# Tumour Phospholipid Metabolism

Franca Podo[1], Silvana Canevari[2], Rossella Canese[1],
Maria Elena Pisanu[1], Alessandro Ricci[1] and Egidio Iorio[1]

[1]*Department of Cell Biology and Neurosciences, Istituto Superiore di Sanità, Roma;*
[2]*Department of Experimental Oncology and Molecular Medicine,*
*Fondazione IRCCS Istituto Nazionale dei Tumori, Milano, Italy.*
*E-mail: franca.podo@iss.it*

## Summary
Abnormalities in phospholipid metabolism represent major hallmarks of cancer cells. Changes in the MRS profiles of aqueous precursors and catabolites of phosphatidylcholine (PtdCho) in cancer lesions allow non invasive monitoring of tumor progression and response to conventional and targeted anti-cancer therapies. Advances and limitations of our present understanding of molecular mechanisms underlying these anomalous metabolic profiles will be here discussed in the light of altered expression and activity of enzymes of the PtdCho cycle and links to dysregulated cell signaling pathways responsible for oncogenesis. An overview will also be provided of a) the role of choline metabolites as possible pharmacodynamic biomarkers of targeted therapies and b) current efforts to identify PtdCho cycle enzymes as possible targets for therapy.

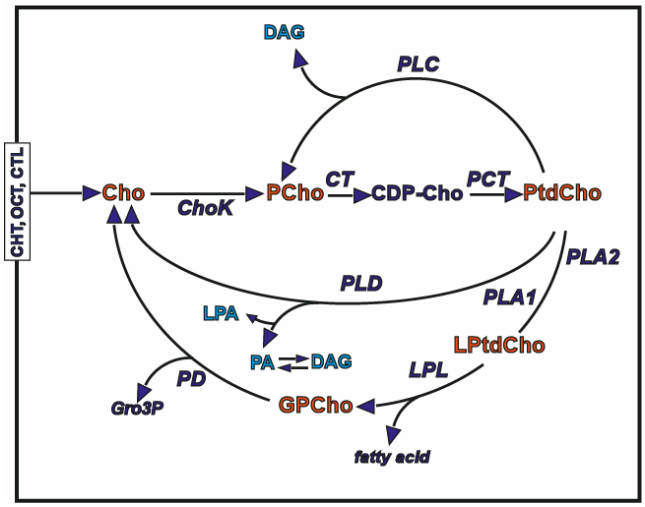
## MRS profiles of phospholipid metabolites in cancer cells
Initial detection in the 90's and characterization of altered MRS profiles of phospholipid metabolites in cancer cells and tissues [1-3] fostered new research areas in cancer cell biology and allowed the identification of novel indicators of in vivo tumor progression at the pre-clinical and clinical level [4-10].

An increase in the $^{1}$H MRS resonance band at 3.2 ppm, mainly due to the trimethylammonium headgroups of choline-containing metabolites (collectively called "total choline" or "tCho peak") is a common feature of a large variety of cancers. Changes in the MRS tCho spectral profile reflect altered contents and metabolic fluxes of phosphocholine (PCho), glycerophosphocholine (GPCho) and free choline (Cho) through the biosynthetic and catabolic pathways of the PtdCho cycle [11]. Alterations in the levels of PCho and GPCho can also be detected in the $^{31}$P MRS frequency ranges respectively typical of phospho-monoester (PME) and phosphodiester (PDE) compounds, together with those of phosphatidylethanolamine (PtdEtn) derivatives, phosphoethanolamine (PEtn) and glycerophosphoethanolamine (GPEtn) [2].

Phospholipids play the dual role of basic structural components of membranes and substrates of reactions involved in key regulatory functions in mammalian cells [12]. Physiologically regulated catabolism of PtdCho, the most abundant phospholipid in eukaryotic cell membranes, generates second messengers and mitogens such as diacylglycerol (DAG), phosphatidic acid (PA), lysophosphatidic acid (LPA), lysophosphatidylcholine (LPtdCho) and arachidonic acid, through three major pathways respectively mediated by phospholipases of the C (PLC), D (PLD) and A (PLA2 and PLA1) type (Scheme below). PCho has also been proposed to be mitogenic [13] by acting as a mediator of growth factor-induced cell proliferation.

A number of links exist between the PtdCho cycle and cell receptor-activated signal transduction pathways [14,15], with implications on the biogenesis and utilization of other lipids and phospholipids [12], under the control of Ras-GTP-stimulated phosphorylation cascades mediated by Raf-1, Ral-GDS and phosphoinositide 3-kinase (PI3K). The interpretation of tCho spectral profiles in terms of individual enzymatic reactions and cell signaling pathways is however limited by the dual role of each tCho component as both substrate and product in PtdCho cycle pathways: in fact, Cho enters the Kennedy pathway by transport from the external medium and is produced in the cell either by PLD-catalyzed PtdCho hydrolysis or phosphodiesterase (PD)-mediated GPCho degradation; PCho, either synthetized by Cho phosphorylation or produced by PLC-mediated PtdCho hydrolysis, is substrate for the second reaction step of the Kennedy pathway, catalyzed by the key-regulatory enzyme cytidylyltransferase (CT); and, finally, GPCho produced at the end of the PtdCho deacylation pathway, is also substrate for PD-mediated hydrolysis into Cho and glycerol 3-phosphate (Gro3P).

**Phosphatidylcholine Cycle**




Abnormal levels of water-soluble choline metabolites are detected by in vitro and in vivo $^{1}$H and $^{31}$P MRS in a large variety of cancer cells, including breast, prostate, colon, ovary carcinomas and neuroepithelial tumors. Examples of absolute metabolites' quantification [16-19] are reported in Table 1. Alterations in intracellular contents of PCho and tCho in cancer cells during tumor progression or in response to therapy, may reflect a multiplicity of modifications taking place at the genetic, epigenetic transcriptional, and post-transcriptional levels.

