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INDIRIZZO DI BIOLOGIA CELLULARE
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SPECIFIC MITOCHONDRIAL BIOGENESIS PATTERNS DRIVE NUTRIENT CHOICE IN BREAST CANCER SUBTYPES

Direttore della Scuola: Ch.mo Prof. Paolo Bernardi

Coordinatore d'indirizzo: Ch.mo Prof. Paolo Bernardi

Supervisore: Ch.mo Prof. Gyorgy Szabadkai

Dottorando: Michela Menegollo

A mio marito, ai miei genitori,
a tutte le persone a me care!!!

Un enorme grazie per la pazienza, il supporto
e l'amore che mi avete dimostrato!!!

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1. SUMMARY

Transformed cells undergo profound reprogramming of cellular metabolism to ensure high rate of proliferation (Hanahan D, 2011). Mitochondria are important for both bioenergetics and biosynthetic pathways, which have to be coordinated. Different cancer types heterogeneously express mitochondrial genes likely reflecting the existence of different mitochondrial pathways providing adaptation to altered needs of cancer cell metabolism. In this work we explored this hypothesis in breast cancer.

Breast cancer is heterogeneous disease classified according to diverse clinical and pathological features, histochemical markers and oncogenic transcriptional programs, that identify five breast cancer subtypes (basal-like, Her2-enriched, Luminal A, Luminal B and normal-like) (Sørli T et al., 2001; Perou CM et al., 2000). So far, the mitochondrial transcriptional patterns were not considered as a basis of classification.

In this work we define for the first time breast cancer Mitochondrial Tumour Subtypes (MTSs), upper fork (UF) and lower fork (LF), according to their nuclear-encoded mitochondrial transcription profile. UF and LF are characterised by two groups of mitochondrial genes co-regulated in opposite way. The classification was performed applying the MCBiclust algorithm (Bentham et al., 2016, preprint) to a mitochondrial gene set (MitoCarta, Pagliarini et al., 2008).

The experimental characterisation of UF and LF revealed distinct bioenergetics and metabolic features. Interestingly, in basal condition UF has higher mitochondrial content, characterised by higher expression of CI and CIV resulting in higher respiration rate. At cellular level metabolism, UF and LF revealed different arrangement of metabolic enzymes belonging to particular pathways of the intermediate metabolism, UF cells express enzymes of which substrates are glucose-derived, whereas LF cells preferentially use enzymes exploiting glutamine-derived substrates. Moreover, metabolic flux analysis confirmed the substrate preference driven by the MTSs to feed into the TCA cycle, in particular

UF cells mitochondria prefer glucose derived pyruvate while LF mitochondria catabolise glutamine.

In addition, experiments in restricted nutrient conditions were carried out. These further confirmed which is the substrate preferentially used by MTSs to sustain cell functionality. Moreover, the same experiments also revealed the activation of different mitochondrial biogenesis programs during deprivation treatments, both looking at mitochondrial protein expression and mtDNA content.

Overall, the mitochondrial biogenesis pattern associated with the MTSs drives nutrient choice and metabolic program, leading to a definition of the concept of substrate preference, new insight in the field of reprogramming of bioenergetics and cancer metabolism.

2. RIASSUNTO

Le cellule tumorali vanno incontro a profonde modifiche del proprio metabolismo cellulare per assicurarsi un alto tasso di proliferazione (Hanahan D, 2011). I mitocondri sono una sede importante sia per la produzione di energia che per i processi biosintetici, pertanto queste vie devono essere opportunamente coordinate. Molti tipi di cancro esprimono geni mitocondriali in modo eterogeneo, questo probabilmente riflette l'esistenza di diverse vie mitocondriali in grado di fornire diverse capacità di adattamento ad un metabolismo alterato, come lo è il profilo metabolico associato ai tumori. In questo lavoro abbiamo esplorato questa ipotesi nel modello tumorale del cancro alla mammella.

Il cancro alla mammella rappresenta una malattia comunemente classificata sulla base di caratteristiche cliniche e patologiche, marcatori istochimici e programmi di trascrizione genica che identificano cinque sottotipi di tumore alla mammella (basal-like, Her2-enriched, Luminal A, Luminal B e normal-like) (Sørlie T et al., 2001; Perou CM et al., 2000). Tuttavia, l'espressione di geni mitocondriali non è stata finora considerata nella classificazione di questi tumori.

In questo lavoro definiamo per la prima volta sottotipi tumorali mitocondriali di cancro alla mammella (MTSs), upper fork (UF) e lower fork (LF), definiti sulla base del loro profilo di trascrizione di geni mitocondriali di codificazione nucleare regolati in modo opposto. La classificazione è stata fatta utilizzando l'algoritmo MCBiclust (Bentham et al., 2016, preprint) su un set di geni mitocondriali (MitoCarta, Pagliarini et al., 2008).

Dalla caratterizzazione sperimentale di UF e LF sono emerse caratteristiche bioenergetiche e metaboliche distintive. In particolare, in condizioni basali UF ha un maggiore contenuto mitocondriale, caratterizzato da una maggiore espressione dei complessi I e IV della catena respiratoria, che culmina in un tasso di respirazione maggiore. A livello di metabolismo cellulare, UF e LF mostrano un diverso assetto di enzimi metabolici appartenenti a particolari vie

del metabolismo intermedio. In particolare le cellule dell'UF esprimono enzimi i cui substrati sono derivati dal glucosio, mentre le cellule di LF preferiscono utilizzare enzimi i cui substrati sono derivati dalla glutammina. Inoltre l'analisi dei flussi metabolici ha confermato la preferenza di substrato determinata dai MTSs per alimentare il ciclo di Krebs. In particolare i mitocondri delle cellule dell'UF preferiscono il piruvato derivato dal glucosio, mentre i mitocondri di LF catabolizzano la glutammina.

In aggiunta, sono stati eseguiti esperimenti con concentrazioni di nutrienti ridotte, i quali, in accordo con i risultati precedenti, hanno ulteriormente confermato i substrati preferenzialmente utilizzati dai MTSs per sostenere la funzionalità cellulare. Inoltre gli stessi esperimenti hanno rivelato l'attivazione di diversi programmi di biogenesi mitocondriale indotti dai trattamenti, sia a livello di espressione di proteine mitocondriali che in termini di contenuto di DNA mitocondriale.

Complessivamente, il pattern di biogenesi mitocondriale associato ai sottotipi tumorali mitocondriali determina la scelta di nutrienti e programmi metabolici, portando così alla definizione del concetto di preferenza di substrato, il quale fornisce una nuova visione nel campo della riprogrammazione bioenergetica e metabolica del cancro e la possibilità di classificare sottotipi tumorali sulla base dell'espressione dei geni mitocondriali.

3. INTRODUCTION

3.1 Cancer: the next generation of hallmarks

Cancer is a heterogeneous disease affecting a large human population; an estimated 14.1 million new cancer cases and 8.2 million cancer deaths occurred in 2012 worldwide. Lung and breast cancers are the most frequently diagnosed cancers and the leading causes of cancer death in men and women, respectively, both overall and in less developed countries (Torre LA et al., 2015).

The alterations in cellular signalling that enable tumour growth and metastatic dissemination are often summarised as hallmarks of cancer, currently used to define tumours. The classic hallmarks, defined in 2000, include sustained proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (Hanahan D et al., 2000). Their acquisition is made possible by two enabling characteristics: genomic instability, causing mutations and chromosomal rearrangement, and inflammation that favour tumour progression (Figure 3.2) (Hanahan D & Weinberg RA, 2011). Most importantly, it has been recently rediscovered that all these hallmarks are intertwined with an altered cancer cell-intrinsic metabolism, either as a consequence or as a cause (Figure 3.1) (Kroemer G & Pouyssegur J, 2008). Indeed, the conceptual progress in the last decade suggests that new hallmarks are involved in the pathogenesis of the majority of cancers: reprogramming of energy metabolism, in order to most effectively support neoplastic proliferation, and the active evasion by cancer cells from attack and elimination by immune cells (Figure 3.2) (Hanahan D & Weinberg RA, 2011). Further complexity is added by the concept of tumour microenvironment, in which a repertoire of cells can contribute to the acquisition of the hallmarks themselves (Figure 3.2) (Hanahan D & Weinberg RA, 2011).

Overall, this scenario appears very complex. Since the main topic of the thesis is cancer metabolism, here I will focus on introducing the concept of metabolic reprogramming.

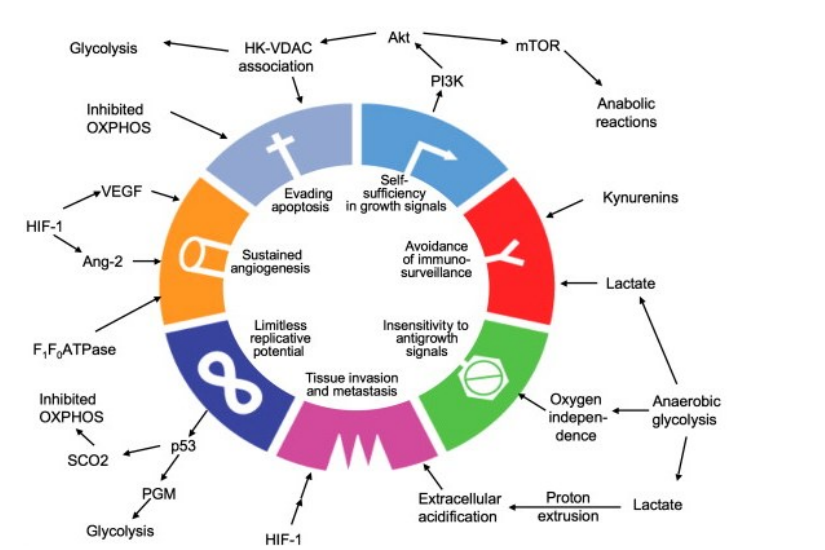


Figure 3.1 The hallmarks of cancer and their link to tumour metabolism (Kroemer G & Pouyssegur J, 2008).

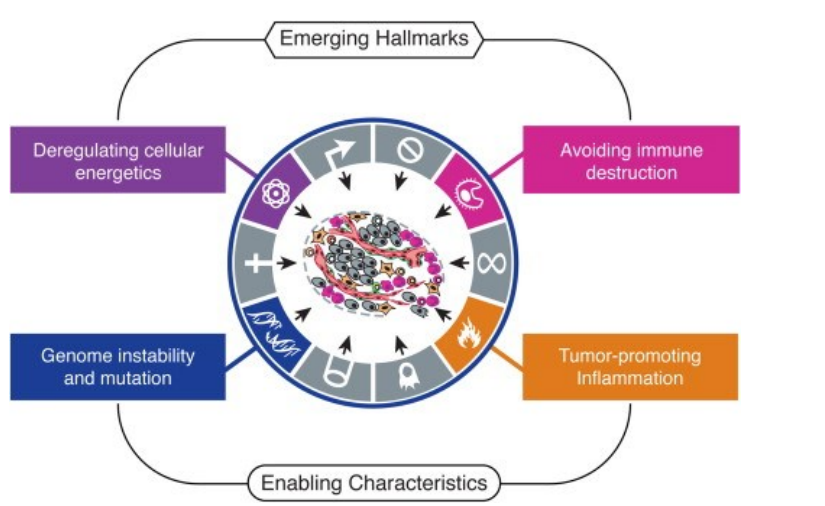


Figure 3.2 Emerging hallmarks, enabling characteristics and tumour microenvironment (Hanahan D & Weinberg RA, 2011).

3.1.1 Reprogramming of cellular metabolism and bioenergetics

Several findings indicate that cancer cells undergo a complex metabolic reprogramming to satisfy the increased requirement of macromolecules and energy necessary for proliferation.

The first alteration of cancer metabolism was observed by Otto Warburg in the 1920s, when he observed that tumour cells consuming large amounts of glucose through glycolysis even in the presence of oxygen, a phenomenon since known as aerobic glycolysis or Warburg effect, whereas normal cells predominantly use respiration as main energy source (Warburg et al., 1927) (Figure 3.3). To explain this phenomenon in which cancer cells limit their bioenergetics to glycolysis, he hypothesized that cancer transformation was caused by defects in mitochondrial function (Warburg, 1956). However, it is now clear that respiration is not impaired in most cancer cells (Wallace DC, 2012; Lunt SY et al., 2011). Hence, the explanation for the switch to aerobic glycolysis in cancer cells is to support the anabolic requirements associate with cell growth and proliferation, providing glycolytic intermediates for biosynthesis (Ward PS and Thompson CB; 2012; Lunt SY et al., 2011; Vander Heiden MG et al., 2009).

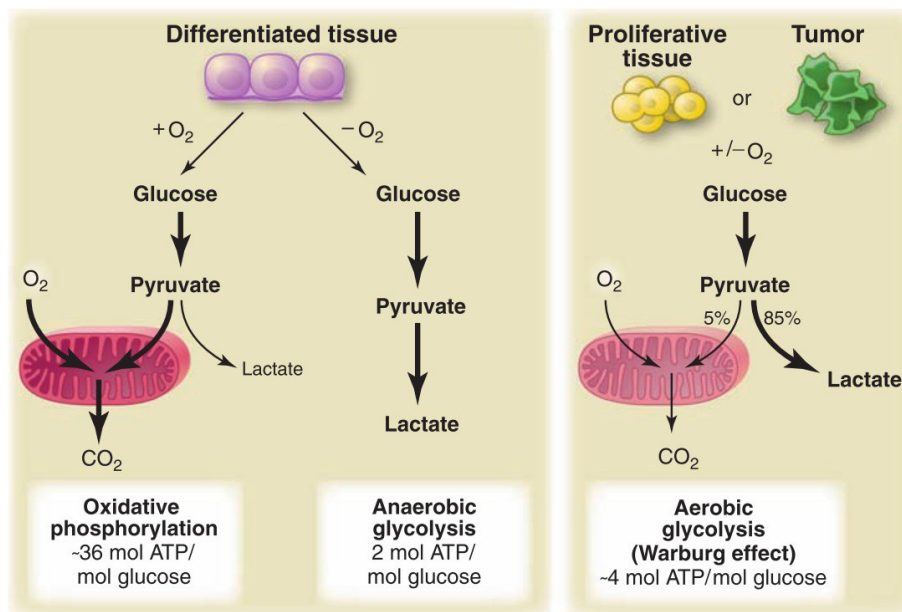


Figure 3.3 Schematic differences between oxidative phosphorylation, anaerobic glycolysis and aerobic glycolysis (Vander Heiden et al., 2009).

The concept has evolved in the following decades highlighting that mitochondrial function is essential for cancer cells (Wallace DC, 2012) and in this context oncogenes (e.g. c-MYC, K-ras, PI3K) play an active role in the metabolic rewiring (reviewed in Ward PS & Thompson CB, 2016; Boroughs LK & DeBerardinis RJ, 2015; Pavlova NN & Thompson CB 2012). Furthermore, metabolites themselves can promote tumorigenesis by altering the epigenome, defining a concept of oncometabolites (reviewed in Nowicki S & Gottlieb E, 2015). To sustain cell growth, cancer cells need to take up nutrients from the environment as source of energy and building blocks, among which glucose and glutamine are fundamental nutrients. Glucose is an energetic source as it supplies carbons, whereas glutamine is apart from providing carbon is also the unique source of nitrogen for cancer cells (DeNicola GM & Cantley LC, 2015; Lunt SY, Vander Heiden MG, 2011) (Figure 3.4). Overall, the concept of cancer metabolism is a leading-edge theme subjected to intense study.

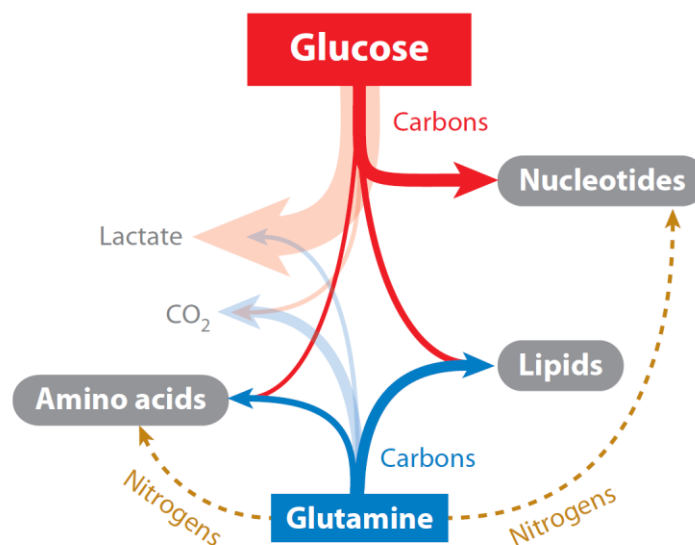


Figure 3.4 Contribution of glucose carbons as well as glutamine carbons and nitrogens to biomass lactate and CO₂ in a proliferating cell (Lunt SY et al., 2011).

3.2 Mitochondrion: a key player in orchestrating metabolism

The intermediate metabolism comprises the catabolic and anabolic reactions happening inside the cells, that produce energy and reducing equivalents (NAD(P)H) to sustain synthesis of new macromolecules. All these reactions support cell proliferation (Figure 3.5).

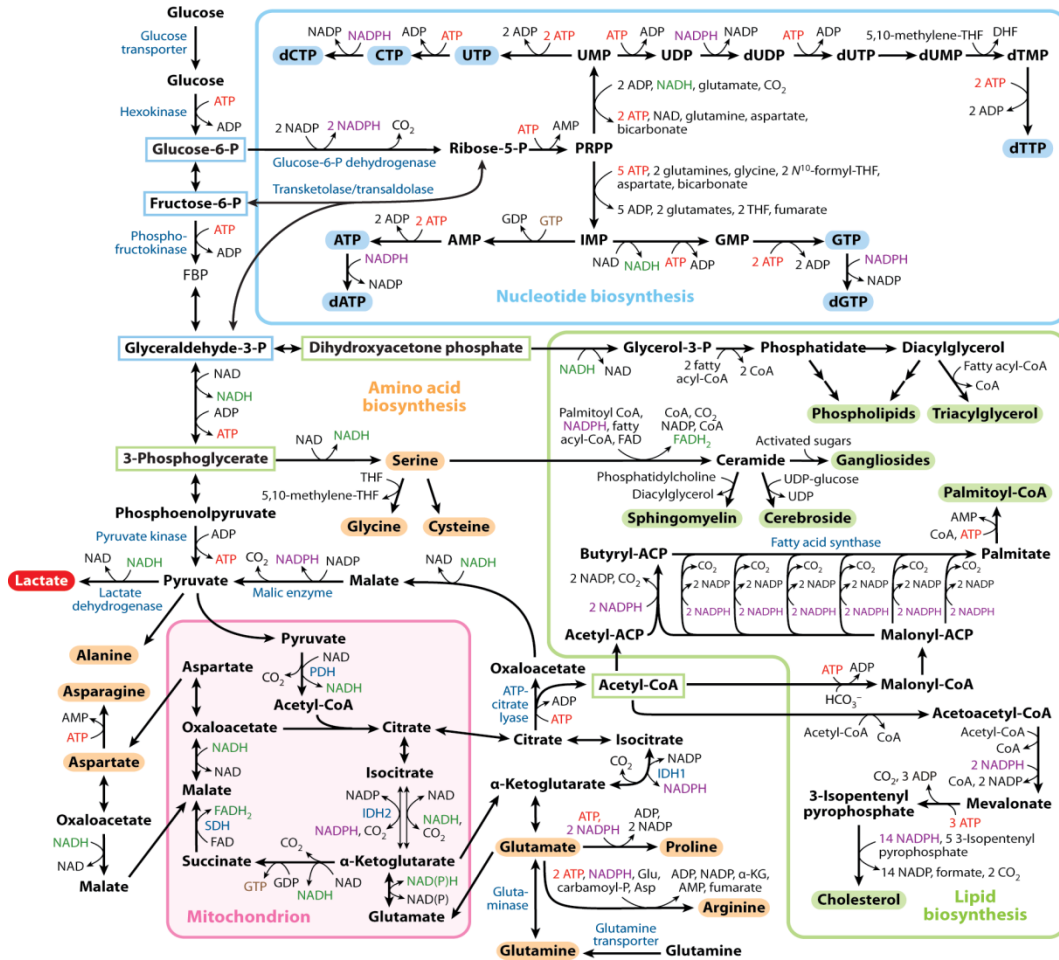


Figure 3.5 Metabolic pathways acting in proliferating cells (Lunt SY et al., 2011).

