-3 PUFA were suggested to be prostate cancer suppressor (268), their beneficial effect is not that clear and more intervention trials or observational studies are still required (269).

-Primary tumour growth and resistance to treatment-

Following up on previous work on the lipidome, that associated the risk of breast cancer to the lipid composition (270,271), a recent pilot study showed that fatty acid composition of breast adipose tissue differed according to breast cancer focality. Low levels of DHA and EPA in breast adipose tissue were associated with tumour multifocality, a marker

of cancer aggressiveness (272). These results could indicate that differences in lipid content may contribute to mechanisms through which peritumoral adipose tissue drives breast cancer aggressiveness.

Developing approaches that can improve the selectivity and efficacy of anticancer drugs, while limiting their side effects, remains a challenge. Long-chain n-3 PUFA have been attributed the potential to increase tumour sensitivity to chemotherapy with no sensitization of normal tissues (264). DHA and EPA have generated intense interest due to their ability to reduce resistance to anthracyclines, taxanes or radiotherapy, without additional side effects, in mammary tumour models. The efficacy of numerous anticancer drugs on breast cancer cell lines and rodent mammary tumours may be enhanced by treatment with DHA.

In the case of breast cancer, n-3 PUFAs supplementations were proposed to have beneficial effects in reducing primary tumour growth (Figure 3). This was attributed to their potency to inhibit cancer cell proliferation (273). Docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3) decreased the proliferation of human MDA-MB-231 breast cancer cells through the regulation of the cell cycle. Both DHA and EPA increased the duration of the G2/M phase, while there was no alteration of G1 or S phases. These two fatty acids decreased the expression of cyclin A, cyclin B1 and cyclin-dependent kinase 1. Upon n-3 PUFA treatment, cyclin B1 phosphorylation was inhibited and the expression of the cell division cycle 25C phosphatase, which dephosphorylates cyclin-dependent kinase 1, was decreased (273).

Different molecular mechanisms such as the amplification of oxidative stress generated by anthracyclines or radiotherapy and increased accumulation of anticancer agents have been proposed to account for the effects of these highly peroxidable fatty acids. In a phase II clinical trial performed with breast cancer patients with severe metastatic disease, a diet

enriched with DHA (1.8 g daily) increased time to progression from 3.5 months for patients not incorporating DHA, to 8.7 months for patients incorporating DHA (Bougnoux et al, 2009). In parallel to this type of nutritional intervention in breast cancer patients under chemotherapy, original modes of action by which DHA can sensitize mammary tumours to anticancer agents have been uncovered in a rat model of N-nitroso-N-methylurea (NMU)-induced mammary tumours. In this breast cancer model, DHA supplementation enhanced DHA tumour content in a time- and dose-dependent manner (274-276). DHA is a natural ligand for the nuclear peroxisome proliferator-activated receptors (PPAR), and in the (NMU)-induced mammary tumour model, a reduced expression level of PPARβ mRNA correlated with regression of mammary tumours. Tumours that most regressed displayed the most reduced PPARβ mRNA expression. In addition, PPARβ regulated DHA-induced growth inhibition of MDA-MB-231 and MCF-7 cells, identifying PPARβ as an important player for the inhibition of breast cancer cell growth and mammary tumour growth with a DHA diet (277).

As already mentioned, an important limitation of cancer treatment is the acquisition by the tumour of a resistance to chemotherapeutic agents. Resistance to taxanes can occur by the induction of signalling pathways such as PI3K/Akt and ERK1/2, which promote survival and cell growth in human cancer cells. In docetaxel-treated MDA-MB-231 cells, phosphorylated-ERK1/2 levels were increased by 60% in both membrane and nuclear compartments, compared to untreated cells and ERK1/2 activation depended on PKCε and PKCδ activation. In DHA-supplemented cells, docetaxel was unable to increase PKCε and PKCδ levels in membrane and nuclear fractions, resulting in both diminished ERK1/2 phosphorylation and increased docetaxel efficacy. Reduced membrane levels of PKCε and PKCδ were associated with significant incorporation of DHA in all phospholipid classes, including in

phosphatidylcholine, which is a major source of phosphatidic acid. Additionally, study of the Akt pathway showed that DHA could repress docetaxel-induced Ser473Akt phosphorylation. In rat NMU-induced mammary tumours, dietary DHA supplementation during docetaxel chemotherapy repressed ERK and Akt survival pathways and, in turn, strongly improved taxane efficacy. P-ERK1/2 levels were negatively correlated with tumour regression. These findings could be of potential clinical importance for the treatment of chemotherapy-resistant cancer (278).