Enhanced levels of PtdEtn derivatives (PEtn and GPEtn) can also be detected in tumors but their values may widely vary in cells according to the concentration of ethanolamine in the extracellular medium. For instance, higher PEtn levels detected in chronic lymphocytic leukaemia cells *versus* normal lymphocytes were associated with a decreased level of ethanolamine in the blood of patients compared with healthy volunteers [20].

Changes in the contents of choline derivatives can be induced by genetic modifications characteristic of cancer cells. Examples are the reported 10-fold increase in PCho in mammary epithelial cells transfected with the human epidermal growth factor receptor 2 (HER2) [16]; the 3-fold increase in PCho in *ras*-transformed NIH 3T3 fibroblasts and its drop following inhibition of the Ras signaling pathway [21]; and the over 2-fold increase in PCho measured in a $p53^{-/-}$ cell variant of a colon cancer cell line [22]. Conversely, a significant decrease in PCho and increase in GPCho were detected in highly metastatic breast carcinoma cells by stable transfection with the metastatic suppressor gene nm23 [23].

The elevated levels of PCho and tCho detected in cancer cells are currently interpreted as fingerprints of tumour progression.

## Molecular mechanisms responsible for altered MRS choline profiles in cancer cells

An increase in PCho, the major tCho component, may derive from a) enhanced Cho transport and choline kinase (ChoK) activation; b) increased PtdCho-PLC activity; c) increased intracellular Cho production by PtdCho deacylation combined with GPCho-PD activity or by PLD-mediated PtdCho catabolism, followed by choline phosphorylation.

***Choline transport*** - Upregulation of the mRNA expression levels of the organic cation transporter-2 (OCT2) and Cho high affinity transporter-1 (CHT1), has been reported to correlate with the intracellular PCho content of breast cancer cells [24]. Similar mRNA expression levels were instead detected for the Cho transporter-like protein 1 (CTL1) and OCT1 in HMEC and the cancer cell lines. No differential mRNA expression levels were instead measured in epithelial ovarian cancer cells (EOC) and in their normal counterparts (ovarian surface epithelial cells, OSE) concerning either CHT1 or the organic cation transporters OCT1 and OCT2, while OCT3, the most highly expressed transporter gene, was substantially downregulated in the cancer cells [19]. Among Cho transporter-like proteins (CTL1-CTL5), only CTL3 showed an increased mRNA expression in EOC cells. Since Cho might also be transported by a $Cho/H^{+}$

antiport system, the overall mRNA expression was measured for $Na^+/H^+$ exchangers (NHE1-NHE5), but no differences were found between EOC and OSE cells.

Overall, these results showed that Cho transport mechanisms may differently affect the size of the PCho pool in breast and ovarian cancer cells, with possible implications for a different response of these cells to inhibitors of choline transport.

**Table 1**. **Quantification of PCho, GPCho and tCho in cancer cells and their non tumoral counterparts.**

| TISSUE OF ORIGIN | PCho[a] (mM) | GPCho[a] (mM) | GPCho/PCho[a] | tCho[a] (mM) | References |
|---|---|---|---|---|---|
| **BREAST** | | | | | |
| **Cancer cells** | 0.5 - 3.2 | 0.05 - 0.7 | 0.1 - 0.2 | 0.5 - 4.7 | Aboagye et al. Cancer Res 1999 [16] |
| **HMEC**[b] | 0 - 0.1 | 0 - 0.2 | 0.2 - 2.7 | 0.05 - 0.3 | |
| **PROSTATE** | | | | | |
| **Cancer cells** | | | | | Ackerstaff et al. Cancer Res 2001 [17] |
| Primary tumor | 3.1 | 1.2 | 0.4 | 4.5 | |
| Metastases | 0.5 - 2.4 | 0.2 - 2.4 | 0.2 - 1.0 | 1.0 - 5.1 | |
| **Non tumoral cells** | | | | | |
| epithelial cells | 0.1 | 0.02 | 0.2 | 0.2 | |
| stromal cells | 0.4 | 0.1 | 0.2 | 0.5 | |
| **OVARY** | | | | | |
| **Cancer cells** | 4.0 - 7.0 | 0.5 - 1.0 | 0.1 - 0.2 | 5.2 - 8.5 | Iorio et al. Cancer Res 2005 [18]; Cancer Res 2010 [19] |
| **Non tumoral cells** | 1.0 - 1.2 | 0.6 - 1.0 | 0.6 - 0.9 | 2.0 - 2.5 | |

[a] Mean value or range of mean values; [b] HMEC, normal or immortalized non tumoral human mammary epithelial cells; tCho = [PCho + GPCho + free choline].

***ChoK activity*** **-** ChoK-mediated phosphorylation of Cho into PCho is the first, highly efficient reaction taking place in the Kennedy pathway of *de novo* PtdCho biosynthesis. ChoK exists in mammalian cells as at least three isoforms ChoKα1, ChoKα2 and ChoKβ, encoded by two genes [14]. The first two isoforms derive from alternative splicing of the same ChoKα gene (*ckα*), while ChoKβ is the product of a separate gene, *ckβ*. ChoK activity depends upon the relative percentages of ChoKα and ChoKβ homo- and heterodimers.

ChoK activation, a critical requirement for induction of DNA synthesis by mitogens and growth factors, is implicated in Ras-dependent and independent carcinogenesis [14,15] and tumor progression [25]. Evidence of hypoxia-regulated expression of ChoK through hypoxia inducible factor 1 (HIF-1) signaling has been reported by Glunde and colleagues in a human prostate cancer cell line [26]. Ras-signaling pathways through Ral-GDS and phosphoinositide 3-kinase (PI3K) effectors can enhance the activity of ChoK during tumor formation and, in turn, ChoK activation and generation of PCho have been proposed as essential events in regulating AKT phosphorylation [27] and activation of Raf-1 and mitogen-activated protein kinases (MAPK) [28]. ChoK has also been proposed as a prognostic factor in breast, non-small cell lung cancer and bladder carcinomas [29-31].