The reactions involve spatially separated enzymes, which localise in the cytoplasm or inside mitochondria. However, the two compartments are kept in connection due to the presence of transporters and shuttle systems.

Normally, glucose is oxidised by glycolysis to produce pyruvate which enters into mitochondria through the mitochondrial pyruvate carriers (MPC1 and 2) (Bricker

DK et al., 2012; Herzig S et al., 2012) and fuels the TCA cycle and consequently oxidative phosphorylation (OXPHOS) culminating in ATP production. Furthermore, the glycolytic intermediates can be shunted to the pentose phosphate pathway (PPP), producing NADPH and ribose-5 phosphate later sorted for nucleotide biosynthesis. Similarly, the TCA cycle intermediates are utilised as substrates for biosynthetic reactions, requiring specific shuttle (malate-aspartate shuttle) and transporters to move outside mitochondria. However, in order to coordinate biosynthetic needs and respiration, they have to be replenished, a phenomenon known as anaplerosis (Metallo CM & Vander Heiden MG, 2013) (Figure 3.6). The main anaplerotic substrates for the TCA cycle are glucose-derived pyruvate and the amino acid glutamine, that act as both catabolic and anabolic substrates.

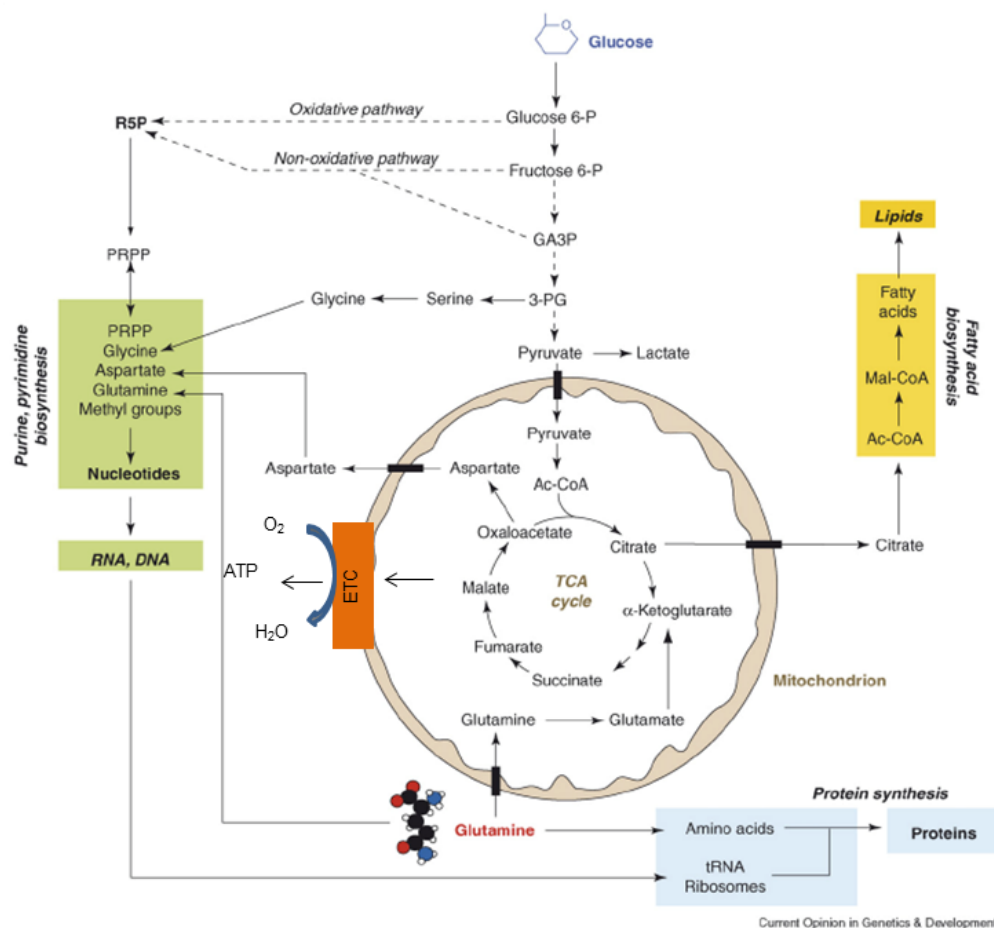


Figure 3.6 The mitochondria connection with the spatially separated reaction of the intermediate metabolism.

Overall, mitochondria play a central role in orchestrating metabolism through a coordination of bioenergetics and biosynthetic pathways.

This concept is particularly important in cancer cells in which a metabolic reprogramming occurs. Indeed, given the importance of mitochondria in bioenergetics and metabolism, cancer cells need to maintain a functional mitochondrial pool to cope with the energetic and metabolic requirements and thus sustain cell proliferation. Mitochondrial biogenesis is the process responsible for the maintenance of the mitochondrial pool.

3.3 Mitochondrial biogenesis

3.3.1 General framework

The mitochondrion represents a unique organelle within the complex endomembrane systems that characterize any eukaryotic cell. Beyond the pivotal role they play in ATP production, a whole new mitochondrial biology has emerged in the last few decades: mitochondria have been shown to participate in many other aspects of cell physiology such as amino-acid synthesis, iron-sulphur clusters assembly, lipid metabolism, Ca^{2+} signaling, reactive oxygen species (ROS) production and cell death regulation.

Hence, many pathological conditions are associated with mitochondrial dysfunction, including neurodegenerative diseases (Alzheimer's, Parkinson's, Huntington's), motoneuron disorders (amyotrophic lateral sclerosis, type 2A Charcot-Marie-Tooth neuropathy), autosomal dominant optic atrophy, ischemia-reperfusion injury, diabetes, ageing and cancer (Duarte et al., 2014; Gaude and Frezza, 2014).

Understanding how mitochondria can sense and decode various signals from the cytosol and other subcellular compartments represents a new exciting challenge in biomedical research.

Mitochondria are defined by two structurally and functionally different membranes: the plain outer membrane (OMM), permeable to ions and

metabolites up to 5000 Da, and the highly selective inner membrane (IMM), characterized by invaginations called *cristae* which enclose the mitochondria matrix. The space between the two membranes is called intermembrane space (IMS). The *cristae* define internal compartments formed by profound invaginations originating from narrow tubular structures called *cristae junctions* (Mannella, 2006) that limit the diffusion of molecules from the intra-*cristae* space towards the IMS, thus creating a micro-environment where the mitochondrial Electron Transport Chain (mETC) complexes are hosted and other proteins are protected from random diffusion. Inside the mitochondrial matrix there is the mitochondrial genome, called mitochondrial DNA (mtDNA); there are also mitochondrial ribosomes and 22 transfer RNAs (tRNAs), the machinery responsible for nucleus-independent protein synthesis.

3.3.2 Mitochondrial biogenesis process

The mitochondrial proteome consists of around 1098 proteins of which expression vary among different tissues (Pagliarini et al., 2008). Mitochondria are continuously remodeled by a dynamic process of fusion and fission, occurring with mtDNA synthesis, protein synthesis inside mitochondria and protein import from the cytosol. Interestingly only 13 proteins, that function as essential subunits of respiratory complexes I, III, IV and V, are encoded by the mtDNA. The mtDNA-encoded proteins are expressed through the bidirectional synthesis of multigenic transcripts, followed by the processing, polyadenylation and translation of individual mRNAs (Taanman JW et al., 1999). Mitochondrial transcription requires a single RNA polymerase (POLRMT), initiation (TFB2M) and stimulatory (Tfam) transcription factors and a family of termination factors (mTERFs) (Bonawitz ND et al., 2006; Scarpulla RC, 2008a). However, the majority of mitochondrial proteins are encoded in the nucleus, rendering the mitochondrial biogenesis process primarily regulated at the nuclear transcriptional level (Scarpulla RC, 2011; Scarpulla RC, 2008a).

A plethora of transcription factors (TFs) and nuclear receptors (NRs) regulates the process in hierarchical manner, among which NRF 1 and 2, CREB, YY1, , c-MYC ERR, PPAR family. The presence of transcriptional co-regulators (TCRs) of the PGC-1 family (PGC-1 α , PGC-1 β and PRC) modulates their activity.

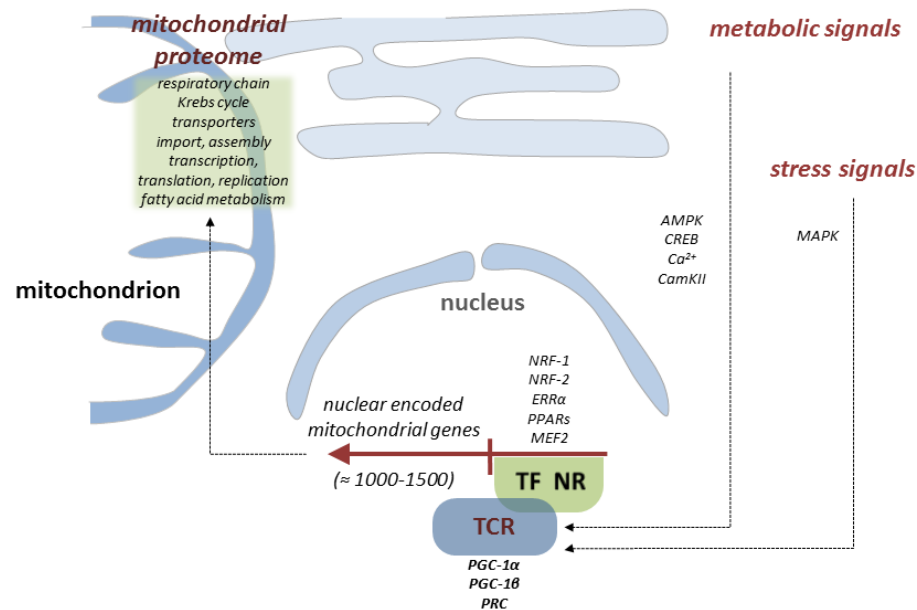


Figure 3.7 Mitochondrial biogenesis a process.

TFs and NRs directly bind DNA promoter regions activating transcriptional programs.

The nuclear respiratory factors 1 and 2 (NRF1, NRF2) are responsible for the induction respectively of the cytochrome c and cytochrome oxidase subunit genes. The two factors frequently work in conjugation in the expression of many nuclear genes specifying essential mitochondrial function (Scarpulla RC, 2008a; Scarpulla RC, 2008b). Moreover, NRFs indirectly control the expression of mtDNA by regulating the Tfam expression.

cAMP response element binding protein CREB, are involved in the expression of mitochondrial genes in response to cyclic adenosine monophosphate (cAMP), a second messenger derivative of ATP used for intracellular signalling. Importantly,

it has been found to bind the PGC-1 α promoter and directly regulate its expression (Herzig et al. 2001). CREB is also involved in general processes such as cell proliferation, differentiation and adaptive responses and much more specific roles such as in the development of memory (Shaywitz 1999).

Yin Yang 1 (YY1) is a transcription factor, that has been implicated in regulation of cytochrome c oxidase subunits (Scarpulla 2008). Moreover, it has been shown by Cunningham et al. (2007) to form a complex with PGC-1 α in muscle to regulate 40 mitochondrial gene expression. Importantly, YY1 requires the activity of the protein mammalian target of rapamycin (mTOR), known to regulate many cellular processes involved in cell growth. In addition, mTOR is often described as a nutrient sensor, providing a link between nutrient sensing pathways and mitochondrial biogenesis.

c-Myc is a transcription factor involved in cell cycle regulation, apoptosis and cellular transformation and has been identified as an oncogene being commonly mutated in many types of cancer promoting cancer metabolic reprogramming (Hsieh AL, et al., 2015, Dang 2012). Myc plays an important role in mitochondrial biogenesis, indeed evidences showed that myc null fibroblast are deficient in mitochondrial content (Li F et al., 2005).

The estrogen related receptors family composed by ERR α , ERR β , ERR γ , known also as orphan receptors because of the structural similarity to the estrogen receptors but lacking of ligands, are differently expressed in the tissues. ERR α is the ubiquitous member, which is known to regulate genes involved in lipid oxidation, OXPHOS, the TCA cycle, mitochondrial import and dynamics as well as response to oxidative stress (Hock 2009). Moreover, the transcriptional activity seems to be dependent on their relationship with PGC-1 α and PGC-1 β (Huss et al. 2015).

The PPAR family is a group of nuclear receptors highly involved in the regulation of mitochondria biogenesis. The PPAR family members, PPAR α , PPAR δ also referred to as PPAR β and PPAR γ , have distinct tissue distributions as well as

physiological functions. PPAR δ and PPAR γ is also thought to regulate co-activator PGC-1 α via a PPAR response element.

The involvement of transcriptional co-regulators in the mitochondrial biogenesis has been appreciated by the discovery of the nuclear co-activator PPAR γ co-activator-1 α (PGC-1 α). Co-regulators function in large multiprotein complexes; they do not directly bind to DNA, but rather they engage TFs and NRs modulating their transcriptional activity (activation or repression).

The PGC-1 family of co-activators are composed by PGC-1 α , which has the first been discovered as co-regulator of PPAR γ and NRs during adaptive thermogenesis in mouse brown adipocytes and skeletal muscle (Puigserver P et al., 2000), and the structurally related transcriptional coactivator PGC-1 β , characterized by having PGC-1 α complementary function, and PGC-1 α related coactivator (PRC). Moreover, new complexity was added by the discovery of alternative splicing variants in the PGC-1 α gene (Ruas JL et al., 2012; Zhang Y et al., 2009). In literature PGC-1 family as referred the 'master regulator' of mitochondrial biogenesis (Jones AW, 2012; Scarpulla 2011).

Interestingly, the PGC-1 family is responsible for the regulation of a broad range of metabolic functions intrinsic, but not only, to mitochondria. In particular, a couple of examples are reported for the main member PGC-1 α , which was found by Mootha et al., 2003 to activate a large set of genes expressing OXPHOS proteins, mediating insulin-dependent glucose disposal; moreover, PGC-1 α coordinates triglyceride metabolism (Zhang Y et al., 2004).

The activity of PGC-1 family are in turn modulated by a range of cellular signaling pathways, through the regulation of PGC-1 expression levels or post-translational modification.

These co-regulators behave like a sensors and they can be influenced by metabolic signals and stress signals, thus modulating the mitochondrial biogenesis. For instance, energy deprivation, represented by decreased ATP/AMP and NADH/NAD⁺ ratios, activates PGC-1 α through both the phosphorylation via AMP activated protein kinase (AMPK) pathway or by sirtuin

(SIRT1) mediated deacetylation. The cellular energy status acts on PGC-1 α also through the mammalian target of rapamycin (mTOR) (Hock MB and Kralli A, 2009).

Interestingly, PGC-1 α has been found altered also in cancer; the promoting tumour growth by inducing gene expression programs supporting lipogenesis (Bhalla K et al., 2011) is only an example.

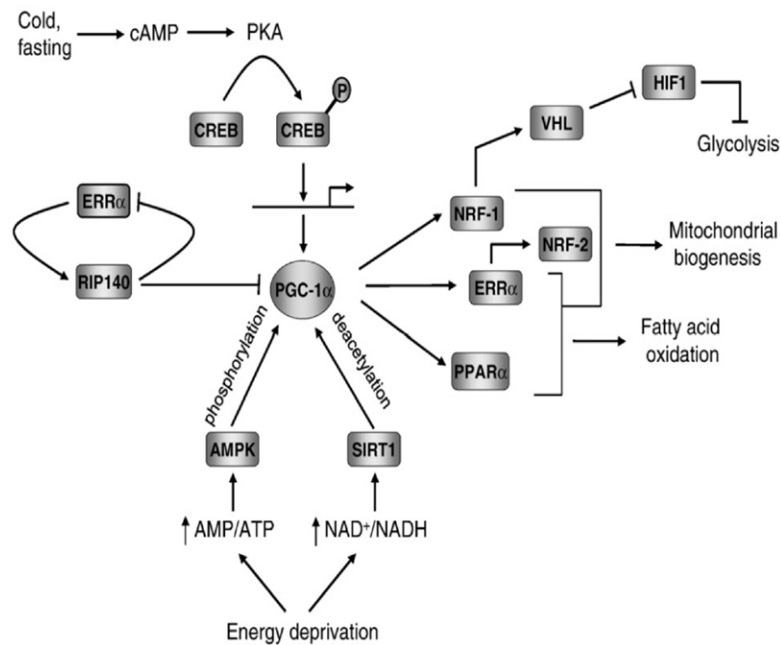


Figure 3.8 The central role of PGC-1 α in the regulation of mitochondrial biogenesis (Scarpulla, 2011).

3.4 Breast cancer

Breast cancer is one of the most common forms of cancer in women worldwide, but it can arise also in men, even if it is rare. Each year, the American Cancer Society estimates the numbers of new cancer cases and deaths that will occur in the United States in the current year and compiles the most recent data on cancer incidence, mortality, and survival. In 2015 Siegel, RL et al. has estimated 249,260 new breast cancer cases, comprising both sex, of which 40,890 estimated death in 2016.