In addition to their antitumour activities reported in epithelial cancer cells, it was demonstrated that n-3 PUFA could increase the efficacy of the chemotherapeutic treatment by remodelling the vascular network in mammary tumours, thereby contributing to more efficient drug delivery (279). Functional vascular parameters (vascularization measured by ultrasounds, interstitial fluid pressure IFP) were determined for two nutritional groups, control and n-3 UFA-enriched diet, of female rats bearing NMU-induced mammary tumours. Whereas docetaxel stabilized tumour growth in the control group, it induced a 50% tumour regression in the n-3 PUFA group. Before the first treatment with docetaxel, there was an apparent remodelling of the tumour vasculature with smaller vessels in the n-3 PUFA group. Ultrasounds parameters were consistently lower in the n-3 PUFA group at all time-points measured, down to 50% at the end of the docetaxel treatment. A single dose of docetaxel in the n-3 PUFA group markedly reduced IFP as early as 2 hours after the first injection of docetaxel and this reduction was maintained for one week, at a time when Evans blue extravasation was increased by 3-fold. A decreased activation of endothelial nitric oxide synthase in tumours of the n-3 PUFA group, and *in vitro*, in human endothelial cells cultured with n-3 PUFA, points toward a PUFA-induced disruption of nitric oxide (NO) signalling pathway (279). Using an original contrast-enhanced ultrasound method (280), the vascular

architecture was analysed during docetaxel treatment of mammary tumours in rats fed with control or n-3 PUFA diets. The vascular network was remodelled in favour of smaller vessels (microvascularization) in the n-3 PUFA tumours, and this correlated with an improved response to docetaxel chemotherapy. Analysis of angiogenesis-related gene expression using PCR arrays showed that the expression of epiregulin and amphiregulin were reduced in tumours of the n-3 PUFA group. This was correlated with tumour regression after chemotherapy. *In vitro* studies showed that epiregulin and amphiregulin expression were strongly induced by VEGF in primary endothelial cells. DHA supplementation repressed only epiregulin gene expression and counteracted VEGF activities on proliferation and pseudo-capillaries formation. The normalization of tumour vasculature under n-3 PUFA diet indicated that such a supplementation, by improving drug delivery in mammary tumours, could be a complementary clinical strategy to decrease anticancer drug resistance (281) (Figure 3).

- *Cancer cell metabolism and autophagy*-

While there are several reports that n-3 PUFA could induce apoptosis in cancer cells, mainly through the formation of ROS, Ca^{2+} accumulation and opening of the mitochondrial permeability transition pore (MPTP) (282) or caspase activation (283,284), there are relatively few data concerning the potential regulation of cancer cell metabolism. It is however tempting to speculate that these long chain n-3 PUFA could also modulate or normalize cancer cell bioenergetics, acting through a modulation of the aerobic glycolysis as well as on the mitochondrial activity.

In a recent study focusing on colorectal carcinogenesis, male Wistar rats received a weekly intraperitoneal injections of ethylenediamine tetra-acetic acid (EDTA) or N,N-dimethylhydrazine dihydrochloride (DMH) for a period of 4 weeks, responsible for the

initiation and post-initiation phase, respectively (285). These groups were subdivided into dietary groups, a control group, a group receiving fish oil and corn oil in similar proportions (1:1) and an n-3 PUFA-enriched group receiving fish oil and corn oil in 2.5:1 proportions. DMH treatment induced a mitochondrial degeneration, disrupted cristae and a significant decrease in electron transport chain complexes activity, reflecting carcinogenetic-associated metabolic reprogramming. This was associated with an increase in cholesterol and cardiolipin levels in post-initiation phase, suggested to provoke a loss of apoptotic activity. Interestingly, fish oil diet in both the ratios stabilized or increased the number of mitochondria. The diet the more enriched in fish oil induced mitophagy, along with modulation of the electron transport chain complexes activity, decreased cholesterol and cardiolipin (a specific phospholipid of mitochondrial membranes containing four acyl chains) levels, thus facilitating apoptosis and attenuating carcinogenesis (285).