Significant 2- to 5-fold ChoK activation has been reported by Eliyahu and coll. in breast cancer cells [24]. Gene expression analyses showed that ChoKβ was the dominant gene in normal mammary epithelial cells, with a ChoKβ/ChoKα expression ratio of about ten. The mRNA expression of ChoKα was 5- to 8-fold higher in breast cancer cell lines than in human mammary epithelial cells (HMEC), while ChoKβ showed a smaller (up to 2-fold) increase only in some investigated cancer cell lines. The higher ChoK activity in cancer cells failed to correlate with the increased PCho level, suggesting that the Chok-mediated reaction is fast and not rate-limiting, although its induction ensures increased PCho levels. Moreover, the activity of CT was found to be several fold lower than that of ChoK in each investigated breast cancer cell line, in agreement with the role of CT as the rate limiting step in PtdCho synthesis and PCho accumulation [24].

A 3 to 4-fold increase in ChoKα (but not ChoKβ) mRNA expression has also been reported in EOC cell lines, compared with non tumoral counterparts [19], in substantial agreement with a 3-fold increased

protein expression. Much higher (12- to 25-fold) increases were found in ChoK activity in EOC cells, suggesting that the activation of this enzyme likely depends upon multiple oncogene-driven and post-translational signaling mechanisms. These results, together with the detected downregulation of enzymes involved in the subsequent steps of the Kennedy pathway, CT (*PCYT1A* and *PCYT1B* genes) and phosphocholine transferase (PCT, *CHPT1* gene) pointed to a major role of ChoK in the build-up of the PCho pool in EOC cells [19]. Since, however, there was a substantial lack of correlation between ChoK activity and intracellular PCho levels, further contributions to the accumulation of this metabolite in EOC cells are expected from activation of phospholipases responsible for PtdCho catabolism.

***PtdCho catabolism*** - The two catabolic pathways responsible for PtdCho hydrolysis at the level of the headgroup phosphodiester bonds can contribute to the intracellular PCho pool in cancer cells, by fueling PLD-produced Cho into the first step of the Kennedy pathway or by directly producing PCho by PLC-catalyzed reaction.

*PtdCho-PLD* - Phospholipase D mediates PtdCho hydrolysis into choline and PA. While the former product re-enters the Kennedy pathway, the latter may act either as pleiotropic second messenger or precursor for the PA phosphohydrolase-mediated production of DAG or LPA, a potent regulator of ovarian cancer cell growth [32]. Two major isoforms of mammalian PLD have been isolated and sequenced, PLD1 (presenting two alternative spliced forms) mainly located in perinuclear and Golgi regions, and PLD2, mainly located in membrane domains [33,34]. PLD is activated in response to hormones and growth factors, as well as to stimulators of vesicle transport, endo- and exocytosis, cell migration and mitosis [35]. Abnormal PLD expression has been reported in some human cancers, in which attention has been focused on the links between the regulatory role played by Ras proteins on PLD activity through Ral-GDS, PI3K and Raf-1 effectors [36], and on effects of PA on Ras-signaling (MAPK, PI3K/AKT and mTOR) and ChoK activity [37,38].

PLD activation has been reported only in one out of five breast cancer cell lines [24], while a lack of differential expression for *PLD1* and only a moderate, if any, overexpression of *PLD2* gene, was associated with a 2- to 4-fold PLD activation only in two out of four investigated EOC cell lines, compared with non tumoral counterparts [19].

Overall, these results point to the conclusion that PLD activation may well contribute to the PCho pool of some cancer cell lines, but it does not seem to play an essential role in the differential PCho levels detected in tumoral versus non tumoral cells.

*PtdCho-PLC* – Although mammalian PtdCho-PLC isoforms have not yet been sequenced nor their genes identified, a growing body of evidence points to the role of this enzyme in processes crucial to cell signaling, such as mitogen- and oncogene-driven triggering of the MAPK/ERK cascade and activation of gene transcription factors, activation of cells of the immune system and programmed cell death [39,40 and references therein]. Notably, PtdCho-PLC is mainly confined to cytoplasmic compartments of non tumoral cells, whereas it is also capable to selectively accumulate on the plasma membrane of ovarian cancer cells [40] and to co-localize with HER2 in membrane raft domains of HER2-overexpressing breast cancer cells [41].

A 3-fold increase in protein expression and 5- to 17-fold increases in PtdCho-PLC activity were measured in EOC cell lines compared with non tumoral counterparts [19]. Cell exposure for 24 h to the PtdCho-PLC inhibitor tricyclodecan-9-yl-potassium xanthate (D609) almost abolished the activity of this enzyme in EOC cells, induced cell growth arrest and reduced to about 60% the intracellular PCho level, maintaining GPCho and free Cho contents practically unaltered. These experiments provided the first direct evidence that PtdCho-PLC activation may substantially contribute to cell proliferation and to the intracellular PCho pool.

*PtdCho deacylation*_- A lower PLA2 expression has been reported for the cytosolic, calcium dependent, group IV PLA2 in a highly malignant (MDA-MB-231) breast cancer cell line, compared with non a tumoral counterpart [42]. $^{31}$P MRS measurements indicated a lower PLA2 activity in EOC compared with OSE cells, while mRNA expression analyses of nineteen PLA2 isoforms showed that the global difference in the overall PLA2 gene expression was not significant [19]. Moreover, the GPCho-PD activity increased only in some, but not in all investigated EOC cells [19]. Overall, these results showed that the deacylation pathway may not be a major source of substrate for the buid-up of the intracellular PCho pool at least in some cancer cells.