Breast cancer is a heterogeneous group of diseases with different histological, prognostic and clinical aspects. Breast tumours are classified according to important clinical features, such as the age of the patient, the tumour stage (I, II, III), the cancer type (ductal, lobular, ductal and lobular, inflammatory), the histological grade, tumour size and the lymph node status that give information on the spreading of cancer cells, finally the tumour subtypes. The three different histological grades are based on the appearance of the cancer cells, grade I refers to cells which look similar to normal cells and are slow growing, grade II refers to cells that are abnormal and are growing at an increased rate, while grade III refers to cells that look very abnormal and are growing quickly. These clinical phenotypes, tumour size, tumour grade and the lymph node status, are used to calculate the Nottingham prognostic index (NPI) which is exploit to assign a probability of 5-year-survival.

In addition, breast cancer is further classified according to the expression of pathological markers, that can be up-regulated in breast cancer and of which expression is evaluated by histochemical analysis: the estrogen receptor (ER), the progesterone receptor (PR) and the human epidermal growth factor receptor 2 (HER2), each driving a particular transcriptional program. According to the expression status, breast cancers are classified as ERs positive or triple negative, that determine a different prognosis.

Further complexity was added by the analysis of gene expression data; according to the expression profile five subtypes have been identified: basal-like, Her2-enriched, Luminal A, Luminal B and normal-like (Sørlie T et al., 2001; Perou CM et al., 2000). Moreover, a set of 50 genes (PAM50) were selected to help clinician in the assignment of samples to belonging to basal-like, luminal A, luminal B, HER2-enriched or normal-like tumours and leading to the choice of the beast treatment (Parker JS et al., 2009).

So far, no studies have involved the analysis of mitochondrial genes in the classification of breast cancer.

3.5 Massively correlated biclustering: MCbiclust

Currently, novel high-throughput technologies have produced a huge amount of transcriptional data, also in the field of breast cancer (T.C.G.A. Network, “Comprehensive molecular portraits of human breast tumours” Nature 2012). The requirement to analyse and extrapolate information from this complex mass of data continue to lead the development of new software. For this purpose, our collaborator Robert Benthams developed a new algorithm named Massively Correlated Biclustering algorithm, MCbiclust (Benthams et al., 2016), which simultaneously clusters genes and samples to find hidden structures in the data. The approach shows advantages compared to the previous ones used until now: (i) it is computationally efficient to be applied to large data matrices containing whole genome transcriptomic data of more than a thousand samples; (ii) it is capable to identify correlated, biologically relevant large gene sets and subsets of heterogeneous samples where the gene set is being differentially regulated. The method addresses key question in genome biology, in particular it has been evaluated using synthetic data and applied to large bacterial and cancer cell datasets. The large biclusters discovered, so far elusive to identification by existing techniques, were found biologically relevant. Thus, MCbiclust has a great potential use in the analysis of transcriptomic data to identify large scale unknown effects hidden within the data (Benthams et al., 2016). Further details on the method are explained in the paper Benthams et al., 2016. The software is available as a Bioconductor package in R.

We exploited this tool in our study on breast cancer, to transcriptionally identify mitochondrial tumour subtypes, which have been characterised in this project.

R package

Custom made algorithm and associated methods for finding, visualising and analysing biclusters in large gene expression data sets. Algorithm is based on with a supplied gene set of size n , finding the maximum strength correlation matrix containing m samples from the data set.

Author: Robert Bentham

Maintainer: Robert Bentham <robert.bentham.11 at ucl.ac.uk>

Citation (from within R, enter citation("MCbiclust")):

Bentham R (2017). MCbiclust: Massive correlating biclusters for gene expression data and associated methods. R package version 0.99.41.

4. AIM OF THE WORK

During tumorigenesis, different stressors, among which oncogenes and metabolic stressors, force cells to adapt. The induction of specific genes can highlight the presence of a specific transcriptional programs that influence cell function and viability.

Recently, cancer metabolism has gained interest, due to the understanding that its alteration might not be simply a consequence of oncogene activation, but might play an active role. Metabolism is tightly linked to cellular bioenergetics, thus reprogramming of cellular metabolism and bioenergetics occurs together during cellular transformation. In this context mitochondria have a central role; indeed in the mitochondria the oxidative phosphorylation with energy production takes place, intimately linked to metabolic pathways, in particular the TCA cycle. In turn, mitochondrial transporters and shuttles move TCA cycle intermediates from mitochondria to cytoplasm and *vice versa*, participating in the whole cell metabolic network.

The hypothesis: Mitochondrial gene expression and function drives tumour adaptation and evolution, so the adaptive process induces specific mitochondrial gene expression pattern and related metabolic programs. Mitochondria act as sensor and play an active and plastic role in the adaptation.

Our work was aimed to distinguish tumour subtypes, characterised by a specific mitochondrial pattern that we call mitochondrial tumour subtypes (MTSs). In order to achieve this aim, we took advantage of the MCBiclust algorithm to analyse breast cancer transcriptomes and identify biclusters of which a pattern could underlie a specific transcriptional program. Following the transcriptional identification of MTSs, we performed functional verification of the MTS subtypes. In particular, cellular metabolism and bioenergetics were analysed in order to identify signalling and metabolic pathways peculiar of MTSs.

My PhD project focused on reprogramming of cellular metabolism and bioenergetics in breast cancer models of the newly identified mitochondrial

tumour subtypes. We evaluated bioenergetics by measuring mitochondrial proteins involved in the energy production and testing the mitochondrial function. We looked at metabolism, analysing the expression of metabolic enzymes at both protein and transcriptional level, finally concluding with metabolic fluxes analysis. In addition we mimic a nutrient-deprived environment to evaluate the acute response and the adaptation. We assessed the mitochondrial health through the analysis of the steady state mitochondrial membrane potential. Then we looked for evidences which could indicate that mitochondrial biogenesis process was turn on. For that, we measured the mtDNA content, the expression of mitochondrial proteins important for the energy production and the ATP production itself.

5. MATERIALS AND METHODS

5.1 Cell cultures

Five breast cancer cell lines were used to perform experiments in this work: MCF7, HCC202, MDA-MB453, Hs578T, MDA-MB436. All the cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) (cat# 41966-029, ThermoFisher), supplemented with 10% Fetal Bovine Serum (FBS) (cat# 10500-064, ThermoFisher) and 10% Normocin (cat# ant-nr-1, Labogene). Cells were maintained in culture in a 37°C incubator set with 5% CO₂ and 95% humidity. The right cell number necessary for each experiment was counted using the hemocytometer.

5.2 Deprivation treatment

For the deprivation treatment, cells were incubated with medium containing reduced either glucose or glutamine, with or without pyruvate. All the media, composed by a combination of different nutrients concentration, were prepared starting from a Dulbecco's modified Eagle's medium (DMEM) no glucose, no phenol red, no glutamine (cat# A14430-01, ThermoFisher), supplemented with 10% Fetal Bovine Serum (FBS) (cat#, 10500-064 ThermoFisher) previously dialysed with SnakeSkin™ Dialysis Tubing, 3,5K MWCO, 35 mm dry I.D., 35 feet (cat# 88244, ThermoFisher) in sterile Phosphate Saline Buffer (PBS1X) (cat#, 20012-019 ThermoFisher) in the proportion of 50mL of serum per 5L of PBS1X. The glucose powder suitable for cell culture (cat# G7021, Sigma) were weight to reach the final concentration of 25mM, 10mM, 0.75mM; the same was done for the Sodium Pyruvate (cat# P2256, Sigma) and Phenol Red powders (cat# P3532, Sigma) to obtain the concentration of use respectively of 1mM and 0.04mM. Finally the sterile stock glutamine solution (cat# 25030-024, ThermoFisher) were diluted to the final concentration of 2mM or 0.2mM. The obtained solutions were filtered using 0.22µm sterile filter to guarantee the medium sterility.

5.3 Cell growth

5.3.1 Counts

To build growth curves, 12-well plates were used. Each plate were seeded with one cell line at a dilution of 4K cells per well, to test three different glucose concentrations in triplicate: glucose 10mM, glucose 0.75mM, galactose 10mM (glucose 0mM), all of them containing normal glutamine (2mM), supplemented or not with pyruvate 1mM. The time course was of 19 days: the seeding at day 1 was executed using a DMEM, high glucose, GlutaMAX™ Supplement, pyruvate (cat# 31966-047 Thermo Fischer); deprivation treatment at day 4 was done by substituting the medium with experimental one after washing twice with sterile PBS1X (cat# 20012-019, Thermo Fischer); cell counting at day 6, 12 and 19 was done trypsinising and determining the cell number by hemocytometer. The entire protocol was repeated for all the five cell lines utilised in this work. Then the average of the three technical replicates for each condition in all the cell types was calculated and the results was plotted on the XY chart as a function of the days; finally a doubling time was estimated, and a ratio between doubling time calculated.

5.3.2 Imaging

Cell proliferation was measured in a time course of 8 days, using 96-well plates; on each plate four cell lines were seeded (MCF7, HCC202, Hs578 T, MDA-MB 436) in triplicate for all the deprivation treatment tested (HH: glucose 25mM, glutamine 2mM; HL: glucose 25mM, glutamine 0.2mM; LH: glucose 0.75mM, glutamine 2mM; LL: glucose 0.75mM, glutamine 0.2mM; all of them supplemented (+) or not (-) with pyruvate 1mM). The seeding and the treatment was done respectively at day -1 and day 0 and the cell number was evaluated at day 2, 4, 6 and 8. Imaging was done using ImageXpress Micro XL high content imaging system. The resulted cell number/fild was plotted as a function of the days.

5.4 Western blotting

This technique was used to evaluate the endogenous expression of proteins in our experimental models in normal culturing conditions and during deprivation treatment. Cells were lysated in RIPA-buffer (150 mM NaCl, 50 mM Tris, 1 mM EGTA, 1% Triton X-100, 0.1%SDS) containing ROCHE inhibitors cocktails; after 30' of incubation on ice the protein lysates were quantified using Pierce BCA protein assay kit (reagent A cat# 23228, reagent B cat# 23224 Thermo Scientific) according to the manufacturer's instructions. Samples were then prepared for loading with loading buffer and denatured by boiling at the appropriate temperature for each antibody used (10' at 60°C for MitoProfile; 5' at 90°C for all the other antibodies). Samples were loaded in the amount of 20µg per well in 4-12% BisTris NuPAGE gel (cat# NP0321BOX Thermo Fischer) and run at 150V in MOPS running buffer until reaching the bottom of the gel. For the blotting, 2h at 30V, a PVDF membrane and the Life Technologies wet system were used. The PVDF was first pre-soak in methanol for the activation and then in the transfer buffer. Successively, a Ponceau-S staining was done to check the protein transferring and the membranes were blocked for one hour at room temperature in Tris-buffered saline (TBS)(Tris 0.5M - NaCl 1.5M)- Tween 0.1% and 5% milk. An appropriate dilution of primary antibody was used to incubate the membrane overnight at 4°C. Horseradish peroxidase-conjugated secondary antibody was applied for 1h at RT. After each antibody incubation, three washes of 5' were done with TBS-Tween 0.1% on an orbital shaker. To reveal the membrane a Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific) was used according to manufacturer's instructions. The obtained images was analysed using ImageJ (downloadable for free).

We used the following antibodies: MitoProfile® Total OXPHOS Rodent WB Antibody Cocktail (1:2000, Miotosciences #MS604,), which recognises one subunit of each respiratory chain complex (CI subunit NDUFB8, CII-30kDa, CIII-Core protein 2, CIV subunit I, CV alpha subunit. The mAbs in the cocktail were chosen because they are against a subunit that is labile when its complex is not

assembled); anti-HK1, anti-HK2, anti-PKM1 and anti-PKM2 (all 1:1000, Cell Signalling respectively #2024, #2867, #4053, #7067); anti-TPI, anti-PDH-E1alpha, anti-IDH2, anti-ME1, anti-PPAT, anti-GLS1, anti-GLUD, anti-GFPT1 and anti GFPT2 (all 1:1000, Abcam respectively #ab135532, #ab110330, #ab55271, #ab97445, #ab128116, #ab93434, #ab166618, #ab125069, #ab190966); anti-IDH1, anti-PSAT1, anti-GOT1 (all 1:1000, Proteintech respectively #12332-1-ap, #20180-1AP, #14986-1AP); anti-PHGDH (1:1000, Santa Cruz #sc292792); anti-GS (1:1000, BD Biosciences #610518); secondary HRP-conjugated antibodies (all 1:5000, BioRad anti-rabbit #170-6515, anti-mouse #170-6516).

5.5 ATP measurements

The ATP levels were evaluated using a Luciferin/Luciferase based approach. In our study, the CellTiter-Glo® Luminescent Cell Viability Assay (cat# G7572, Promega) was used. The method determines the number of viable cells in culture based on the quantitation of the ATP present, which signals the presence of metabolically active cells, in our case it was chosen with the aim to estimate the ATP produced by a known number of seeded cells and compare the results among cell types. It consists in a one-shot reaction; indeed, after the addition of the reconstituted reagent, cell lysis and generation of a luminescent signal proportional to the amount of ATP present occur. The assay relies on the properties of a thermostable luciferase (Ultra-Glo™ Recombinant Luciferase), which generates a stable “glow-type” luminescent signal. The luciferase reaction consists in the mono-oxygenation of beetle luciferin catalysed by Ultra-Glo™ Recombinant Luciferase in the presence of Mg²⁺, ATP and molecular oxygen with a production of oxyluciferin AMP, PP, CO₂ and light emission. The manufacturer’s instructions suggested the following technical protocol: 1- after adding the test compound, equilibrate the plate and its contents at room temperature (RT) for approximately 30 minutes, avoiding temperature gradients which could cause uneven signals; 2- add a volume of CellTiter-Glo® Reagent equal to the volume of cell culture medium present in each well (e.g., add 100µl of reagent to 100µl of

medium containing cells for a 96-well plate); 3- mix contents for 2 minutes on an orbital shaker to induce cell lysis; 4-to incubate the plate at RT for 10 minutes to stabilize luminescent signal; 5- record luminescence.

In this work, the ATP production was evaluated after 30' and 24h of deprivation treatment with the following media: HH -/+; HL -/+; LH -/+; LL -/+. In details, at day -1 5K cells of MCF7, HCC202, Hs578 T, MDA-MB-436 cell lines were seeded in opaque black 96-well plate in double, two plate-lines per cell type except for the last column of the plate, on which the blank (for removing the medium background during data analysing) and the ATP standard of 10mM (on which the samples luminescent was normalised to) were loaded. At day 0 the treatment was applied for 30' and 24h per each condition (every plate with one combination of glucose and glutamine, with and without pyruvate, e.g., HH+ and HH-). After the deprivation treatment and before measuring the ATP levels, in order to discriminate the contribution of mitochondria and glycolysis to the ATP production, cells were treated with the ATP synthase inhibitor oligomycin at a concentration of 10µM, or with the glucose analogue 2-deoxiglucose (cat# D8375, Sigma) (2-DG, lacking of the 2-hydroxyl group replaced by hydrogen) which is not processed through glycolysis, at a concentration of 10mM. Moreover the combination of both compounds was applied to completely abolish the ATP production and the resulted values used to exclude the internal background during the analysis. The media containing inhibitors was prepared using a serum-free DMEM no glucose, no phenol red, no glutamine, supplemented with HEPES 10mM at pH7.4. The glucose and glutamine concentration and the presence or not of pyruvate was determined by the deprivation treatment previously done on each plate; moreover when the glycolytic inhibitors 2-DG was used, glucose was not added to the medium containing inhibitor. The treatment with the inhibitors was done for 30' at room temperature, allowing in this manner also the RT equilibration of the plate, as suggested by the manufacturer's instructions as described at the beginning of this paragraph. The luminescence was recorded using PerkinElmer EnVision plate

reader. Finally, protein content of each well were evaluated using the BCA protein assay as index of the cell content per well.

For each plate the triplicate values for the total ATP produced (no inhibitors added), the amount of ATP after oligomycin and 2-DG treatments was obtained; the duplicate values was recorded for the oligomycin/2-DG inhibition; triplicate values for the ATP standard and five replicates of medium luminescence for the background were acquired. The average of the replicates was calculated and the background was subtracted. The resulted values were normalised to the ATP standard previously adjusted for the background and further normalised to the protein content.

Data analysis showed fluctuation in the values produced by the treatment of the combination of the two inhibitors oligomycin and 2-DG, consequently these data couldn't be used to remove the internal background. To overcome the problem the data were analysed as described in the scheme of the figure 6.4.2D.

5.6 Oroboros assay

Oxygen consumption rates were measured in collaboration with Cathy Qin, an undergraduate medical student at UCL London, using an Oroboros Oxygraph-2k high resolution respiratory system. Cells were grown to confluency in 10cm plates for 48 hours prior to the assay. The cells were diluted to 1 million cell/ml in a respiration buffer (DMEM powder (8.3g/L), sodium pyruvate (110mg/L), glucose (1000mg/L), Glutamax 100x (10ml/L), Sodium bicarbonate (3.7g/L), FBS 10% and media adjusted to 7.4pH and filter sterilised with a 0.22 Stericup). Prior to the assay, electrode air calibration was performed with the respiration buffer, as suggested by manufacturer's protocol. 2ml of the cell suspension were added to each of the two chambers, and the O₂ flow signal allowed to stabilise to the basal respiration rate. Drugs were added to the chambers using Hamilton syringes at the following concentrations and order: oligomycin (2.5µM), carbonylcyanide-p-(trifluoromethoxy)-phenylhydrazone (FCCP) (titrated 1µl at a time from a 1mM stock, to produce maximal respiratory capacity), and antimycin

A (2.5 μ M). Oligomycin inhibits adenosine triphosphate (ATP) synthase and therefore blocks the main proton channel into the mitochondrial matrix, the resulting respiration rate is due to the proton leak in the inner mitochondrial membrane. FCCP uncouples the inner mitochondrial membrane allowing protons to freely pass across the membrane. This equalises the mitochondrial membrane potential but also leads to the flow of electrons in the ETC not being dependent on the number of protons in the mitochondrial matrix. This results in a maximal respiration rate where the ETC is not limited by the number of protons in the mitochondrial matrix. Antimycin A inhibits cytochrome C reductase otherwise known as Complex III in the ETC. This stops all oxygen consumption from the mitochondrial and gives us a value for non-mitochondrial respiration that can be subtracted from the basal, leak and maximal rates to give mitochondrial specific rates. Data were then extracted and analysed using O2K cell analysis template to give oxygen consumption per unit cells. Significance between different groups was then tested by one-way analysis of variance (ANOVA).