In PC3 prostate cancer cells, treating the cells with fish oil, containing 90% ethyl ester n-3 fatty acid among which 40% DHA, 40% EPA, and 10% other n-3 PUFA, induced important phosphoproteomic changes as compared to a control group treated with oleic acid. Particularly relevant are the phosphorylation levels of the two regulatory serine residues in pyruvate dehydrogenase alpha 1 (PDHA1), serine-232 and serine-300, which were significantly decreased upon fish oil treatment, resulting in the increase of pyruvate dehydrogenase (PDH) activity. Results brought by this study supported the protective role of n-3 PUFA in prostate cancer through the suppression of aerobic glycolysis by restoring PDH activity, and in controlling the balance between lipid and glucose oxidation (286).

Metabolic investigation were also performed in breast cancer cells showing respiratory (BT-474 cell line) or glycolytic (MDA-MB-231 cell line) phenotypes, compared to non-cancerMCF-10 mammary cells, and revealed that DHA supplementation significantly

diminished the cancer cell metabolism and glycolytic profile of malignant cell lines in a dose-dependent manner (287). DHA enrichment decreased HIF-1α levels and transcriptional activity in the malignant cell lines but not in the non-transformed cell line. Consequently, downstream targets of HIF-1α, such as GLUT-1 and LDH, were decreased by DHA treatment in the BT-474 cell line, as well as in the MDA-MB-231 cell line. Correlatively, DHA supplementation decreased glucose uptake and oxidation, glycolytic metabolism, and lactate production. DHA induced a decrease of intracellular ATP in both cancer cell lines. This resulted in the phosphorylation of AMP-activated protein kinase (AMPK, at Thr172) as a metabolic stress marker. These important results indicated that DHA could alter cancer cell growth and survival by modulating cancer cell metabolism, while not affecting the one of non-cancer cells (287). By specifically interfering with cancer cell metabolism, the supplementation with n-3 PUFA (such as DHA) could be considered as an opportunity to inhibit cancer cell survival and tumour progression (288).

Recent data have shown that n-3 PUFA could regulate autophagy, which could be involved in both survival and apoptosis of cancer cells, depending on the carcinogenetic phase and on the treatment context. In a study conducted in cervical, breast and lung cancer cells, DHA was found to induce apoptosis and to increase the number of autophagic vacuoles without impairing autophagic vesicle turnover. The induction of autophagy was mediated by p53. DHA-induced autophagy was associated with p53 loss, and to the activation of AMPK and the decrease in the activity of mTOR. Autophagy inhibition suppressed apoptosis, and further autophagy induction enhanced apoptosis in response to DHA treatment (289).

Another study performed in the context of colon carcinogenesis studied the effects of DHA on cancer cell differentiation or programmed cell death involving mitochondrial pathway

induced by sodium butyrate. Sodium butyrate induced autophagy both in HT-29 and HCT-116 cells, which are sensitive and insensitive to the induction of differentiation, respectively. However, autophagy supported cell survival only in HT-29 cells. DHA promoted cell death and activated PPARγ in both cell types. The inhibition of autophagy both attenuated differentiation and enhanced apoptosis in HT-29 cells treated with sodium butyrate and DHA, but was ineffective in HCT-116 cells. PPARγ silencing decreased differentiation and increased apoptosis only in HT-29 cells. Thus, authors suggested that diverse responses of colon cancer cells to fatty acids might rely on their level of differentiation, which may in turn depend on distinct engagement of autophagy (290).

DHA treatment induced oxidative stress in SW620 and Caco-2 colon cancer cells. DHA inhibited the growth of SW620 cells and induced transcriptional regulation of genes involved in oxidative stress response and autophagy. However, the oxidative stress response was not the cause of DHA-induced cytotoxicity in SW620 cells. Inhibition of autophagy sensitized both SW620 and Caco-2 cells to DHA. Autophagy stimulation resulted in decreased DHA-sensitivity of SW620 cells that have a low basal level of autophagy, while inhibition of autophagy in Caco-2 cells, displaying a higher level of basal autophagy, resulted in increased DHA-sensitivity. Taken together, these results indicated that autophagy was important for the DHA sensitivity of colon cancer cells (291).