## MRS profiles as biomarkers of response to targeted therapeutic agents

An increased understanding of the molecular etiology of cancer supports the development of innovative therapies based on the use of compounds specifically designed to target key molecular mechanisms

responsible for the malignant phenotype. Unique challenges of a targeted therapy compared with chemotherapy include the need to identify appropriate pharmacodynamic markers to guide dose and schedule and to utilize robust biomarkers enabling the selection of patient populations that are most likely to benefit from the treatment. The capability of the PCho MRS signal to act as pharmacodynamic biomarker to detect and monitor the effectiveness of specific inhibitors of reactions involved in Ras-effector activated signaling pathways in cancer cells is summarized in Fig. 1.

***Inhibition of Ras and Ras/c-Raf/MEK1/2/ERK1/2 signaling*** - The MAPK signaling is involved in key cellular functions including proliferation, motility and apoptosis. A major component of the MAPK signaling is represented by the Ras/c-Raf/MEK1/2/ERK1/2 pathway which, following Ras mutation, results in an aberrant activation of target proteins and transcriptional factors in about 30% of human cancers.

Time-dependent decreases in PCho have been reported in breast and colon cancer cells upon cell exposure to U0126, a noncompetitive MEK inhibitor [43]. The results could be interpreted as linked to multiple pathways, likely including ChoK- and CT–mediated reactions in *de novo* PtdCho biosynthesis and an altered balance with phospholipase-mediated PtdCho degradation. An additional note of interest was that in some cases PCho has been found to be required for activation of c-Raf and ERK1/2 (and DNA synthesis), while inhibition of ChoK activity was sufficient to cause blockade of MAPK signaling [28], suggesting double directionality of the links between the PtdCho cycle and cell signaling pathways.

***Inhibition of PI3K*** - The type I PI3K pathway triggered by tyrosine kinase receptor (TKR) stimulation and Ras activation mediates the conversion of phosphatidylinositol 4,5-bisphospate (PIP2) into phosphatidylinositol 3,4,5-trisphosphate (PIP3), which in turn acts as effector of downstream cell signaling including AKT phosphorylation. An aberrant PI3K pathway and altered expression of the antagonizing phosphatase PTEN are involved in tumor progression, angiogenesis, invasion and cell survival. PI3K is therefore held as an attractive target for novel mechanism-based anticancer treatments. The PI3K inhibitor LY294202 caused a significant up to 2-fold decrease in PCho and 5-fold increase in GPCho levels in breast cancer cells which were characterized by increased expression of the epidermal growth factor receptor (EGFR) and by different *ras* and *p53* mutational status [44]. Concentration- and time-dependent decrease in PCho was also induced on prostate and colon cancer cells by PI-103, a class I PI3K and mammalian target of rapamycin (mTOR) inhibitor [45]. The observed decrease in PCho was not considered to be specific for PI3K inhibition, as it was also seen in cells treated with the MAPK inhibitor U0126, suggesting that the effects on PCho are downstream of both PI3K and Raf/MEK/ERK signaling pathways [44].

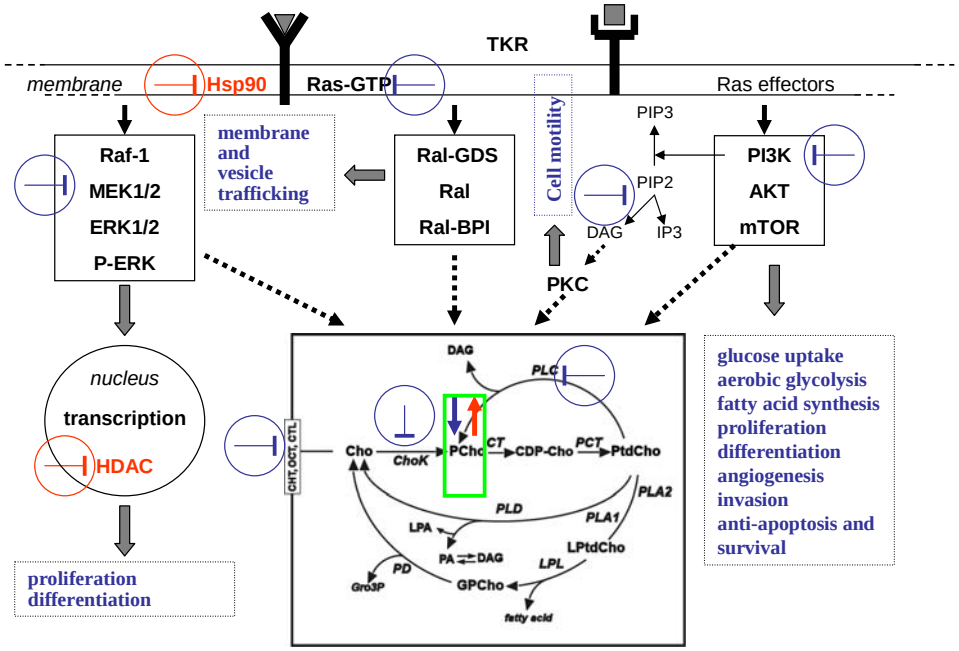
***Inhibition of PI-PLCγ1*** - Phosphoinositide specific phospholipase Cγ1 (PI-PLCγ1) is activated by phosphorylation downstream of many TKR and is implicated in the regulation of cell adhesion, motility and angiogenesis. PI-PLCγ1 mediates the hydrolysis of PIP2 into inositol trisphosphate (IP3), which mobilizes $Ca^{2+}$ from intracellular stores, and DAG, involved in protein kinase C (PKC) activation, which is in turn implicated in cytoskeletal rearrangement at the leading edge necessary for cell motility. PI-PLCγ1 inactivation *via* an inducible short hairpin (sh) RNA caused in prostate cancer cells decreases in PCho, tCho and NTP, along with rounded-up cell morphology, indicative of reduced adhesion and migration [46].