5.7 Mitochondrial membrane potential measurement

The measurement of mitochondrial membrane potential is based on the distribution of the mitochondrion-selective lipophilic cation "tetramethyl rhodamine methyl ester" dye (TMRM, Life Technologies). It is fluorescent and membrane permeable and its distribution into intracellular compartments is triggered by electrochemical gradients. Hence, at low concentrations, its accumulation into mitochondria was shown to be driven by mitochondrial membrane potential (almost -180mV). In order to promote the correct distribution of the probe, cells were loaded with the dye-solution at very low concentration. Changes in mitochondrial membrane potential cause a redistribution of the dye between mitochondria and cytoplasmic environment. In our work, $\Delta\psi_m$ of four cell lines (MCF7, HCC202, MDA436, Hs578T), treated with 8 different deprivation treatment (HH -/+; HL -/+; LH -/+; LL -/+), was evaluated. The same time course protocol of 8 days used for imaging the cell

growth (paragraph 5.3.2) was applied also in this case. Cells were loaded with TMRM solution. The probe was excited at 560 nm and the emission light was recorded in the 590-650 nm range. FCCP (carbonyl cyanide p-trifluoromethoxyphenylhydrazone, 10 μ M), an uncoupler of oxidative phosphorylation, was added to completely collapse the electrical gradient established by the respiratory chain ($\Delta\Psi$). Data are expressed as difference between the TMRM fluorescence before and after FCCP depolarization. ImageXpress Micro XL high content imaging system was used in TMRM experiments.

5.8 Mitochondrial biogenesis determination

Mitochondrial biogenesis can be evaluated at different levels among which mitochondrial proteins and mitochondrial DNA (mtDNA) content.

The first aspect was evaluated by measuring the protein levels of respiratory chain complexes at normal culturing condition and after glucose deprivation treatment. The seeding protocol, the time course and the media was the same of which used to build the growth curve by counts (paragraph 5.3.1); at day 19 cells were collected and processed by western blotting analysis using the MitoProfile antibody cocktail (paragraph 5.4).

The mtDNA content was determined by imaging. The protocol used was the same applied for the imaging of cell growth and $\Delta\Psi_m$: time course of 8 days and 8 different deprivation treatments (HH -/+; HL -/+; LH -/+; LL -/+). Before imaging by ImageXpress Micro XL high content imaging system, cells were labelled with Hoechst, TMRM and picoGreen to see respectively the nuclei, mitochondria and dsDNA, in this case mtDNA. From the Hoechst/picoGreen ratio dsDNA spots, net of nuclear DNA, were revealed. From the TMRM/picoGreen overlay the overlap of green spots of mtDNA and mitochondria were clearly observed.

5.9 Metabolomics: Gas chromatography mass spectrometry (GC-MS)

Cells were grown at standard culturing condition as described in cell culture methods, but with carbon-13 labelled glucose/glutamine to the media. The metabolomics analysis and the procedure described below, was done in collaboration with Dr Mariia Yuneva at the Crick Institute.

Before metabolite extraction, cell plates were taken to a cold room 500µL of medium from each plate was put into 1.5mL tubes, for later analysis, and frozen. The remaining media was removed and the plates placed in Fan ice/water bath before washing two times with 5ml of ice-cold PBS.

To extract the metabolites the following process was used: 800µL ice-cold methanol, containing an internal standard of 1mM scyllo-inositol, was added to the plates; cells were then detached from the plate by scraping with a cell scraper. This mixture was added to a 15ml tube, and the plate then washed with 400µL of methanol and 400µL of H₂O which was also added to the tube. Then 400µL of ice-cold chloroform was added to each tube. The tubes were placed in a water bath sonicator in a cold room for one hour, with 3x8 minute pulses of sonication and then centrifuged for 10 minutes at 16,000rpm at a temperature of 0°C. The supernatant was extracted and dried in a vacuum concentrator. The cell pellet was then re-extracted with 200µL of methanol and 100µL of H₂O, this was sonicated, spun and the supernatant added to previous supernatant tube and dried again in a vacuum concentrator. The remaining cell pellet was used for estimating dry weight and measuring total protein. The dried supernatant was resuspended in 50µL chloroform, 150µL methanol and 150µL H₂O and spun for 5 minutes at 0°C and 16,000rpm. The extract is then in a biphasic partition, with the upper phase containing the polar metabolites and the lower phase containing lipidic metabolites. The polar phase portions of each extract were then transferred to GC-MS vial inserts and dried in a vacuum concentrator. Separate vial inserts had 10µL of the saved cell culture medium added, with 1mM scyllo-inositol, which were also dried in a vacuum concentrator. Each vial insert then had 30µL of methanol added, containing 1µL of 5mM nor-leucine as

another internal standard, followed by 30 μ L of methanol without nor-leucine, with the vials being dried in a vacuum concentrator after each addition. Before running samples on the mass spectrometer, derivatisation was done to improve GC separation. 20 μ L methoxyamine (30mg/mL in pyridine) was added to each insert and this was vortexed briefly and then incubated at room temperature overnight. Silylation was then done by adding 20 μ L of BSTFA + TCMS reagent to each insert and incubating for 1 hour at room temperature. An Agilent 7890A GC with a 5975C triple axis detector MSD (Agilent Technologies, Santa Clara, CA) was used to analyse the samples. Metabolites were separated on an Agilent J&W 122-5532G DB-5ms capillary column (30m x 0.25mm, 0.25 μ m film thickness), in splitless mode. The injector and transfer line temperatures were 270 and 280°C, respectively. The flow rate of helium carrier gas was 0.7 mL/min. The oven temperature was programmed to hold at 70°C for 2 min, increased to 295°C at a 12.5°C/min ramp rate, increased from 295°C to 320°C at a 25°C/min ramp rate, and held at 320°C for 3 minutes. The mass spectrometer was operated in scan mode, after a 6 minute solvent delay with a range of 50–565 mass/charge (m/z) and a scan-rate of 2.8 scans per second. Metabolites were identified by matching retention times and fragmentation patterns to commercially available standards. Metabolite peaks were integrated at each isotopologue m/z using MassHunter Workstation software (Agilent Technologies). Peak areas were quantified based on peak areas of known standards using nor-leucine as an internal standard, and then metabolite levels were normalised to protein content. Mass isotopologues were stripped of the contribution from natural abundance, based on the chemical formula of derivitised fragment quantified. Percent enrichment for an isotopologue was calculated by dividing the corrected intensity by the sum of corrected intensities of all isotopologues for that metabolite. Significance of metabolite enrichment between different samples was calculated with one-way ANOVA.

6. RESULTS

6.1 Identification of two Mitochondrial Tumour Subtypes characterised by a specific nuclear encoded mitochondrial gene expression pattern

We exploited as bioinformatics approach the MCbiclust algorithm (Bentham et al., 2016) to measure correlated expression.

It was applied to the breast cancer (BRCA) microarray dataset, from the The Cancer Genome Atlas, for mitochondrial genes (MitoCarta, Pagliarini et al., 2008) in a group of cancer samples, to see whether there was a coordinated/correlated expression of these genes. The gene expression levels were compared one to each other in all the samples and a correlation value was attributed to every gene tested on the basis of its expression profile. High correlation was found only in a subset of cancer samples in which the presence of 2 highly correlated groups of genes emerged. The presence of two groups of nuclear encoded mitochondrial genes in breast cancer samples suggests the existence of a specific transcriptional program (Figure 6.1A).

To confirm the hypothesised transcriptional program found by MCbiclust, in collaboration with Robert Bentham, PhD in Szabadkai lab in London, cancer samples were plotted according to the correlation strength and gene expression levels, using Principal Component (PC) analysis looking for the presence of grouped samples. Interestingly, the first hundred samples, with highly correlating genes, split into 2 forks. Samples belonging to the upper fork highly expressed gene group1 and showed low expression of gene group2; conversely, in lower fork samples the gene expression pattern was the opposite. On the other hand, samples located at the stem of the fork showed low correlation of the same genes (Figure 6.1B).

The clustering of breast cancer samples, based on their mitochondrial gene expression profile confirmed the presence of a specific transcriptional program, characterised by two groups of nuclear-encoded mitochondrial genes co-regulated in opposite way. We refer to this specific pattern as *fork pattern* which

represent a mitochondrial biogenesis pattern (Figure 6.1B-C-D). Two Mitochondrial Tumour Subtypes (MTSs) characterised by a specific nuclear-encoded mitochondrial transcriptional signature, corresponding to the upper fork (UF) and lower fork (LF) were identified.

The fork pattern was also partially predictive of tumour type and pathology currently defined and used, indeed UF samples are primarily Luminal B whereas LF are mainly Luminal A. Interestingly, the MTSs classification (UF and LF) did not show an absolute correlation with the PAM50 intrinsic subtypes of breast cancer samples, highlighting the potentiality of MCbiclust-based classification to ameliorate the metabolic stratification of breast cancer by including the aspects related to the nuclear-encoded mitochondrial genes through a definition of specific mitochondrial transcriptional signature (UF and LF).

For this purpose it was necessary to functional verify MTSs, testing them *in vitro*, in order to identify signalling and metabolic pathways representative of the UF and LF. For this reason, exploiting again the MCbiclust, breast cancer cell lines representative of the fork pattern were selected, similarly as MCbiclust was applied previously to the CCLE dataset for the MitoCarta genes (Bentham et al., 2016).

Table 1. Source, clinical and pathological features of tumours used to derive breast cancer cell lines used in this study, associated with the fork pattern.

Fork Pattern	Cell Line	Gene cluster	ER	PR	HER2	TP53	Source	Tumour Type	Age years	Ethnicity
Upper Fork (UF)	MCF7	Lu	+	[+]		+/- ^{WT}	PE	IDC	69	W
	HCC202	Lu	-	[-]	+	[-]	P.Br	Duc.Ca	82	W
	MDA-MB 453	Lu	-	[-]		- ^{WT}	PF	AC	48	W
Lower Fork (LF)	Hs578T	BaB	-	[-]		+ ^M	P.Br	IDC	74	W
	MDA-MB 436	BaB	[-]	[-]		[-]	PE	IDC	43	W

AC, adenocarcinoma; BaB, Basal B; Duc.Ca, ductal carcinoma; IDC, invasive ductal carcinoma; Lu, luminal; P.Br, primary breast; PE, pleural effusion; W, White.

ER/PR/HER2/TP53 status: ER/PR positivity, HER2 overexpression, and TP53 protein levels and mutational status (obtained from the Sanger web site; M, mutant protein; WT, wild-type protein) are indicated. Expression data are derived from mRNA and protein levels. Square brackets indicate that levels are inferred from mRNA levels alone where protein data is not available.

Adapted from Neve RM et al., Cancer Cell. 10: 515-27 (2006).

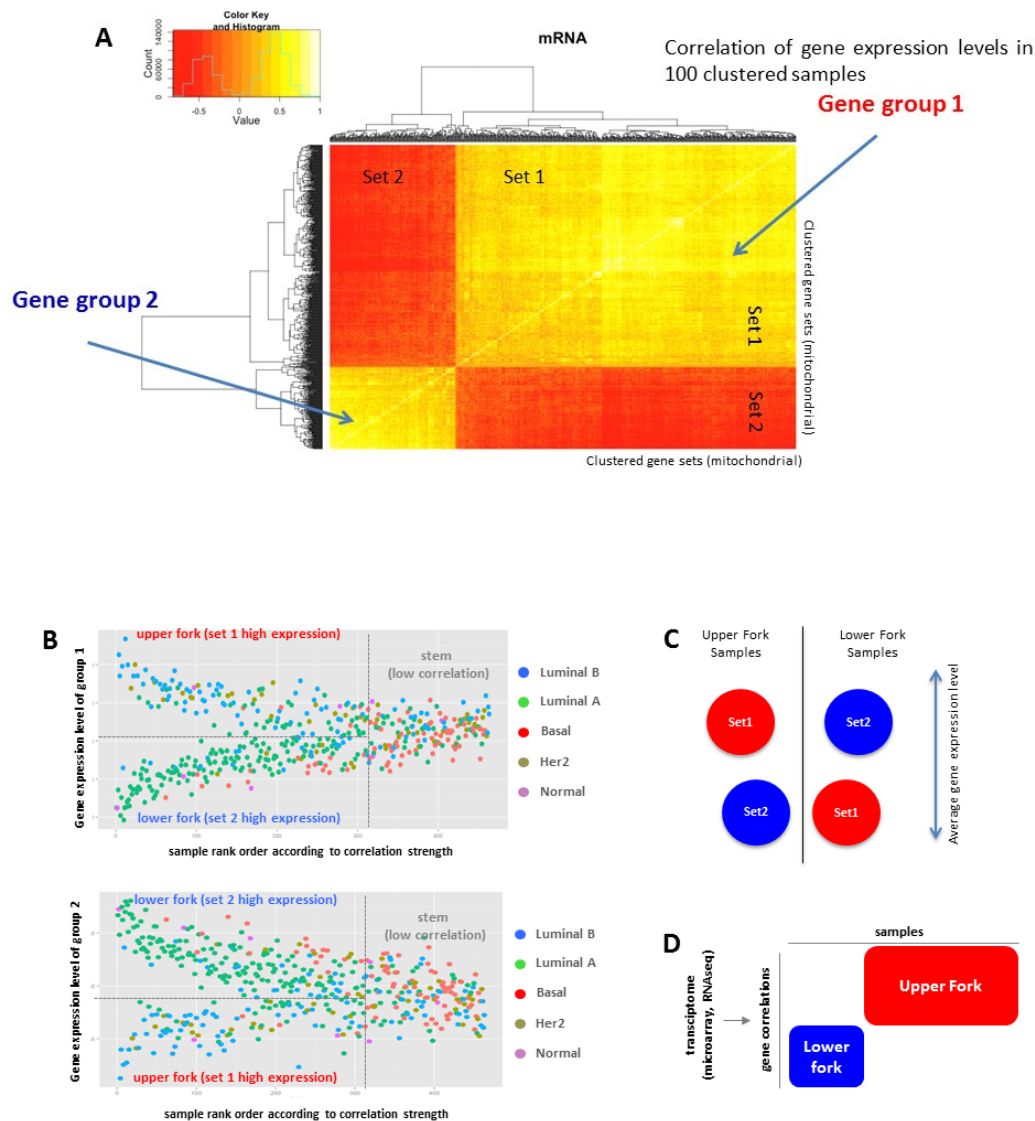


Figure 6.1 Transcriptional signature of mitochondrial biogenesis (or MCbiclust and the resulted Fork Pattern) **A:** Correlation of gene expression levels in 100 clustered samples. The gene-gene heatmaps of the BRCA bicluster showing the division of the genes into two groups with different regulation in the upper and lower fork samples. Figure produced by Robert Benthams. **B:** Fork pattern. Ranking of the samples according to the correlation strength of gene group1 and gene group2. Figure produced by Gyorgy Szabadkai. **C:**Transcriptional program: co-regulation in opposite way of gene group1 and gene group2. **D:** Mitochondrial tumour subtypes (MTSS): characterised by nuclear encoded mitochondrial transcriptional signature.

6.2 Mitochondrial tumour subtypes differentially express respiratory chain complexes and this is reflected in mitochondrial function

We decided to characterise the mitochondrial biogenesis pattern associated with the MTSs, upper fork and lower fork, starting from the proteins crucial for ATP production.

We kept the breast cancer cell lines representative of the fork pattern (UF: MCF7, HCC202, MDA-MB 453; LF: Hs578T, MDA-MB 436) at standard culturing condition (glucose 25mM, glutamine 2mM and pyruvate 1mM). Cells were collected and the protein expression profile of the respiratory chain complexes was evaluated by western blotting analysis, using the MitoProfile antibody cocktail which recognises one subunit of each respiratory chain complex (CI subunit NDUF8, CII-30kDa, CIII-Core protein 2, CIV subunit I, CV alpha subunit. The mAbs in the cocktail were chosen by the company, because they are against a subunit that is labile when its complex is not assembled). We interpreted the variation of protein expression of the single subunit as a variation of the entire complex. We normalised the results obtained for each subunit to β -tubulin, the mitochondrial heat shock protein grp75 and the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH), to highlight differences related to the cytoplasm/cytoskeleton, the mitochondrial content and glycolytic function (Figure 6.2A). Interestingly, significant differences were found in ETC protein expression between UF and LF; in particular looking at the normalisation to the general state of the mitochondria, we observed complex I and IV more expressed in the UF compared to the LF (Figure 6.2C-D). Moreover, we analysed also the ratio between the proteins on which complexes were normalised to. Interestingly, the grp75/ β -tubulin ratio indicated that UF has more mitochondrial content at protein level than LF and the same result was found also for the glycolytic enzyme gapdh (Figure 6.2B). In summary, these results showed differences in expression of OXPHOS component between the two forks, in particular UF revealed higher mitochondrial content of CI and CIV as compared to the LF.

To verify if these differences in respiratory chain protein expression could reflect a different mitochondrial function between UF and LF, we followed the mitochondrial respiration by measuring the oxygen consumption rate with Oroboros assay. The experiment was done in collaboration with Cathy Qin, an undergraduate medical student at UCL London. The levels of oxygen consumed by mitochondria in order to produce ATP gave information on the working status of the ETC. Cells were cultured at standard culturing condition; the oxygen consumption was evaluated at: basal respiration, when ETC and ATP synthase are coupled; Leak (uncoupled) state, when ATP synthase is inhibited by oligomycin and the oxygen consumption is due to the proton leak through the inner mitochondrial membrane; maximal respiration, in which there is uncoupling between the ETC and ATP synthase obtained after addition of the uncoupler FCCP and consequently protons can easily flow inside mitochondrial matrix dissolving the membrane potential and rendering ETC independent from the proton gradient. The results (Figure 6.2E) showed higher respiration (higher oxygen consumption) of the UF in basal, Leak (uncoupling) and maximal respiration states compared to the LF and the differences were statistical significant. These results confirmed that the proteomic differences observed by western blotting analysis cause functional mitochondrial differences.