-EMT and Invasive properties –

As already mentioned, EMT is a key step in the acquisition of aggressiveness in cancer cells, through increased invasive properties and resistance to treatments. To date, there is no potent pharmacological strategy to prevent EMT. Again, there is evidence that n-3 PUFA may

inhibit the expression of EMT markers and reduce associated invasive properties in cancer cells (Figure 3).

A recent study indicated that the supplementation of colorectal cancer cells with DHA, inhibited the expression of the serine protease Granzyme B, characterized as being an EMT inducer, and therefore inhibited the expression of Snail1 and N-cadherin while overexpressing E-cadherin. DHA treatment also resulted in a reduction of colorectal cancer cell invasiveness (292). These results supported the use of DHA, a dietary compound without toxic effects, as an adjuvant in colorectal cancer therapy.

Similarly, it was found that increasing the endogenous levels of n-3 PUFA, using n-3 fatty acid desaturase (fat-1) transgenic mice, was associated with a reduction in the growth rate of melanoma xenografts. This reduction in melanoma growth in fat-1 mice, compared with wild-type mice, was associated with an increased expression of E-cadherin and the reduced expression of its transcriptional repressors, Zeb-1 and Snail-1 (293). It also significantly repressed the EGFR/Akt/β-catenin signalling pathway and induced the formation of significant levels of anti-inflammatory n-3 PUFA-derived lipid mediators, such as resolvin D2 and E1, maresin 1 and 15-hydroxyeicosapentaenoic acid (293).

While it is commonly accepted, since a long time now, that n-3 PUFA have anti-invasive and anti-metastatic properties (264,294-297), molecular details for these effects are not fully described. One possible explanation is the repression of EMT, but again, the clear mechanism for this remains to be elucidated. Importantly, n-3 PUFA, through the activation of PPARγ, have been shown to down-regulate the expression of NHE1 and to reduce cancer colony growth (298). This inhibition of NHE1 expression could also be responsible for the anti-invasive activity of these fatty acids. What is known is that these fatty acids can, in their

native non-peroxidated or peroxidated forms, modulate the activity of NHE exchangers (299,300), and of ion channels (301,302) that have been demonstrated to be key regulators of cancer cell invasiveness (295). An important mode of regulation by n-3 PUFA is the change of physico-chemical properties of cell membranes after their incorporation into phospholipids, notably in lipid raft domains (303). Indeed, DHA and EPA have been shown to have protective effects by affecting NHE1 activity trough a non-genotoxic pathway associated with plasma membrane remodelling (304).

This is the case for the $Na_V1.5$ channel, importantly regulating the invasive properties of breast and colorectal cancer cells (170,305,306), which activity was initially found to be inhibited in expression systems or in native rat cardiomyocytes (307,308). As such, n-3 PUFAs have been proposed to exert their beneficial effect on cancers through a reduction of the activity of $Na_V1.5$, such as it would have beneficial cardiac anti-arrhythmic effects (295,309). In initial studies performed in Alexander Leaf's group, n-3 PUFA were found to directly bound to the channel and to inhibit its activity (310,311). However, contrasting results were obtained in human breast cancer cells in which $Na_V1.5$ channel, which is expressed under the form of a neonatal splice variant (167), was not inhibited by acute application of n-3 PUFA, even at high concentrations (30-50 µM) (312). In fact, growing breast cancer cells in the presence of low doses of DHA (0.5 to 10 µM) reduced *SCN5A* gene expression and levels of $Na_V1.5$ proteins and related sodium current (312,313). The inhibition of *SCN5A* expression was mediated by the lipid-sensitive nuclear receptor PPARβ. This inhibition of $Na_V1.5$ activity was responsible for a reduced activity of NHE1, decreasing H^+ efflux, resulting in a limited extracellular acidification, a reduced proteolytic activity and the inhibition of breast cancer cell mesenchymal invasion (312). In this context, one could postulate that DHA would also limit cell migration and resistance to treatment that are

properties related to pH regulation, in all cancer cells showing functional $Na_V1.5$-NHE1 complexes.