***Inhibition of fatty acid synthase (FASN)*** - The activity of FASN, a key enzyme in *de novo* biosynthesis of long-chain fatty acids, is generally low in normal tissues, but is significantly increased in cancer, including prostate, breast, ovarian and colon carcinomas. Inhibition of FASN by pharmacological agents, such as Orlistat or cerulenin, or small interfering RNA (siRNA), induced cell cycle arrest and apoptosis of transformed cells, along with decreases in the PCho MRS signal, attributed to reduced synthesis, rather than to increased utilization. [47]. The PCho levels correlated with fatty acid synthesis.

***Inhibition of Heat Shock Protein 90 (Hsp90)*** - Inhibition of Hsp90 results in proteasomal degradation of several client oncogenic proteins, offering the possibility of simultaneously acting against several signaling pathways responsible for malignant progression. The Hsp90 inhibitor 17AAG, reported to act as anticancer agent in absence of antiproliferative-related cytotoxicity, induced in colon cancer cell lines significant 2- to 20-fold increases in PCho, associated with depleted levels of Hsp90, c-Raf-1 and Cdk4 and decreased cell proliferation, compared with the respective vehicle-treated controls [48]. The increase in PCho induced by this inhibitor was opposite to what would be expected on the basis of the simplified assumption that the elevated PCho levels characteristic of cancer cells are mere indicators of enhanced cell proliferation. The spectral changes detected by MRS in cancer cells in vitro were confirmed by $^{31}$P MRS examinations of colon cancer cell xenografts and their tissue extracts, in which elevated values of the PME/PDE ratio were also detected in response to 17AAG [48].

***Inhibition of histone deacetylase*** - Histone acetylation plays an important role in transcriptional regulatory mechanisms and in DNA replication and repair. Acetylation is regulated by the opposing effects of two families of enzymes, histone acetyltransferases and histone deacetylase (HDAC). Inhibition of HDAC can represent a new strategy for cancer therapy, by affecting the expression of genes responsible for cell proliferation and cell cycle progression, angiogenesis, cell differentiation and apoptosis. $^{31}$P MRS analyses of colon cancer cells treated either with HDAC inhibitors (LAQ824, SAHA or FSAHA) showed up to 2-fold

increases in PCho (with no other spectral change), associated with marked increases in acetyl histone H3 levels, decreases in c-Raf (Hsp90 client protein), antiproliferative effects and alterations in cell cycle profiles [49]. In agreement with these in vitro results, examinations of colon carcinoma xenografts showed increases in the PME/(total phosphorus) signal area ratio, associated with a compromised bioenergetic status (high Pi, low NTP levels), likely due to reduced perfusion of the treated tumor. Elucidation of the mechanisms underlying increases in the levels of PCho in cancer cells treated with HDAC inhibitors requires further investigations, as discussed above for the effects of Hsp90 inhibition.




**Phosphatidylcholine Cycle**

**Fig 1**. Schematic representation of links between the PtdCho cycle and some Ras-effector-mediated signal transduction pathways in cancer cells. Inhibitors (encircled —| symbol) are indicated in blue or red according to their capability to induce a decrease or an increase in the PCho signal of exposed cancer cells, respectively [Ref. 19,21,43-46,48-56].

**Enzymes of the PtdCho cycle as potential anticancer therapy targets**

The dual directionality of links between the aberrant PtdCho cycle and several oncogene-activated cell signaling pathways in cancer cells strongly support the interest of exploring the possible use of enzymes involved in choline metabolism as targets for antitumor therapies.

The reported evidence on the role of ChoK in cell proliferation, transformation and carcinogenesis suggested the use of this enzyme as a potential novel target for treatment of different types of tumors [50-56]. In particular, a Chok inhibitor ( MN58b) and ChoKα depletion were found to induce non reversible cytotoxic effects on tumoral cells, including increase in ceramides and apoptosis [54,55]. $^{1}$H and $^{31}$P MRS experiments on human breast and colon carcinoma cell lines before and after treatment with MN58b showed a decrease in PCho and tCho levels, followed by a significant drop in cell proliferation [56], correlated with ChoK activity. Furthermore, inhibition of ChoK showed a strong in vivo antitumor activity against human breast cancer in nude mice [25], associated with a decrease in the $^{31}$P MRS PME resonance [56].

RNA interference (RNAi) -mediated ChoK knockdown induced a significant decrease in PCho level in breast cancer cells, along with reduced proliferation and promotion of cell differentiation [51]. A combination of siRNA-Chokα with a chemotherapeutic drug, 5-FU, resulted in a larger reduction of cell viability/proliferation in breast cancer cells than in non tumoral counterparts [52]. Furthermore, preclinical evaluation of lentiviral vector-mediated downregulation of ChoK using shRNA in established breast cancer

xenografts in SCID mice confirmed the capability of MRS approaches to monitor the effectiveness of ChoK downregulation in vivo, by measuring the tumoral PCho levels [53].

The simultaneous activation of both ChoK and PtdCho-PLC in ovarian cancer cells [19] suggested this phospholipase as a potential target for antitumor therapy. The hypothesis was supported by the reported role of PtdCho-PLC in mitogenesis and by the strong delay induced by PtdCho-PLC inhibition on the recovery of the S-phase fraction in these cancer cells re-stimulated by growth factors (serum, LPA) after serum deprivation [40]. Further support to this hypothesis has been given by experiments showing that 24-h cell incubation with D609 induced in EOC cells proliferation arrest and decrease in MRS-detected PCho level similar to those associated with serum deprivation [19].