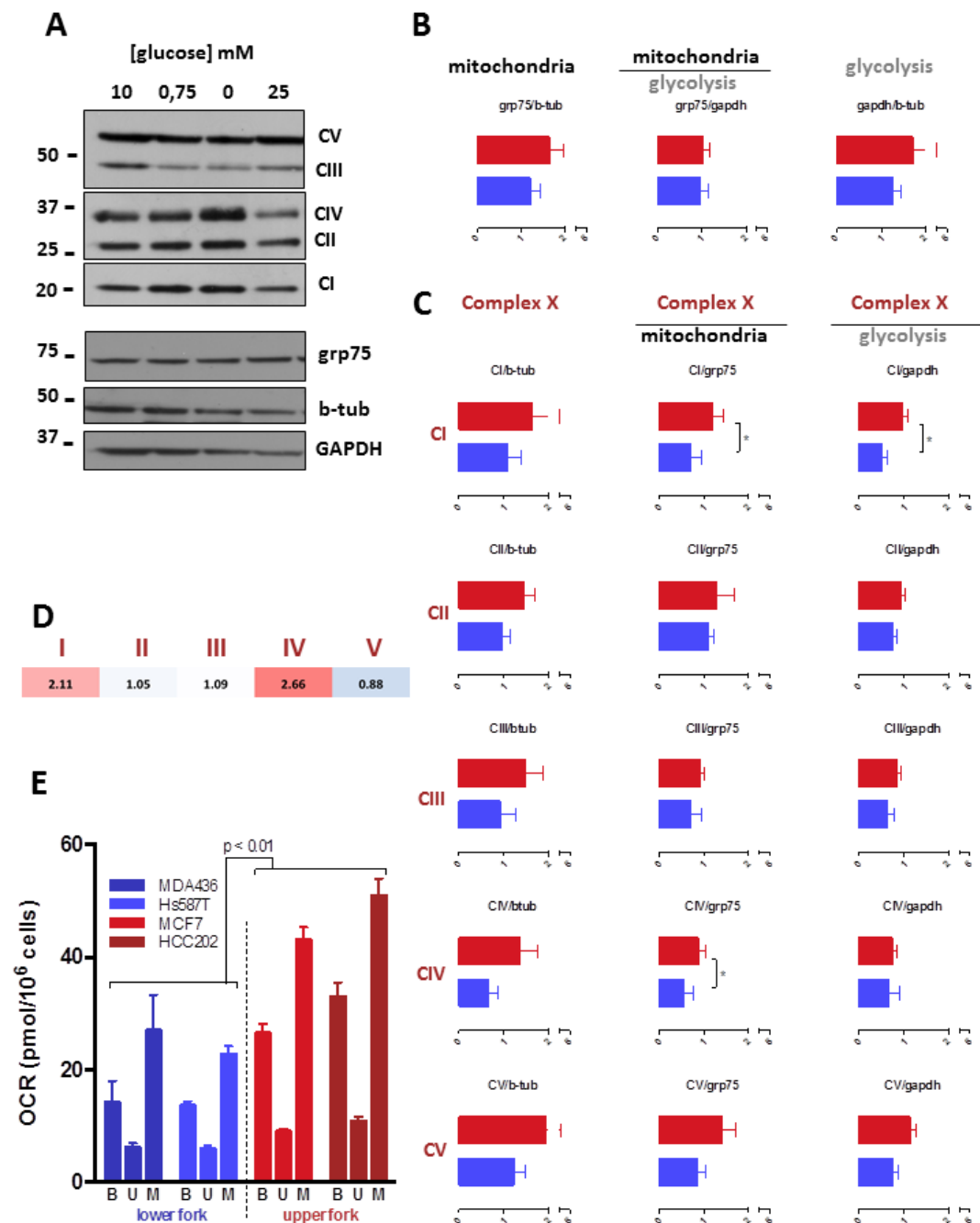


Figure 6.2 Expression profile of respiratory chain complexes and related function **A:** Representative western blots image. Results obtained from UF cell lines (MCF7,HCC202, MDA-MB-453) and LF (Hs578 T, MDA-MB-436) using MitoProfile antibody cocktail to see Complex I, II, III, IV, V (CI,CII,CIII,CIV,CV). **B:** Mitochondria content: grp75/ β -tubulin ratio is higher in UF. **C:** Quantification: CI and CIV are significantly more expressed in the UF. n=3, t-test; significance with p-value < 0.05. **D:** Complexes relative changes: UF/LF ratio shows CI and CIV more expressed in UF compared to LF. **E:** Oxygen consumption rate: Differing oxygen consumption rates in the

cancer cell lines, B=basal rate, U=uncoupling rate and M=maximal rate. The difference between the upper and lower fork cell lines was found to be significant with a p-value < 0.05.

Figure produced by Gyorgy Szabadkai.

6.3 The nuclear-encoded mitochondrial expression pattern associated to different metabolic pathways can drive different substrate preference between upper fork and lower fork

It is known that many enzymes important for the intermediate metabolism localise inside mitochondria; moreover, the presence of mitochondrial shuttles and transporters links the metabolic reactions occurring in the mitochondrion to which ones happening in the cytosol, highlighting the role of this organelle in orchestrating metabolism. Interestingly, we found a different expression profile of the respiratory chain complexes, between UF and LF, that could influence the mitochondrial oxidative metabolism and leading to specific metabolic programs characteristic of each fork.

Given these premises, with the aim to understand the metabolic arrangement of the UF and LF, we focused our attention on global cellular metabolism using different approaches: protein expression analysis, bioinformatics and metabolomics.

6.3.1 Metabolic enzymes involved in different pathways are differently expressed at protein level in the fork pattern

We evaluated the protein expression level of metabolic enzymes belonging to different pathways of energetic and intermediate metabolism: glycolysis (HK1, HK2, TPI, PKM1, PKM2); TCA cycle (PDH-E1a, IDH, IDH2), malate shuttle and pyruvate metabolism (ME1, ME2, ME3), purine pyrimidine biosynthesis (PPAT), serine metabolism (PHGDH, PSAT1), glutamine/glutamate metabolism (GLS1, GLUD, GOT1, GS), hexosamine pathway (GFPT1, GFPT2) (Table2). It is known that some enzymes preferentially use glucose-derived substrates, others glutamine-

derived substrate, others exploit the pyruvate produced inside the cell favouring pyruvate anaplerosis.

The possible differences in protein expression existing between the two forks, could predict which pathway is preferentially used by the two MTSs to sustain the metabolic needs of the cells and indicate which substrate could be better utilised to sustain cell viability and functionality. We evaluated the protein expression in breast cancer cell lines representative of the fork pattern by western blotting (Figure 6.3.1A). For normalisation we referred to the housekeeping protein α -actin and the mitochondrial heat shock protein grp75. The expression profile of each cell line and the results grouped for UF and LF were evaluated. For some enzymes the expression pattern of cells belonging to the same fork was identical, making the differences in the grouped results statistically significant, highlighting metabolic features of the forks. In other cases, the differences among the cell lines belonging to the same fork did not give significance to the grouped outcomes.

Starting the analysis from the housekeeping proteins, the grp75/ α -actin ratio showed that UF cells had more grp75 than LF (Figure 6.3.1B); the difference was statistically significant, indicating a status in which UF generally has higher mitochondrial content compared with LF (Figure 6.3.1B); this is in agreement with the result emerged from grp75/ β -tubulin ratio showed in the previous paragraph (Figure 6.2B). Successively, going through the results of metabolic enzymes, we found differences in protein expression between UF and LF, which were statistically significant. In particular, two enzymes, GLUD and GLS1, involved in the glutamine/glutamate metabolism and both localising at mitochondrial level, were expressed in opposite way in the two forks; GLUD that catalyses the oxidative deamination of glutamate to α -ketoglutarate and ammonia showed increased expression in the UF, whereas GLS1 that hydrolyses glutamine to glutamate and ammonia was more expressed in the LF (Figure 6.3.1C). Interestingly, these two enzymes characterised by the opposite expression in the two forks, share the substrate/product glutamate (Figure

6.3.1D) indicating that glutamine/glutamate metabolism could be differently arranged between MTSs. Others enzymes, GOT1 and GS, belonging to the same metabolic pathway were tested, but no significant differences has emerged (Figure 6.3.1C).

Next, we focused on other pathways in which glutamine and glutamate were involved as reactive substrates. In the purine/pyrimidine biosynthesis the enzyme PPAT, which uses glutamate to convert the 5-phosphoribosylamine-diphosphate in 5-phosphoribose-1-diphosphate and glutamine in the first step of the pathway, did not shown differences between UF and LF at grouped level, probably because of the variability in protein expression between the two LF cell lines (Figure 6.3.1E). We evaluated also pathways in which glucose and glutamine metabolism are linked: serine metabolism and the hexosamine pathway. In serine metabolism two enzymes were tested; from the comparison of the grouped results between UF and LF, we found significant differences in the protein expression of the enzyme PSAT1 (Figure 6.3.1F). The enzyme, which catalyses the reaction between phospho-serine (or phospho-threonine) and α -ketoglutarate to produce 3-phospho-pyruvate (or phosphor-butanoate) and glutamate, was more expressed in the LF and this profile was confirmed also looking at the level of the single cell line. Instead, we did not find differences for the enzyme PHGDH, which converts the 3-phospho-glycerate into 3-phospho oxypyruvate with a reduction of NAD^+ to NADH, between UF and LF at grouped level; however, looking at the protein expression of each cell lines, we observed variability among cells of the UF (Figure 6.3.1F). For the hexosamine pathway, we tested two enzymes GFPT1 and GFPT2, which both control the glucose flux into the pathway. In particular, the reaction involves D-fructose-6-phosphate (a glucose-derived substrate) and glutamine which are converted into D-glucosamine-6-phosphate and glutamate. We found a specific pattern for GFPT2 which was highly expressed in the LF, both looking at the single cell line expression and the grouped results, which were statistical significant (Figure 6.3.1G).

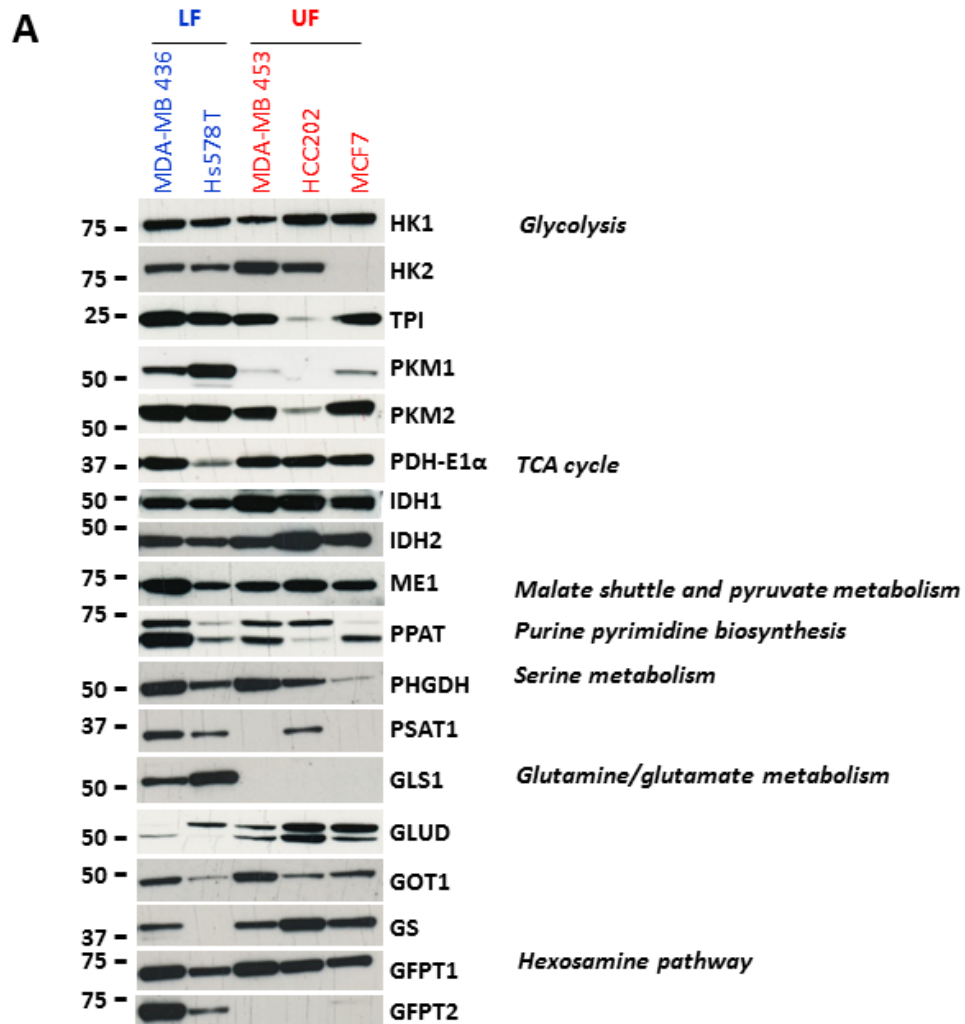
Next, enzymes involved in glucose metabolism were also analysed, in order to evaluate if the MTSs could different exploit the glucose substrate. Concerning the glycolysis, we measured the expression of HK1 and HK2 proteins, the last one is the isoform usually associated with cancer; moreover, both enzymes catalyse glucose phosphorylation to produce glucose-6-phosphate. We found no differences between MTSs in HK1 and HK2 expression (Figure 6.3.1H). We analysed also TPI, which catalyses the isomerization of glyceraldehydes 3-phosphate (G3P) and dihydroxy-acetone phosphate (DHAP). When we looked at the protein expression individually of the cell lines, we found variability in the UF, in particular HCC202 cell line expressed less enzyme compared to the other two UF cell types. However, when we observed the grouped results, TPI was significantly more expressed in the LF, but only when the normalisation was done to the mitochondrial protein grp75 (Figure 6.3.1H); the content of this glycolytic enzyme is higher in LF only related to the mitochondrial content, indicating again that LF has less mitochondria then UF. The last glycolytic enzymes we evaluated were PKM1 and PKM2; both enzymes catalyse the transfer of a phosphoryl group from phosphoenolpyruvate to ADP, generating ATP and pyruvate. PKM2 isoform, usually associated to cancer metabolism (ref), did not show differences between UF and LF. However there was variability in the UF cell lines (Figure 6.3.1H). Surprisingly, we found PKM1 significantly more expressed in LF, looking at the expression of both single cell lines and grouped results (Figure 6.3.1H).

Pyruvate is the end product derived from glycolysis, and it can undergo three different fates: it can be transported into mitochondria through the mitochondrial pyruvate carrier and fuel the TCA cycle or converted into lactate by the action of lactate dehydrogenase, or it can be carboxylated by pyruvate carboxylase to produce oxaloacetate favouring mitochondrial anaplerosis. Here, the route of the TCA cycle was considered. PDH-E1 α , IDH1 and IDH2 protein expression was measured. PDH-E1 is part of the pyruvate dehydrogenase complex (PDC), a nuclear-encoded mitochondrial multi-enzyme complex that

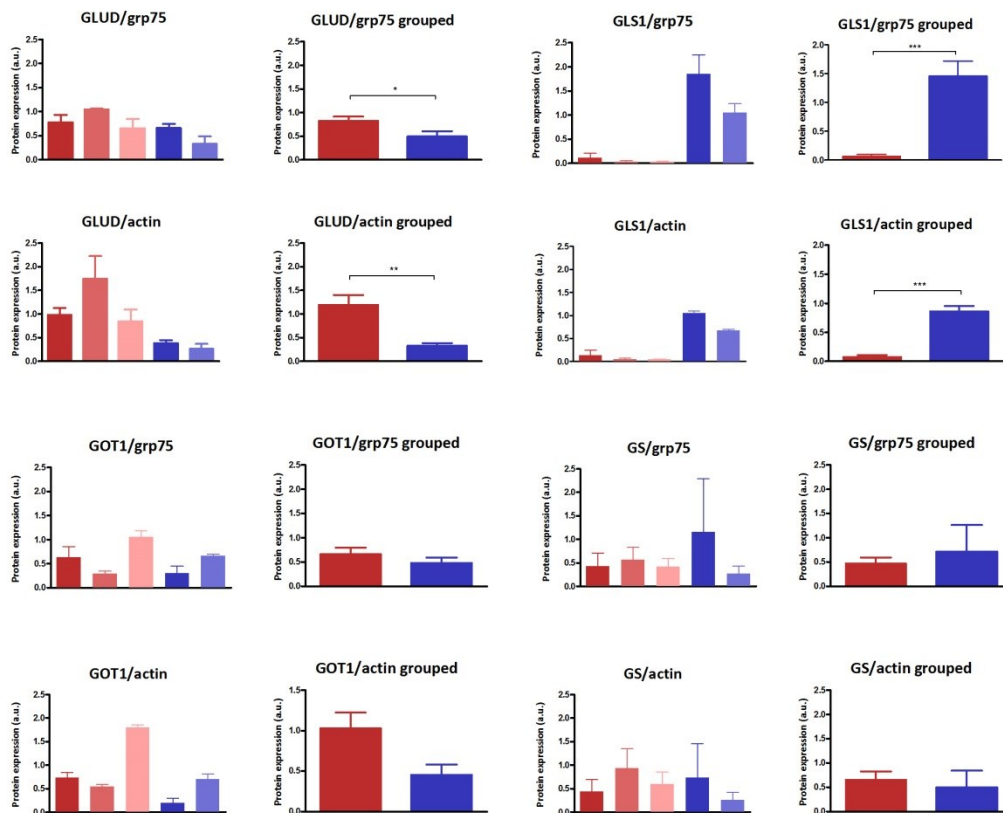
catalyses the oxidative decarboxylation of pyruvate to acetyl-CoA and CO₂, providing the primary link between glycolysis and TCA cycle. PDH-E1 α is a subunit of the pyruvate dehydrogenase enzyme (PDH-E1), a hetero-tetramer of two alpha and two beta subunits. We found no significant difference in protein expression between UF and LF (Figure 6.3.1I); however the enzymatic activity is controlled by phosphorylating-(inactivation)/dephosphorylating-(activation) events to work of PDKs (pyruvate dehydrogenase kinases) and PDPs (pyruvate dehydrogenase phosphate phosphatase) on PDH-E1 α specific serine residues, meaning that despite of the PDH-E1 α subunit was equally expressed on both forks, we do not know if there was differences in its activity. Further analysis need to be done to clarify this question. The enzyme IDH2 is a mitochondrial protein which acts in the TCA cycle catalysing the oxidative decarboxylation of isocitrate to α -ketoglutarate; IDH1 is the cytosolic counterpart that promotes the same reaction in the cytoplasm, for this reason we treated IDH1 in this section. We found both enzymes more expressed in the UF when they were normalised to α -actin (Figure 6.3.1I). Finally, we analysed enzymes involved in the malate shuttle and pyruvate metabolism. Three isoforms of malic enzymes (MEs) are known, all of them catalysing the oxidative decarboxylation of malate in the presence of NAD(P)⁺ and the decarboxylation of oxaloacetate. The ME1 is the cytosolic isoform NADP-dependent, ME2 NAD-dependent and ME3 NADP-dependent isoforms localise both in the mitochondria. We found no differences in ME1 expression between UF and LF (Figure 6.3.1L); whereas for the ME2 and ME3 we have some preliminary results (data not shown), but more experiments need to be done in order to obtain conclusive data.

Table 2. Pathway, enzyme acronym and full name, reaction and localisation.