Altogether, these studies provide mechanistic, pre-clinical and clinical information that dietary supplementations of n-3 PUFA might represent new powerful opportunities to normalise cancer cell metabolism, limit the aggressive progression of some cancer types and prevent the resistance to anticancer treatments (Figure 3).

4. Conclusions

Conflict of interests

Authors declare no conflict of interest.

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Figure legends:

Figure 1: The gain of invasive capacities in carcinoma cells

The acquisition of an invasive phenotype in carcinoma cells is associated with the epithelial-to-mesenchymal transition (EMT), a process characterized by the loss of functional apico-basal polarity, the loss of cell-to-cell adhesions (loss of ZO-1 and E-cadherins), the remodelling of the actin cytoskeleton (with the loss of the submembrane cortical F-actin network to the profit of actin stress fibers), the secretion of extracellular matrix (ECM)-degrading proteases (such as matrix metalloproteinases (MMP) and cathepsins). Invasive carcinoma cells that underwent the EMT display a rear-leading-edge polarity, representative of mesenchymal cells, and develop invasive structures, called invadopodia, where is performed the proteolysis of the ECM. In this new cell phenotype, there is redistribution of existing ion channels and transporters, such as the Na^+-H^+ exchanger type 1 (NHE1) expressed in baso-lateral side of normal epithelial cells that is translocated to the leading edge and to invadopodia of invasive carcinoma cells. There is also the over-expression of some ion channels such as voltage-gated gated sodium channels (Na_V) in invadopodia. Na_V activity promotes invadopodial formation and ECM proteolytic activity through the Src-dependent phosphorylation of the actin-nucleating promoting factor cortactin (at Y421), resulting in actin polymerisation, and the increase of H^+ efflux through NHE1, the acidification of the extracellular microenvironment, resulting in protease activation.

Figure 2: Cancer cell metabolism and the increased production of protons promote tumour progression

Under hypoxia, cancer cells are selected on their capacity to produce ATP through the glycolysis. They generally keep this preferred metabolism even in normoxic conditions, a situation called "aerobic glycolysis" (or Warburg effect). This is associated with the increase in glucose transporter 1 (GLUT1) and glycolytic enzymes expression, that is mediated through the stabilization of the hypoxia-sensitive transcription factor HIF1α, and the increase in the production of H^+. HIF1α also promotes the epithelial-to-mesenchymal transition (MET) through the expression of EMT transcription factors (Zeb1/2, Snail 1/2, Twist, …), leading to the down-regulation of epithelial markers (such as Zonula Occludens 1, ZO-1, and epithelial cadherin, E-Cadh) and the up-regulation of mesenchymal markers (such as vimentin and the neuronal cadherin, N-Cadh). High amounts of CO_2-derived protons are generated by the mitochondrial activity (tricarboxylic acid (TCA) cycle) and the pentose phosphate pathway. The efflux of H^+, mediated by several plasma membrane transporters (such as NHE1, V-ATPase, MCT…), permits cancer cell survival and proliferation, favours the acquisition of migrative and invasive properties and inhibits anti-cancer immune response. Voltage-gated sodium channels ($Na_V1.5$ and $Na_V1.7$) increase the activity of NHE1 and promote cancer cell invasiveness through the proteolytic degradation of the ECM. Protons that are intracellularly produced are also transported to autophagolysosomes to support autophagy and cancer cell survival under nutrient deprivation or anticancer treatment. Extracellular acidification reduces chemotherapy efficacy by limiting the diffusion of some chemotherapeutic agents (such as anthracyclines) and by overactivating the activity of the plasma membrane efflux pump P-glycoprotein (P-gp). Chemotherapy efficacy is also limited by the expression of P-gp at mitochondrial membranes and by the mitochondrial bioenergetics activity (oxidative phosphorylation, OXPHOS).

Figure 3: Proposed anti-tumour effects of dietary n-3 polyunsaturated fatty acids (n-3 PUFA)

Dietary n-3 PUFA can, directly or after their being stored under the form of triacylglycerids in adipose tissues, interfere with the carcinogenesis of epithelial tissues and tumour progression. They are proposed to inhibit tumour progression through the inhibition of tumour inflammation, the inhibition of primary tumour growth, the reduction of local invasion, the prevention of metastases development or relapse, and to improve anticancer treatment by limiting chemoresistance.