**Future directions**

MRS analyses of in vitro cultured cancer cells provide new insights on elucidation of mechanisms involved in tumor-specific metabolic alterations and allow identification of useful pharmacodyamic biomarkers of cancer response to targeted therapy. The current in vitro MRS studies, integrated with gene expression and biochemical analyses on cancer cells and tissues, fuel novel information into molecular oncology and may shed light on the mechanisms responsible for both altered MRS profiles of cancer lesions and changes in the standardized uptake value (SUV) of radiolabeled choline in positron emission spectroscopy (PET) examinations of cancer patients.

Interpretation of in vivo MRS profiles requires the additional consideration of effects exerted by the tumor microenvironment in terms of pH, hypoxia and angiogenesis, and on the influence of altered metabolism and physiology of host cells (e.g. stromal, endothelial and immune cells). Furthermore, analysis of MRS profiles in terms of biomarkers of tumor response to therapy requires the use of suitable approaches to overcome the problems of susceptibility-broadened and spatially averaged signal profiles of multi-component tumor lesions. Some of these limitations may however be at least partially compensated using the higher spatial selectivity provided by multivoxel MRSI approaches. The use of suitable preclinical models support the feasibility of in vivo quantitative MRS approaches and their possible transferability to the clinical setting.

In view of the high levels of specificity achieved in the characterization of some metabolic changes induced in cancer cells by the abnormal activation of oncogene-driven signaling cascades and by their inhibition under the action of targeted therapies, MRS is expected to continue to represent a valuable first-line method to test the efficacy of new therapeutic approaches in cancer, in the frame of an integrated platform of multimodal imaging approaches.

*The authors acknowledge support by: Associazione Italiana per la Ricerca sul Cancro AIRC 2007-2009 (F.P.); Integrated Oncology Program RO 06.5/N.ISS/Q0, Oncology Program OncOrd 37/07/N. ISS/70CF/4 and Special Program Alleanza Contro il Cancro 2006, ACC3-AC5/D, Ministry of Health, Italy (F.P.); Accordo di Collaborazione Italia-USA ISS/530F/0F29 (F.P.); Special Project of Health Ministry "Progetto Oncologico di Medicina* Molecolare: i Tumori Femminili" (S.C.); and FONDAZIONE CARIPLO 2007n. 2007.5376 (S.C.).

## References

1 Negendank W. Studies of human tumors by MRS: a review. NMR Biomed 1992;5(5):303-324.

2 de Certaines JD, Larsen VA, Podo F, Carpinelli G, Briot O, Henriksen O. In vivo $^{31}$P MRS of experimental tumours. NMR Biomed 1993;6(6):345-365.

3 Podo F. Tumour phospholipid metabolism. NMR Biomed 1999;12(7):413-439.

4. Ackerstaff E, Glunde K, Bhujwalla ZM. Choline phospholipid metabolism: a target in cancer cells? J Cell Biochem 2003;90(3):525-533.

5 Gillies RJ, Morse DL. In vivo magnetic resonance spectroscopy in cancer. Annu Rev Biomed Eng 2005;7:287-326.

6 Glunde K, Ackerstaff E, Mori N, Jacobs MA, Bhujwalla ZM. Choline phospholipid metabolism in cancer: consequences for molecular pharmaceutical interventions. Mol Pharm 2006;3(5):496-506.

7 Glunde K, Serkova NJ. Therapeutic targets and biomarkers identified in cancer choline phospholipid metabolism. Pharmacogenomics 2006;7(7):1109-1123.

8. Beloueche-Babari M, Chung YL, Al-Saffar NM, Falck-Miniotis M, Leach MO. Metabolic assessment of the action of targeted cancer therapeutics using magnetic resonance spectroscopy. Br J Cancer 2010;102(1):1-7.

9 Glunde K, Artemov D, Penet MF, Jacobs MA, Bhujwalla ZM. Magnetic resonance spectroscopy in metabolic and molecular imaging and diagnosis of cancer. Chem Rev. 2010;110(5):3043-3059.