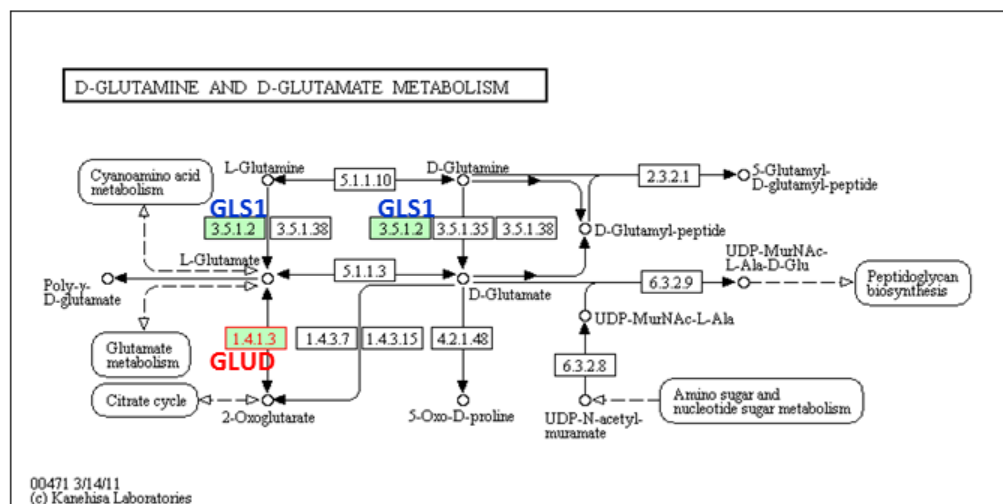
Pathways	Symbol	Full name	Reaction	Localization
<i>Glycolysis</i>	HK1	Hexokinase 1	ATP + D-hexose = ADP + D-hexose 6-phosphate	cytoplasm
	HK2	Hexokinase 2	ATP + D-hexose = ADP + D-hexose 6-phosphate	cytoplasm
	TPI	triosephosphate isomerase	D-glyceraldehyde 3-phosphate = glyceralone phosphate	cytoplasm
	PKM1	Pyruvate kinase M1	ADP + phosphoenolpyruvate = ATP + pyruvate	cytoplasm
<i>TCA cycle</i>	PKM2	Pyruvate kinase M2	ADP + phosphoenolpyruvate = ATP + pyruvate	cytoplasm
	PDH-E1alpha	Pyruvate Dehydrogenase E1 Component Subunit Alpha	Pyruvate + CoA = Acetyl-CoA + CO ₂	mitochondrion
	IDH1	Isocitrate dehydrogenase 1	isocitrate + NADP+ = 2-oxoglutarate + CO ₂ + NADPH + H+	cytoplasm
	IDH2	Isocitrate dehydrogenase 2	isocitrate + NADP+ = 2-oxoglutarate + CO ₂ + NADPH + H+	mitochondrion
<i>Malate shuttle and pyruvate metabolism</i>	ME1	Malic enzyme 1	(1) (S)-malate + NADP+ = pyruvate + CO ₂ + NADPH (2) oxaloacetate = pyruvate + CO ₂	cytoplasm
	ME2	Malic enzyme 2	(1) (S)-malate + NAD+ = pyruvate + CO ₂ + NADH (2) oxaloacetate = pyruvate + CO ₂	mitochondrion
	ME3	Malic enzyme 3	(1) (S)-malate + NADP+ = pyruvate + CO ₂ + NADPH (2) oxaloacetate = pyruvate + CO ₂	mitochondrion
<i>Purine and pyrimidine Biosynthesis</i>	PPAT	Phosphoribosyl pyrophosphate amidotransferase	5-phospho-beta-D-ribose 1-diphosphate + L-glutamine + 5-phospho-alpha-D-ribose 1-diphosphate + H ₂ O	cytoplasm
<i>Serine metabolism</i>	PHGDH	phosphoglycerate dehydrogenase	3-phospho-D-glycerate + NAD+ = 3-phosphonoxy pyruvate + NADH + H+	cytoplasm
	PSAT1	Phosphoserine Aminotransferase 1	(1) O-P-L-serine + 2-oxoglutarate = 3-phosphonoxy pyruvate + L-glutamate (2) 4-phosphonoxy-L-threonine + 2-oxoglutarate = (3R)-3-hydroxy-2-oxo-4-oxophonoxybutanoate + L-glutamate	cytoplasm
<i>Glutamine/ glutamate metabolism</i>	GLS1	Glutaminase	2 L-glutamate + NADP+ = L-glutamine + 2-oxoglutarate + NADPH + H+	cyt/mit
	GLUD	Glutamate dehydrogenase	L-glutamate + H ₂ O + NAD+ = 2-oxoglutarate + NH ₃ + NADH + H+	mitochondrion
	GOT1	Aspartate Aminotransferase 1	L-aspartate + 2-oxoglutarate = oxaloacetate + L-glutamate	cytoplasm
	GS	Glutamine synthase	ATP + L-glutamate + NH ₃ = ADP + phosphate + L-glutamine	cytoplasm
<i>Hexosamine pathway</i>	GFPT1	Glutamine-Fructose-6-Phosphate Transaminase 1	L-glutamine + D-fructose 6-phosphate = L-glutamate + D-glucosamine 6-phosphate	cytoplasm
	GFPT2	Glutamine-Fructose-6-Phosphate Transaminase 2	L-glutamine + D-fructose 6-phosphate = L-glutamate + D-glucosamine 6-phosphate	cytoplasm



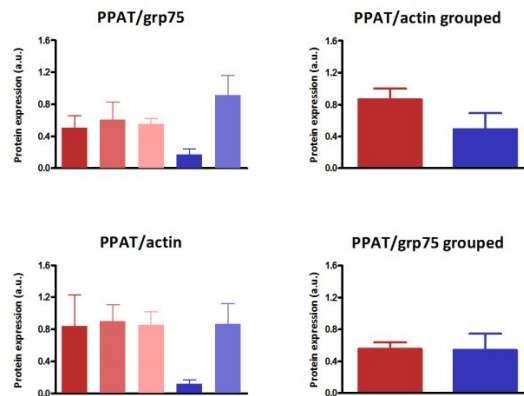
C Glutamine/Glutamate metabolism



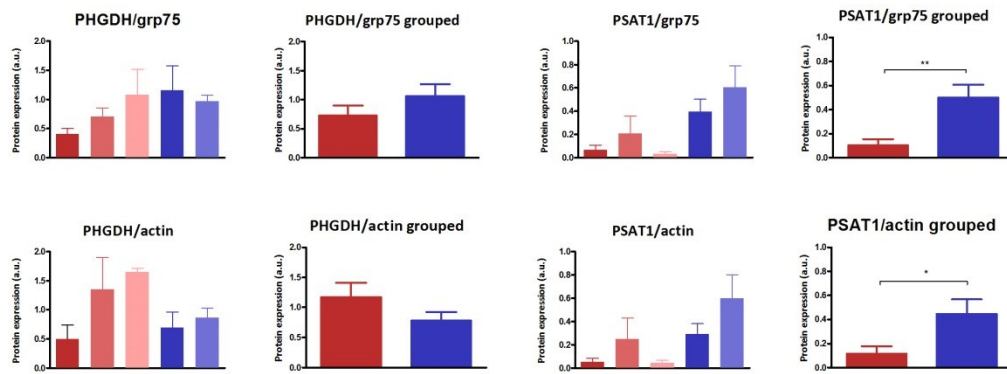
D



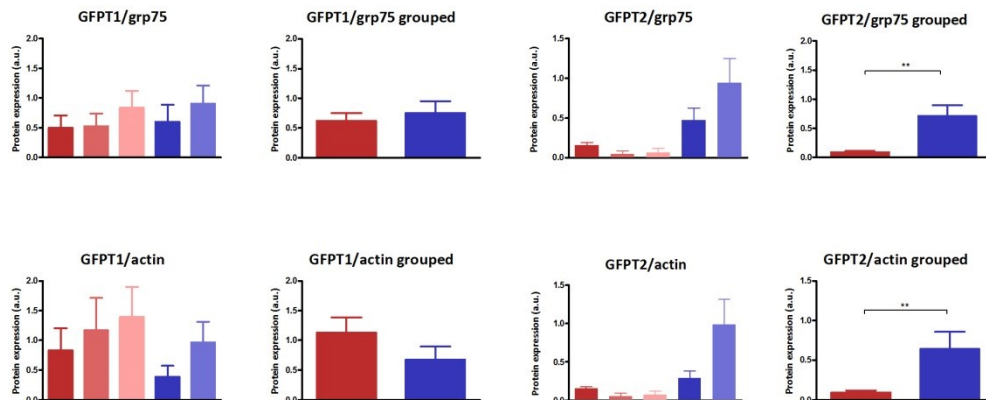
E *Purine Pyrimidine biosynthesis*



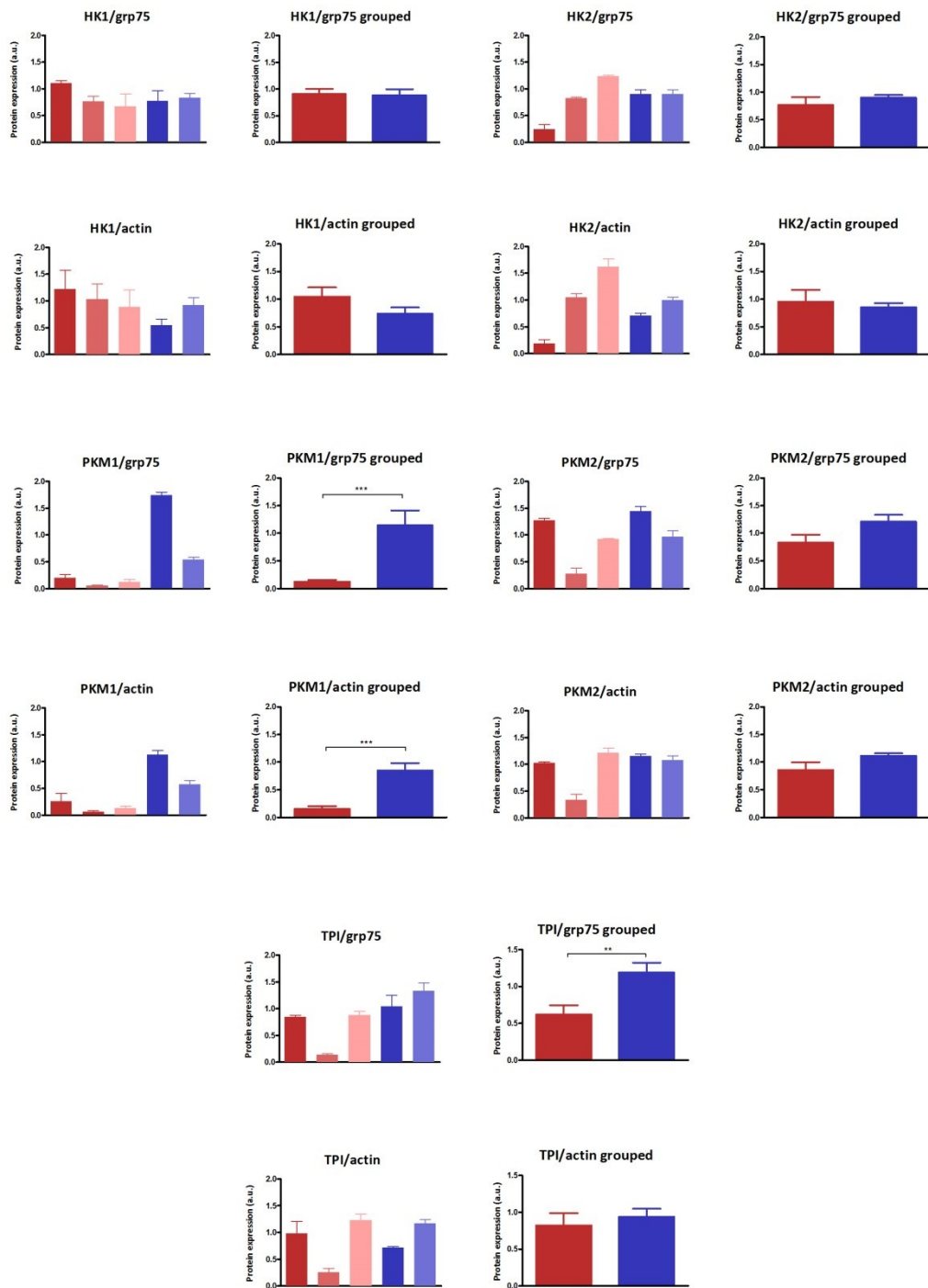
F *Serine metabolism*



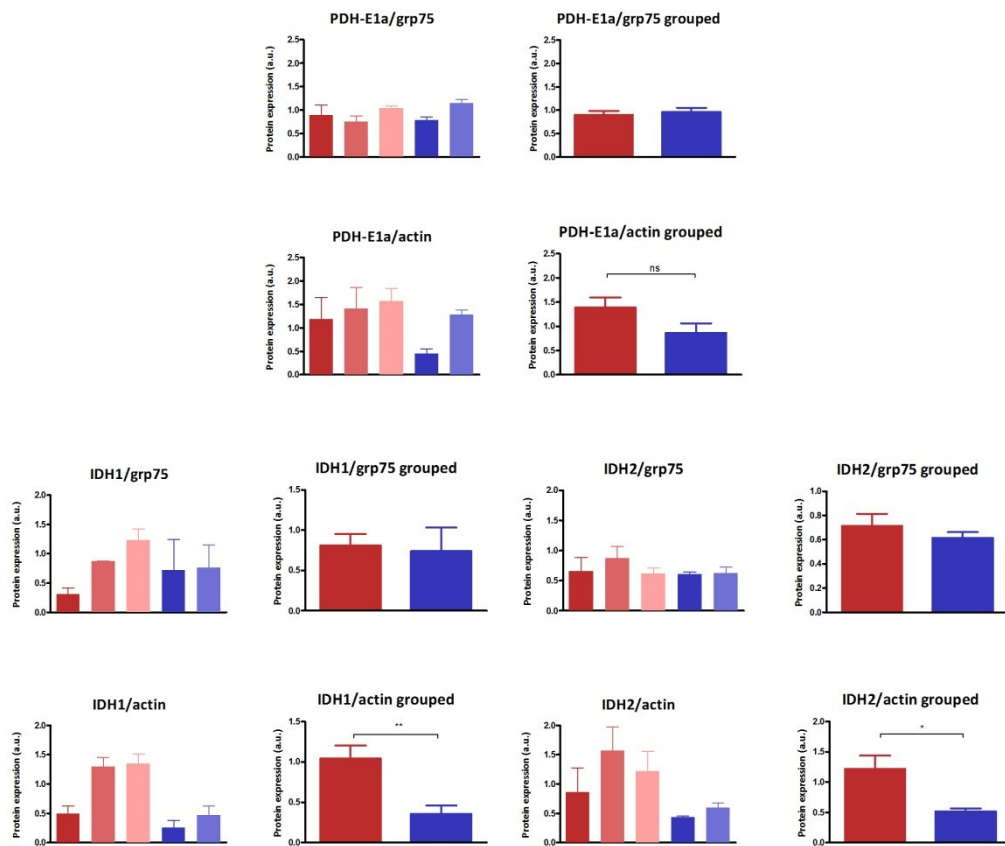
G *Hexosamine pathway*



H Glycolysis



I TCA cycle



L Malate shuttle

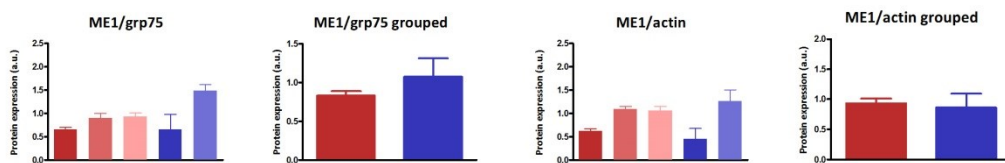


Figure 6.3.1 Different protein expression profile of metabolic enzymes between UF and LF in breast cancer cell lines **A:** Representative western blots image and mitochondrial content: the protein expression of metabolic enzymes belonging to different pathways were evaluated in UF and LF breast cancer cell lines. **B:** Mitochondria content: grp75/ α -actin ratio is higher in UF; n=3; t-test with a significance ***<0.0001. **C:** Glutamine/glutamate metabolism: quantification; n=3; t-test with a significance *p-value <0.05; ** p-value <0.01; ***p-value < 0.0001. **D:** Kegg scheme of glutamine and glutamate metabolism: GLS1 and GLUD were highlighted according to the fork pattern; glutamate is the common substrate/product shared by the two enzymes. **E:**

Purine/pyrimidine biosynthesis: quantification; n=3. **F**: *Serine metabolism*: quantification; n=3; t-test with a significance *p-value <0.05; ** p-value <0.01. **G**: *Hexosamine pathway*: quantification; n=3; t-test with a significance ** p-value <0.01. **H**: *Glycolysis*: quantification; n=3; t-test with a significance ** p-value <0.01; *** p-value <0.0001. **I**: *TCA cycle*: quantification; n=3; t-test with a significance *p-value <0.05; ** p-value <0.01. **L**: *Malate shuttle and pyruvate metabolism*: quantification; n=3. “red colour: Upper Fork; blue colour: Lower Fork”.

6.3.2 Correlation between metabolic gene expression levels and the fork pattern

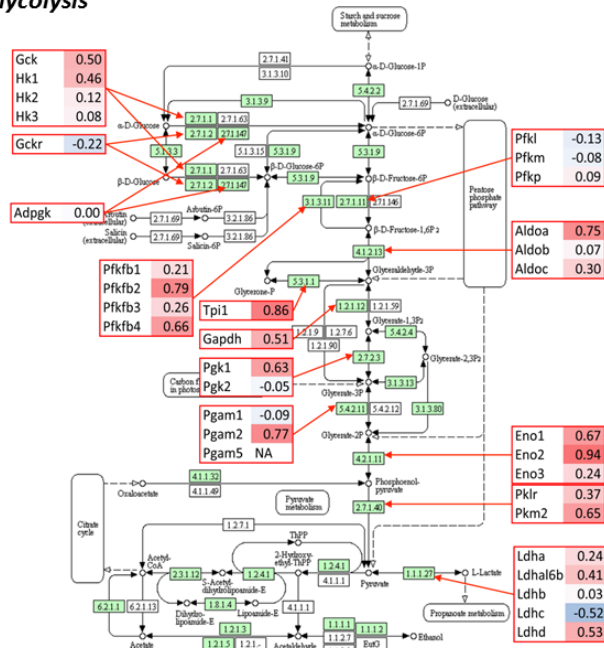
The differences we found in the protein expression profile of metabolic enzymes in breast cancer cell lines representative of the fork pattern suggested that the MTSs might have a different arrangement of the metabolic pathways through which the substrates glucose and glutamine are metabolised. Indeed, the results obtained so far by western blotting analysis seem to suggest that LF is more prone to use reactions in which glutamine and glutamine-derived substrates are involved.