10 Podo F. Canevari S, Canese R, Pisanu ME, Ricci A, Iorio E. Magnetic resonance evaluation of response to targeted treatment in cancer cells. NMR Biomed 2010, in press.
11 Pelech SL, Vance DE. Signal transduction via phosphatidylcholine cycle. Trends Biochem Sci 1989;14:28-30.
12 Vance JE, Vance DE. Phospholipid biosynthesis in mammalian cells. Biochem Cell Biol 2004;82(1):113-128.
13 Cuadrado A, Carnero A, Dolfi F, Jimenez B, Lacal JC. Phosphorylcholine: a novel second messenger essential for mitogenic activity of growth factors. Oncogene 1993;8(11):2959-2968.
14 Aoyama C, Liao H, Ishidate K. Structure and function of choline kinase isoforms in mammalian cells. Prog Lipid Res 2004;43(3):266-281.
15 Janardhan S, Srivani P, Sastry GN. Choline kinase: an important target for cancer. Curr Med Chem 2006;13(10):1169-1186.
16 Aboagye EO, Bhujwalla ZM. Malignant transformation alters membrane choline phospholipid metabolism of human mammary epithelial cells. Cancer Res 1999;59(1):80-84.
17 Ackerstaff E, Pflug BR, Nelson JB, Bhujwalla ZM. Detection of increased choline compounds with proton nuclear magnetic resonance spectroscopy subsequent to malignant transformation of human prostatic epithelial cells. Cancer Res 2001;61(9):3599-3603.
18 Iorio E, Mezzanzanica D, Alberti P, Spadaro F, Ramoni C, D'Ascenzo S, Millimaggi D, Pavan A, Dolo V, Canevari S, Podo F. Alterations of choline phospholipid metabolism in ovarian tumor progression. Cancer Res 2005;65(20):9369-9376.
19 Iorio E, Ricci A, Bagnoli M, Pisanu ME, Castellano G, Di Vito M, Venturini E, Glunde K, Bhujwalla ZM, Mezzanzanica D, Canevari S. Activation of phosphatidylcholine cycle enzymes in human epithelial ovarian cancer cell. Cancer Res 2010;70(5):2126-2135.
20 Franks SE, Smith MR, Arias-Mendoza F, Shaller C, Padavic-Shaller K, Kappler F, Zhang Y, Negendank WG, Brown TR. Phosphomonoester concentrations differ between chronic lymphocytic leukemia cells and normal human lymphocytes. Leuk Res 2002;26(10):919-926.
21 Ronen SM, Jackson LE, Beloueche M, Leach MO. Magnetic resonance detects changes in phosphocholine associated with Ras activation and inhibition in NIH 3T3 cells. Br J Cancer 2001;84(5):691-696.
22 Mori N, Delsite R, Natarajan K, Kulawiec M, Bhujwalla ZM, Singh KK. Loss of p53 function in colon cancer cells results in increased phosphocholine and total choline. Mol Imaging 2004;3(4):319-323.
23 Bhujwalla ZM, Aboagye EO, Gillies RJ, Chacko VP, Mendola CE, Backer JM. Nm23-transfected MDA-MB-435 human breast carcinoma cells form tumors with altered phospholipid metabolism and pH: a $^{31}$P nuclear magnetic resonance study in vivo and in vitro. Magn Reson Med 1999;41(5):897-903.
24 Eliyahu G, Kreizman T, Degani H. Phosphocholine as a biomarker of breast cancer: molecular and biochemical studies. Int J Cancer 2007;120(8):1721-1730.
25 Ramirez de Molina A, Bañez-Coronel M, Gutierrez R, Rodriguez-Gonzalez A, Olmeda D, Megias D, Lacal JC. Choline kinase activation is a critical requirement for the proliferation of primary human mammary epithelial cells and breast tumor progression. Cancer Res 2004;64(18):6732-6739.
26 Glunde K, Shah T, Winnard PT, Jr., Raman V, Takagi T, Vesuna F, Artemov D, Bhujwalla ZM. Hypoxia regulates choline kinase expression through hypoxia-inducible factor-1 alpha signaling in a human prostate cancer model. Cancer Res 2008;68(1):172-180.
27 Chua BT, Gallego-Ortega D, Ramirez de Molina A, Ullrich A, Lacal JC, Downward J. Regulation of Akt(ser473) phosphorylation by choline kinase in breast carcinoma cells. Mol Cancer 2009;8:131.
28 Jimenez B, del Peso L, Montaner S, Esteve P, Lacal JC. Generation of phosphorylcholine as an essential event in the activation of Raf-1 and MAP-kinases in growth factors-induced mitogenic stimulation. J Cell Biochem 1995;57(1):141-149.
29 Ramirez de Molina A, Gutierrez R, Ramos MA, Silva JM, Silva J, Bonilla F, Sanchez JJ, Lacal JC. Increased choline kinase activity in human breast carcinomas: clinical evidence for a potential novel antitumor strategy. Oncogene 2002;21(27):4317-4322.
30 Ramirez de Molina A, Sarmentero-Estrada J, Belda-Iniesta C, Taron M, Ramirez de Molina V, Cejas P, Skrzypski M, Gallego-Ortega D, de Castro J, Casado E, Garcia-Cabezas MA, Sanchez JJ, Nistal M, Rosell R, Gonzalez-Baron M, Lacal JC. Expression of choline kinase alpha to predict outcome in patients with early-stage non-small-cell lung cancer: a retrospective study. Lancet Oncol 2007;8(10):889-897.
31 Hernando E, Sarmentero-Estrada J, Koppie T, Belda-Iniesta C, Ramirez de Molina V, Cejas P, Ozu C, Le C, Sanchez JJ, Gonzalez-Baron M, Koutcher J, Cordon-Cardo C, Bochner BH, Lacal JC, Ramirez de Molina A. A critical role for choline kinase-alpha in the aggressiveness of bladder carcinomas. Oncogene 2009;28(26):2425-2435.