To see whether these observations were confirmed also in breast cancer samples, we looked for the correlation between metabolic gene expression levels and the fork pattern in transcriptomics data in breast tumour samples; the correlation values of mRNA expression ranged from -1 to +1: positive values, indicating a positive correlation, were associated with the UF, whereas negative values (negative correlation) were related to the LF. By exploiting the transcriptional data available and Kyoto encyclopedia of genes and genomes (Kegg) database, we attributed a correlation value to the metabolic enzymes belonging to glycolysis, TCA cycle and pyruvate/acetyl-CoA metabolism. We found that metabolic enzymes differently correlated with the fork pattern in breast cancer samples at gene expression level; in particular in the glycolysis most enzymes had positive correlation indicating they were more expressed in the UF (Figure 6.3.2A); in the TCA cycle (Figure 6.3.2B) and in the pyruvate/acetyl-CoA metabolism (Figure 6.3.2C) we observed different parts of both pathways correlating with UF whereas other parts with LF, indicating that

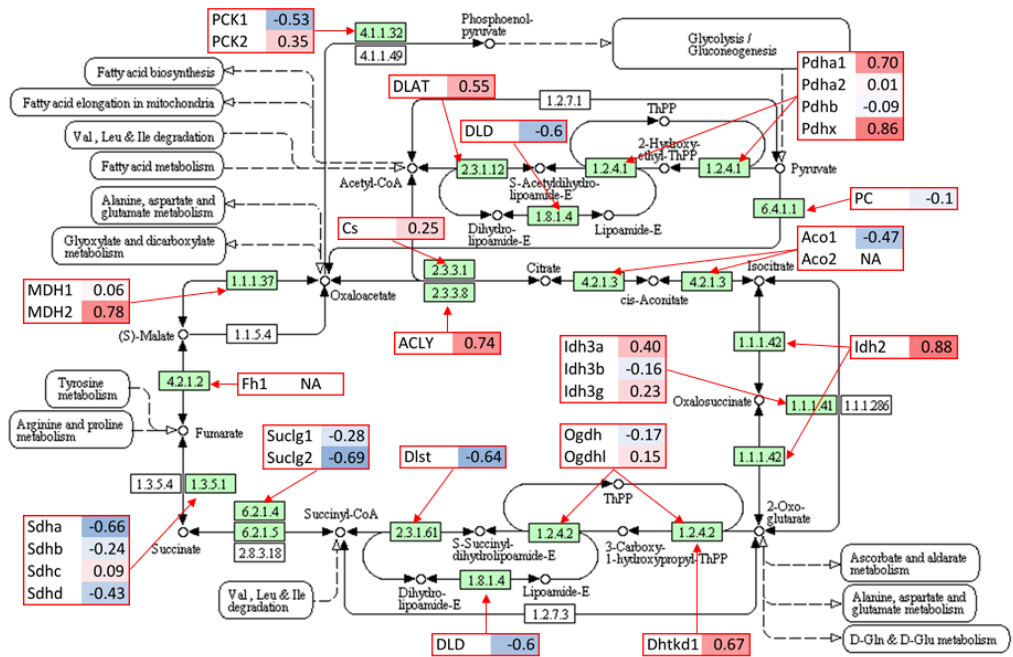
some enzymes were more expressed in the UF and others in the LF. Moreover, based on the results obtained by western blotting analysis, that highlighted differences in protein expression of GLS1 and GLUD enzymes between UF and LF in breast cancer cell lines, we looked also at the glutamine/glutamate metabolism. Again, we observed that some enzymes of the pathway correlated more with the UF and other with the LF (Figure 6.3.2D). Interestingly, the results for GFPT2 was confirmed, it was more expressed in the LF at transcriptional level of breast cancer samples as well, perfectly in agreement with the western blotting data; however for the enzyme GLUD we found the opposite behaviour compared to the proteomic analysis, indeed in this correlation experiment GLUD was associated with the LF and not with UF.

Overall, these correlation results obtained from gene expression data originated from breast cancer samples, confirmed the indication we already found at protein level obtained from breast cancer cell lines: the differences at genes expression level of metabolic enzymes in the fork pattern suggest which pathway is preferentially used by each fork or, more precisely, which is the enzymatic arrangement of a specific pathway that confer the best yield to that pathway and thus might predict the substrate, glucose or glutamine, that are better exploit by UF or LF.

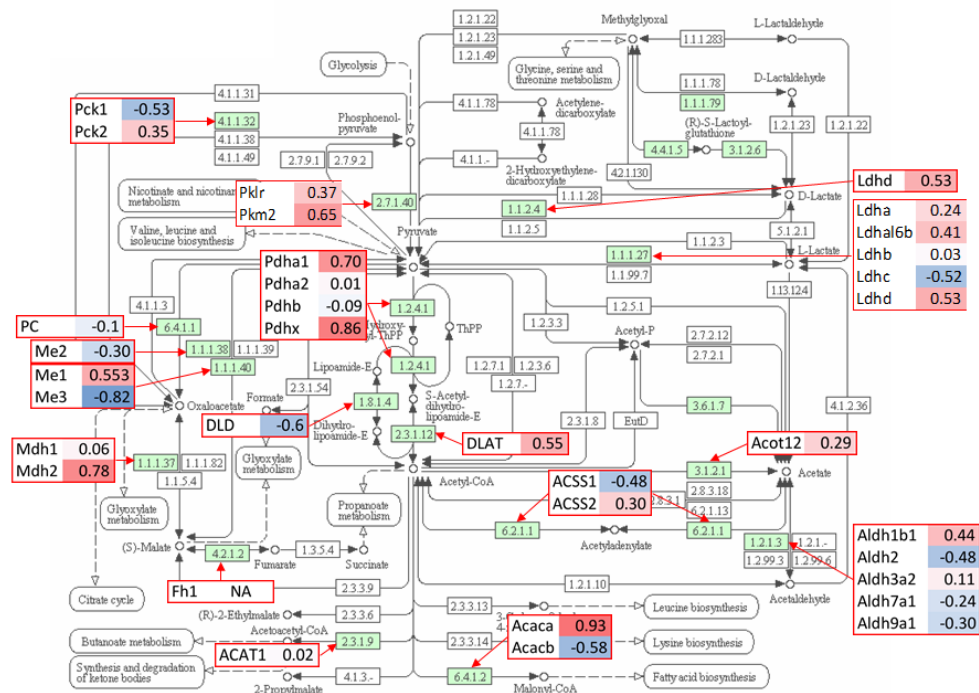
A Glycolysis



B TCA cycle



C Pyruvate and acetyl-CoA metabolism



D Glutamine/glutamate metabolism

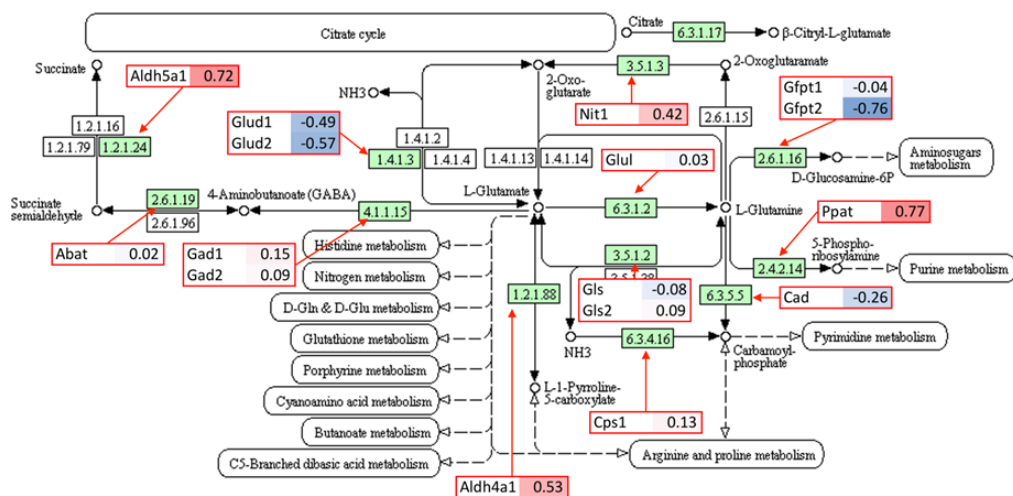


Figure 6.3.2 Different metabolic arrangement between UF and LF at gene expression level in breast cancer samples A: Glycolysis. B: TCA cycle. C: Pyruvate/acetyl CoA metabolism. D: Glutamine/glutamate metabolism.

“Metabolic schemes are taken from Kegg; red colour is associated with Upper Fork and blue with Lower Fork; the red box includes the acronym of the enzymes flanked by the correlation value”.

6.3.3 Metabolomics

In the analysis done until now to understand the metabolic arrangement of the UF and LF, both the approaches we used (proteomic and transcriptomic analysis respectively on breast cancer cell lines and breast cancer samples) indicated that the two mitochondrial tumour subtypes differently utilise metabolic pathways.

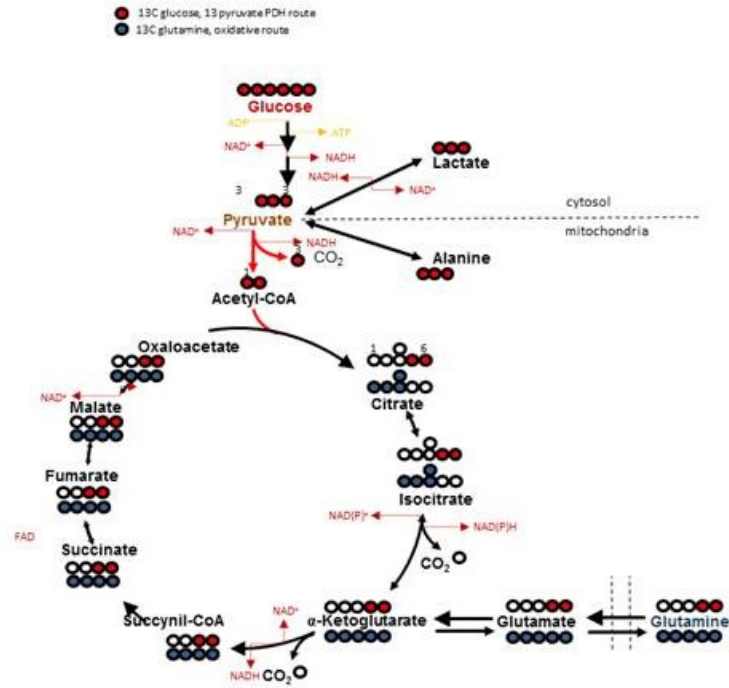
To verify if these results could reflect different overall metabolic flow of mitochondrial cancer subtypes, in collaboration with Dr. Mariia Yuneva of the Crick Institute, metabolomics and flux analysis of the representative breast cancer cell lines was performed. Cells were treated with medium containing labelled 13-carbon glucose, 13-carbon glutamine or 13-carbon pyruvate and the derived metabolites were analysed using the gas-chromatography mass spectrometry. If glucose utilisation in the TCA cycle is considered, one round of the cycle produces two labelled carbon (M+2) atoms for each TCA cycle intermediates, instead glutamine utilisation produces metabolites with four labelled carbons (M+4) (Figure 6.3.3A). It is of note that more rounds of the cycle usually occur producing isotopologues with one (M+1), three (M+3), five (M+5) labelled carbons; moreover the cycle instead of developing in the canonical oxidative direction can turn back in the reductive mode, but these scenarios were not considered in text. Indeed, further analysis are still in progress to better elucidate all the possible pathways.

We tried to understand the efficiency of glucose and glutamine utilisation in the TCA cycle analysing the cell lines representative of the fork pattern. The nutrient concentration of the experimental media, prepared with 13-carbon labelled substrates, was glucose 10mM and glutamine 1mM, supplemented or not with pyruvate (HH+, HH-). The overall incorporation of labelled carbons highlighted that UF incorporated more labelled carbons from glucose than LF, while LF generally incorporated more labelled carbons from glutamine than UF. Moreover, LF incorporates labelled carbon from pyruvate more than UF (Figure 6.3.3B). Next, we focused on the fractional enrichment of some labelled metabolites alanine and malate. The graph of alanine relative incorporation

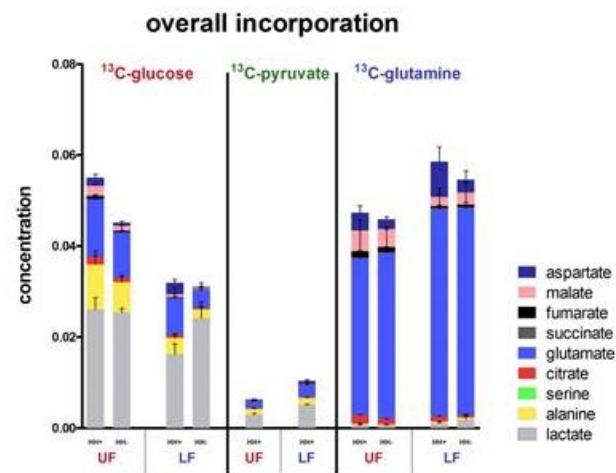
showed that the alanine labelling derives almost completely from pyruvate-derived glucose for both UF and LF, however looking at the fractional enrichment we observed that UF produces more alanine derived from glucose compared to the LF (Figure 6.3.3C). On the other hand, the graph of malate relative incorporation showed the usage of both glucose (M+2, M+3) and glutamine (M+4) to produce malate. Moreover, we observed also that the majority of labelled-malate derives from glutamine and even if UF had more malate and the isotopologue M+4 was comparable between the two forks, the fractional enrichment showed that the labelling from glutamine was higher in the LF (Figure 6.3.3D). In summary, we observed that upper fork cells use more efficiently glucose whereas lower fork cells prefer glutamine as carbon source in the TCA cycle. This is in agreement with the results obtained so far, indicating that the differences in the enzymatic arrangement could determine a substrate preference.

A

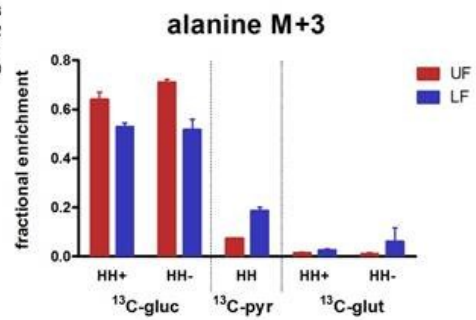
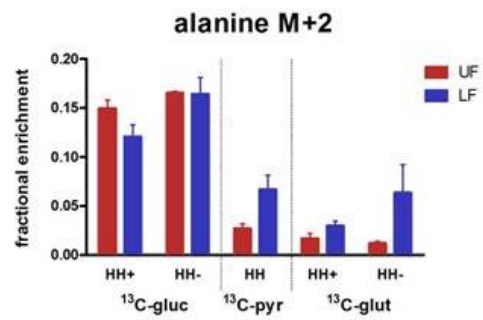
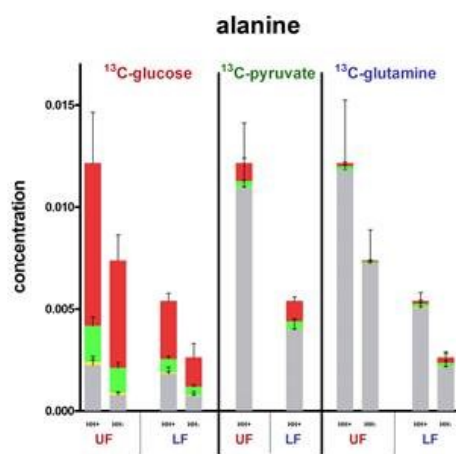
Fluxes



B



C



D

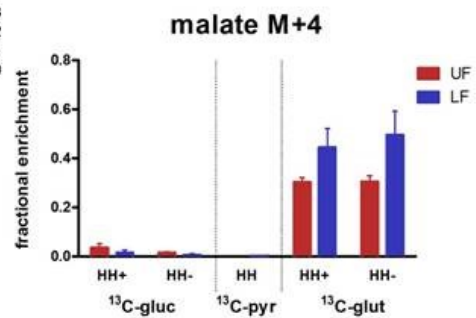
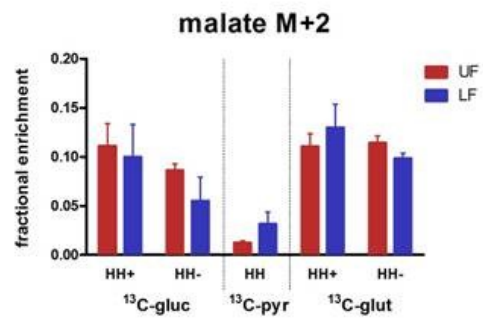
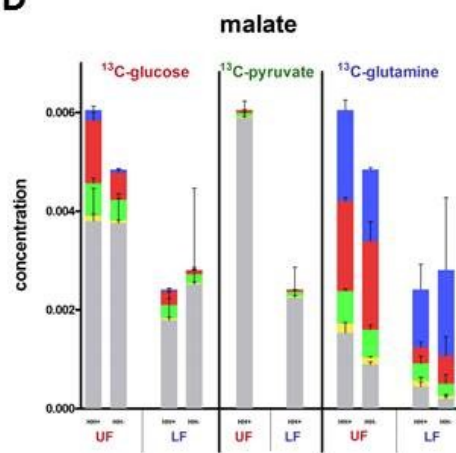


Fig.6.3.3 Substrate preference observed by gas chromatography mass spectrometry **A: Flux scheme:** the picture represents the TCA cycle, fuelled by the glucose derived-pyruvate and glutamine. Each circle correspond to a carbon atom that can be labelled or not (white circle). Red colour is the labelling derived from glucose which produces isotopologue substrates M+2, whereas the blue colour represents the labelling obtained from glutamine and the isotopologues are M+4. **B: Overall incorporation of labelled carbons:** the labelling derived from glucose is higher in the UF, the labelling derived from glutamine is higher in the LF. **C: Alanine relative incorporation and fractional enrichment:** the graphs show the usage of glucose-derived pyruvate more efficiently in the UF to produce alanine. **D: Malate relative incorporation and fractional enrichment:** malate is labelled from both glucose and glutamine. The labelling from glutamine is more efficient in the LF compared to the UF.

6.4 Deprivation effect: acute response and adaptation

The results obtained so far have indicated a different mitochondrial biogenesis pattern associate to UF and LF; this was highlighted by the protein expression profile of the respiratory chain complexes, indeed CI and CIV were increased in the UF, causing a different function in terms of oxygen consumption between the forks; moreover the differences in expression of metabolic enzymes, at both transcriptional and proteome level, and the nutrients utilisation by the fork pattern assessed by metabolomics, suggested that MTSs may diversely exploit metabolic pathways to adopt to a variable nutrients availability.

Our results, indicating that the mitochondrial tumour subtypes (UF and LF) we identified have different substrate preferences and since nutrients supply of cancer cells *in vivo* can vary and consequently tumours evolve in order to adapt to the microenvironment, we decided to test the sensitivity of the representative breast cancer cell lines to deprivation of different nutrients. For this reason, we reduced either glucose or glutamine concentration. Moreover, we tested whether the primary TCA cycle substrate pyruvate has any effect. We evaluated the contribution of these nutrients to different aspect of cell viability: cell growth, metabolic changes (ATP production, mitochondrial membrane potential), adaptive mitochondrial biogenesis (mitochondrial protein expression and mtDNA content).