32 Lee Z, Swaby RF, Liang Y, Yu S, Liu S, Lu KH, Bast RC, Jr., Mills GB, Fang X. Lysophosphatidic acid is a major regulator of growth-regulated oncogene alpha in ovarian cancer. Cancer Res 2006;66(5):2740-2748.
33 Hammond SM, Jenco JM, Nakashima S, Cadwallader K, Gu Q, Cook S, Nozawa Y, Prestwich GD, Frohman MA, Morris AJ. Characterization of two alternately spliced forms of phospholipase D1. Activation of the purified enzymes by phosphatidylinositol 4,5-bisphosphate, ADP-ribosylation factor, and Rho family monomeric GTP-binding proteins and protein kinase C-alpha. J Biol Chem 1997;272(6):3860-3868.
34 Colley WC, Sung TC, Roll R, Jenco J, Hammond SM, Altshuller Y, Bar-Sagi D, Morris AJ, Frohman MA. Phospholipase D2, a distinct phospholipase D isoform with novel regulatory properties that provokes cytoskeletal reorganization. Curr Biol 1997;7(3):191-201.
35 Foster DA, Xu L. Phospholipase D in cell proliferation and cancer. Mol Cancer Res 2003;1(11):789-800.
36 Lucas L, Penalva V, Ramirez de Molina A, Del Peso L, Lacal JC. Modulation of phospholipase D by Ras proteins mediated by its effectors Ral-GDS, PI3K and Raf-1. Int J Oncol 2002;21(3):477-485.
37 Toschi A, Lee E, Xu L, Garcia A, Gadir N, Foster DA. Regulation of mTORC1 and mTORC2 complex assembly by phosphatidic acid: competition with rapamycin. Mol Cell Biol 2009;29(6):1411-1420.
38 Yalcin A, Clem B, Makoni S, Clem A, Nelson K, Thornburg J, Siow D, Lane AN, Brock SE, Goswami U, Eaton JW, Telang S, Chesney J. Selective inhibition of choline kinase simultaneously attenuates MAPK and PI3K/AKT signaling. Oncogene; 2010;29(1):139-149.
39 Ramoni C, Spadaro F, Barletta B, Dupuis ML, Podo F. Phosphatidylcholine-specific phospholipase C in mitogen-stimulated fibroblasts. Exp Cell Res 2004;299(2):370-382.
40 Spadaro F, Ramoni C, Mezzanzanica D, Miotti S, Alberti P, Cecchetti S, Iorio E, Dolo V, Canevari S, Podo F. Phosphatidylcholine-specific phospholipase C activation in epithelial ovarian cancer cells. Cancer Res 2008;68(16):6541-6549.
41 Paris L, Cecchetti S, Spadaro F, Abalsamo L, Lugini L, Pisanu ME, Iorio E, Natali PG, Ramoni C, Podo F. Inhibition of phosphatidylcholine-specific phospholipase C downregulates HER2 overexpression on plasma membrane of breast cancer cells. Breast Cancer Res 2010;12(3):R27.
42 Glunde K, Jie C, Bhujwalla ZM. Molecular causes of the aberrant choline phospholipid metabolism in breast cancer. Cancer Res 2004;64(12):4270-4276.
43 Beloueche-Babari M, Jackson LE, Al-Saffar NM, Workman P, Leach MO, Ronen SM. Magnetic resonance spectroscopy monitoring of mitogen-activated protein kinase signaling inhibition. Cancer Res 2005;65(8):3356-3363.
44 Beloueche-Babari M, Jackson LE, Al-Saffar NM, Eccles SA, Raynaud FI, Workman P, Leach MO, Ronen SM. Identification of magnetic resonance detectable metabolic changes associated with inhibition of phosphoinositide 3-kinase signaling in human breast cancer cells. Mol Cancer Ther 2006;5(1):187-196.
45 Al-Saffar NM, Jackson LE, Raynaud FI, Clarke PA, de Molina AR, Lacal JC, Workman P, Lach MO. The phosphoinosiide 3-kinase inhibitor PI-103 downregulates choline kinase alpha leading to phosphocholine and total choline decrease detected by magnetic resonance spectroscopy. Cancer Res 2010; 70:5507-5517.
46 Beloueche-Babari M, Peak JC, Jackson LE, Tiet MY, Leach MO, Eccles SA. Changes in choline metabolism as potential biomarkers of phospholipase C{gamma}1 inhibition in human prostate cancer cells. Mol Cancer Ther 2009;8(5):1305-1311.
47 Ross J, Najjar AM, Sankaranarayanapillai M, Tong WP, Kaluarachchi K, Ronen SM. Fatty acid synthase inhibition results in a magnetic resonance-detectable drop in phosphocholine. Mol Cancer Ther 2008;7(8):2556-2565.
48 Chung YL, Troy H, Banerji U, Jackson LE, Walton MI, Stubbs M, Griffiths JR, Judson IR, Leach MO, Workman P, Ronen SM. Magnetic resonance spectroscopic pharmacodynamic markers of the heat shock protein 90 inhibitor 17-allylamino,17-demethoxygeldanamycin (17AAG) in human colon cancer models. J Natl Cancer Inst 2003;95(21):1624-1633.
49 Chung YL, Troy H, Kristeleit R, Aherne W, Jackson LE, Atadja P, Griffiths JR, Judson IR, Workman P, Leach MO, Beloueche-Babari M. Noninvasive magnetic resonance spectroscopic pharmacodynamic markers of a novel histone deacetylase inhibitor, LAQ824, in human colon carcinoma cells and xenografts. Neoplasia 2008;10(4):303-313.
50 Lacal JC. Choline kinase: a novel target for antitumor drugs. IDrugs 2001;4(4):419-426.
51 Glunde K, Raman V, Mori N, Bhujwalla ZM. RNA interference-mediated choline kinase suppression in breast cancer cells induces differentiation and reduces proliferation. Cancer Res 2005;65(23):11034-11043.

52 Mori N, Glunde K, Takagi T, Raman V, Bhujwalla ZM. Choline kinase down-regulation increases the effect of 5-fluorouracil in breast cancer cells. Cancer Res 2007;67(23):11284-11290.
53 Krishnamachary B, Glunde K, Wildes F, Mori N, Takagi T, Raman V, Bhujwalla ZM. Noninvasive detection of lentiviral-mediated choline kinase targeting in a human breast cancer xenograft. Cancer Res 2009;69(8):3464-3471.
54 Bañez-Coronel M, Ramirez de Molina A, Rodriguez-Gonzalez A, Sarmentero J, Ramos MA, Garcia-Cabezas MA, Garcia-Oroz L, Lacal JC. Choline kinase alpha depletion selectively kills tumoral cells. Curr Cancer Drug Targets 2008;8(8):709-719.
55 Rodriguez-Gonzalez A, Ramirez de Molina A, Fernandez F, Lacal JC. Choline kinase inhibition induces the increase in ceramides resulting in a highly specific and selective cytotoxic antitumoral strategy as a potential mechanism of action. Oncogene 2004;23(50):8247-8259.
56 Al-Saffar NM, Troy H, Ramirez de Molina A, Jackson LE, Madhu B, Griffiths JR, Leach MO, Workman P, Lacal JC, Judson IR, Chung YL. Noninvasive magnetic resonance spectroscopic pharmacodynamic markers of the choline kinase inhibitor MN58b in human carcinoma models. Cancer Res 2006;66(1):427-434.