6.4.1 Cell growth depend on the presence of glucose and glutamine

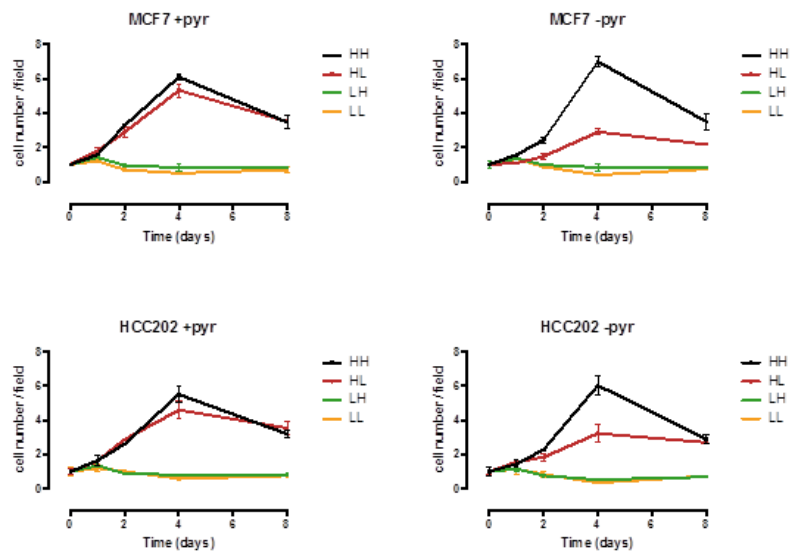
With the purpose to evaluate the efficiency to utilise substrates for the biosynthesis, we started to set up a protocol of proliferation assay to measure the cell growth at different nutrients availability. Initially we followed the cell growth in a time course of 19 days: at day 1 cells were seeded, at day 4 the experimental medium was added and finally cells were counted 3 times at day 6, 12 and 19. As first step, we tested the glucose contribution to the cell growth giving to the cells media with different glucose concentration (high 10mM or low 0.75mM) or galactose (10mM) instead of glucose, supplied with or without pyruvate (1mM). After counting, the doubling time (dt) and a ratio high/low dt, high/gal dt were calculated. We obtained some preliminary results which showed a general slowing down of the doubling time of both forks cell lines in low glucose and in galactose condition; nevertheless the high/low dt ratios indicated that cells were able to survive in condition of glucose deprivation suggesting an adaptation.

We invested around six months in the setting-up of the protocol, but despite of the promising results, the method had many technical problems; indeed to build comparable growth curves it was necessary to seed the same cell number for all the cell lines, but the intrinsic features of some lines made them sensitive to the cell dilution, so in some experiments we didn't obtain results for some of the cell lines tested, moreover the variability was high. Furthermore, for each experiment the protocol and the analysis required almost one month. For these reasons, we moved to another approach.

We decided to evaluate cell proliferation by imaging, in a time course of 8 days, testing the sensitivity of the cells to the combination of reduced glucose or glutamine concentration, in presence or absence of pyruvate. We observed that glucose-deprived cells did not growth despite of the presence of glutamine (Figure 6.4.1 A-B: green trace), indicating that both forks rely on aerobic glycolysis; moreover cell proliferation slowed down consequently of glutamine reduction as well even if glucose was still in the medium (Figure 6.4.1 A-B: red

trace); interestingly, we also observed that if pyruvate was present, cell growth was rescued in the UF but not in the LF (Figure 6.4.1 A-B: red trace), showing that these two substrate, pyruvate and glutamine, have a different impact of the forks. In conclusion, not surprisingly we found that proliferation of cells from both groups was dependent on the presence of high glucose in the medium, showing that both cell types use aerobic glycolysis for biosynthesis (Figure 6.4.1 A-B). We also observed that glutamine as well influence the cell growth, but interestingly the presence of this substrate was more important for LF. Moreover, in situation of glutamine deprivation, only UF was able to exploit pyruvate to sustain the cell growth.

A Upper Fork



B Lower Fork

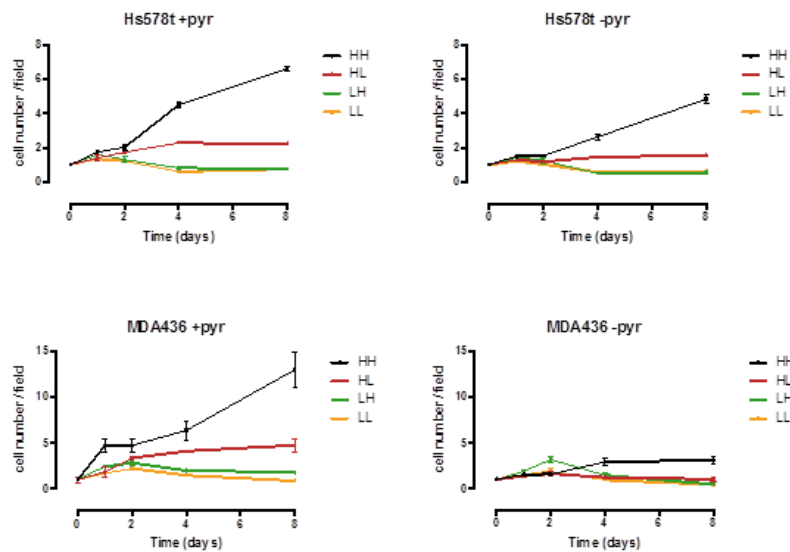


Figure 6.4.1 Cell growth depends on glucose and glutamine A: Cell growth imaging of the upper fork. B: Cell growth imaging of the lower fork. “HH: high glucose, high glutamine (black trace); HL: high glucose, low glutamine (red trace); LH: low glucose, high glutamine (green trace); LL: low glucose, low glutamine (yellow trace). Glucose: high correspond to 25mM and low to 0.75mM. Glutamine: high correspond to 2mM and low to 0.2mM”.

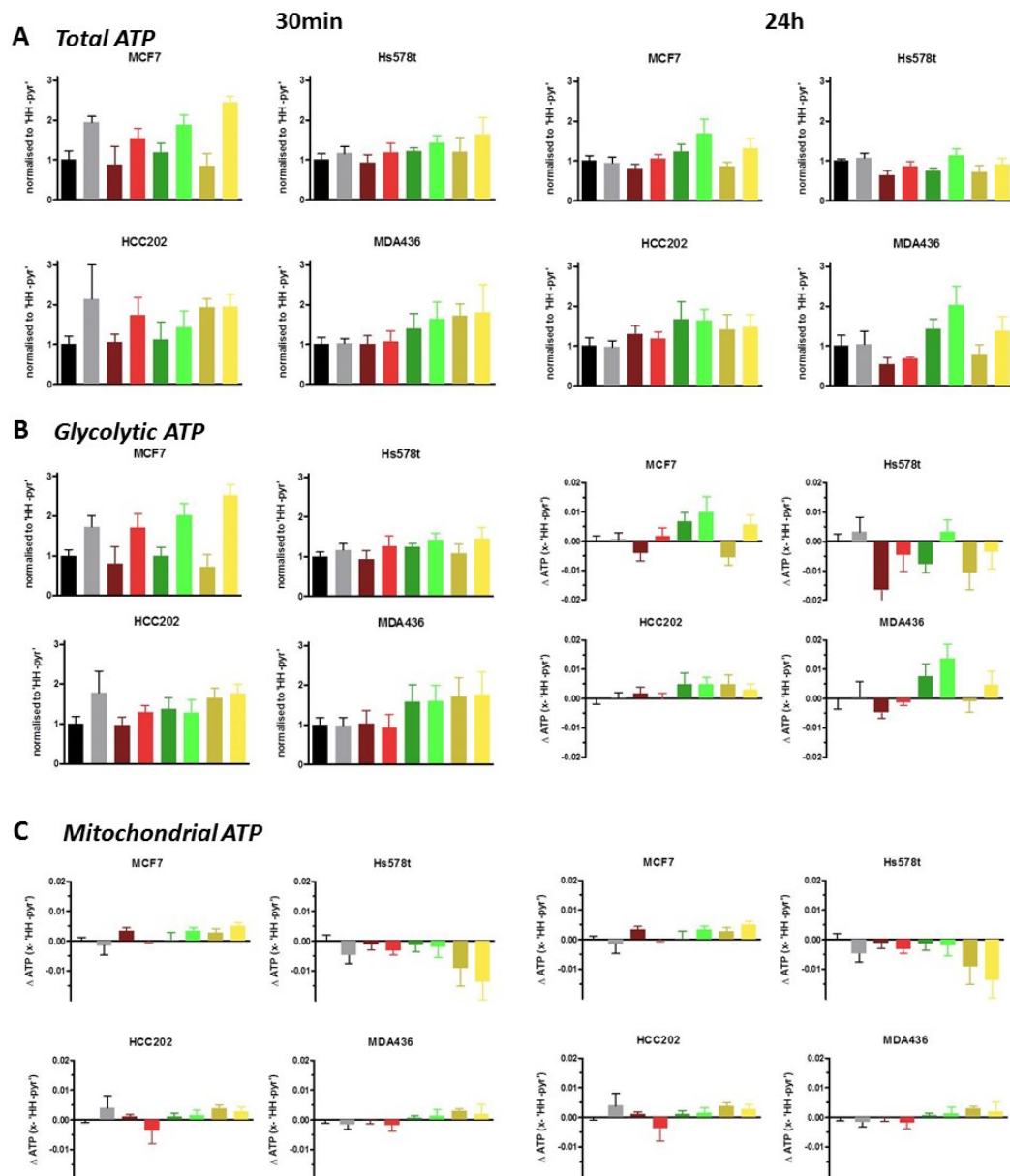
6.4.2 The ATP production and the mitochondrial membrane potential are influenced by substrate preferences

Mitochondria are sensors of cell health, consequently parameters like mitochondrial membrane potential ($\Delta\psi_m$) and ATP production give information about the general status of the cell. For this reason, we evaluated these two parameters in our cellular models representative of the fork pattern.

We measured the ATP levels after 30 minutes and 24 hours of deprivation, to assess the energy production during the acute response and the adaptation. To discriminate the contribution of mitochondria and glycolysis to this process, we treated cells with oligomycin (ATP synthase inhibitor) or 2-deoxiglucose (glucose analogue which is not processed through glycolysis). The glycolytic ATP pattern at 30' and 24h of deprivation was essentially the same found for the amount of total ATP (Figure 6.4.2 A-B); the contribution of mitochondria to the ATP production appears very limited (Figure 6.4.2 C), indicating that both UF and LF cells are primarily glycolytic, limiting the energy production to this pathway, suggesting also that the major role of mitochondria is to supply metabolic intermediates. Moreover, the two forks used different substrates for the ATP production: when glutamine was reduced (HL-/+) the ATP production of LF decreased (Figure 6.4.2 A-B), indicating that LF is sensitive to glutamine deprivation independently from the presence of glucose; interestingly, pyruvate increased the ATP production only in the UF during the acute response (30min) (Figure 6.4.2 A-B).

The second aspect of mitochondrial health we evaluated is the steady-state mitochondrial membrane potential $\Delta\psi_m$, which was measured using the cationic fluorescent dye TMRM. The status of $\Delta\psi_m$ give information on the OXPHOS activity and the contribution of mitochondria to the cell proliferation process. For this part, cells were cultured with media containing reduced concentration of either glucose or glutamine, in presence or absence of pyruvate; then $\Delta\psi_m$ was recorded by imaging. We observed a striking difference between the MTSs. UF cells depolarised in the absence of glucose, while LF cells were able to maintain a

membrane potential. On the other hand, in glutamine deprivation UF depolarised despite of the presence of pyruvate, whereas LF did not. This confirms that upper fork relies on glucose for the mitochondrial activity, whereas lower fork cells utilise other substrates than glucose derived pyruvate, such as glutamine present in the medium, more efficiently to feed into the TCA cycle and provide electrons for maintaining OXPHOS activity. Moreover, in LF pyruvate preserved the $\Delta\psi_m$ in glutamine deprivation, but it wasn't able to rescue the cell growth in the same type of cell, recovery that was done in the UF, meaning that pyruvate acts at different levels in the MTSs.



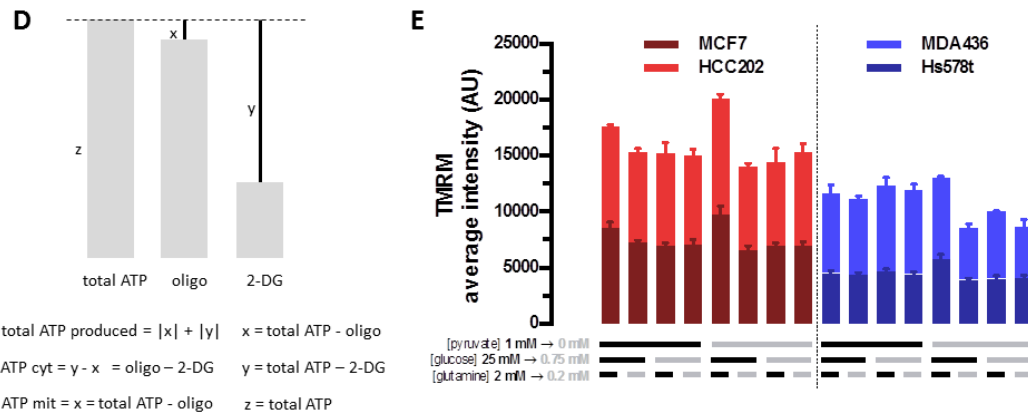


Figure 6.4.2 Mitochondrial physiology status.

Energy production: glycolysis versus mitochondria **A:** Total ATP: produced after 30' and 24h of deprivation. **B:** Glycolytic ATP: produced after 30' and 24h of deprivation, showing a similar pattern of the total ATP. **C:** Mitochondrial ATP: produced after 30' and 24h of deprivation, showing a minimal contribution of mitochondria to the ATP production. **D:** Method: Total represents ATP values produced by cells not treated with inhibitors. Oligo indicates ATP recorded after oligomycin treatment (ATP synthase inhibitor), meaning that glycolysis is the main source of ATP. 2-DG corresponds to the ATP produced after glycolysis inhibition, so the ATP is mitochondrial-derived. Total, Oligo and 2-DG data comprise the background. Data analysis: the mitochondrial ATP and glycolytic ATP, net of the background, were calculated subtracting respectively Oligo and 2-DG to the Total values. The total ATP produced, not comprising the background, was determined as the sum of glycolytic ATP and mitochondrial ATP in absolute values. "black: HH -pyr; gray: HH +pyr; dark red: HL -pyr; light red: HL +pyr; dark green: LH -pyr; light green: LH +pyr; dark yellow: LL -pyr; light yellow: LL +pyr. HH: high glucose, high glutamine; HL: high glucose, low glutamine; LH: low glucose, high glutamine; LL: low glucose, low glutamine. Pyruvate supplementation: +pyr (1mM) , -pyr (0mM). Glucose: high correspond to 25mM and low to 0.75mM. Glutamine: high correspond to 2mM and low to 0.2mM. Oligo = oligomycin; 2-DG= 2-deoxyglucose"

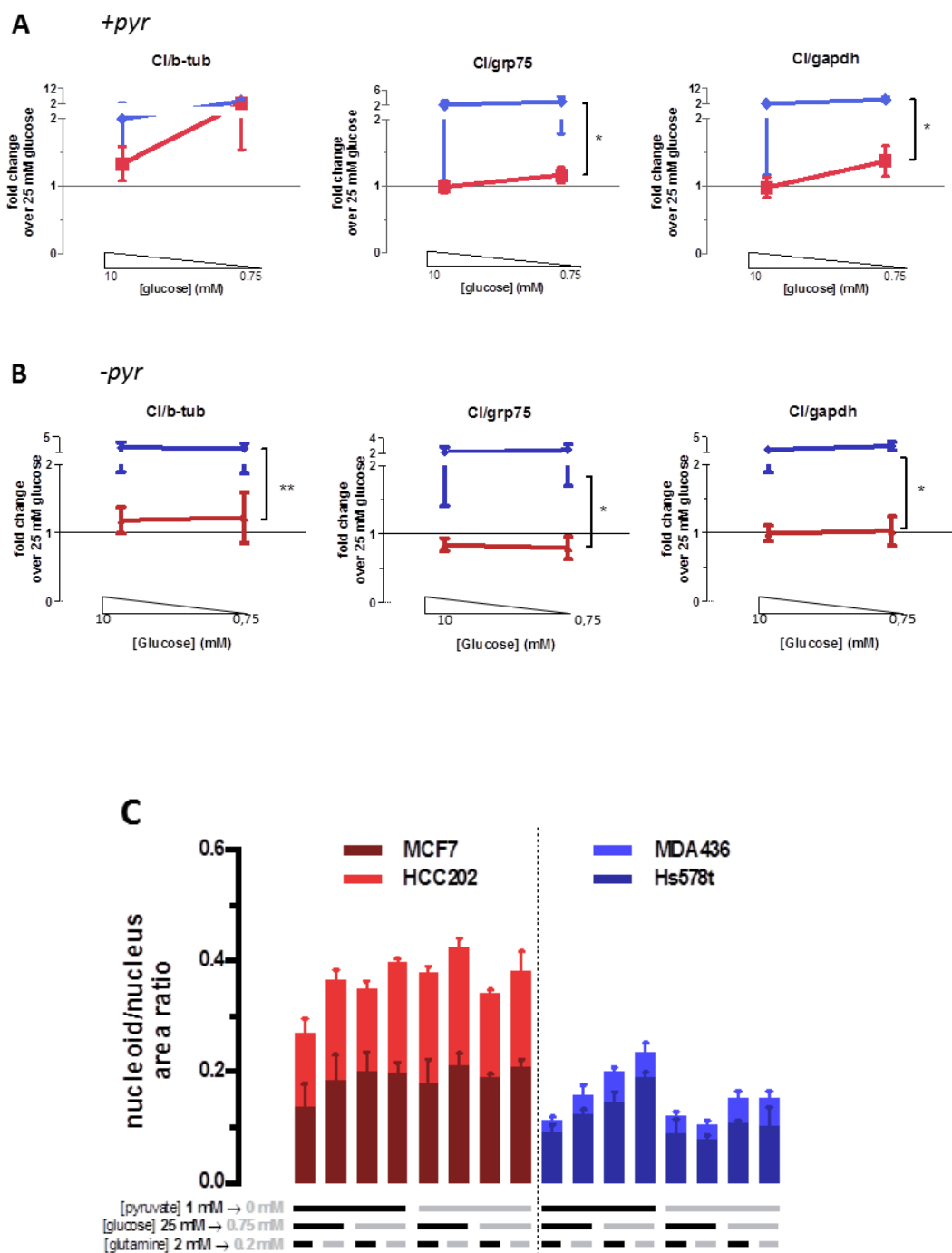
Mitochondrial membrane potential **E:** Steady state mitochondrial membrane potential ($\Delta\psi_m$): plots produced by Gyorgy Szabadkai.

6.4.3 Different mitochondrial biogenesis programs provide adaptation to different nutrient supply

The results emerged from imaging the cell growth during deprivation of different nutrients, highlighted the importance of glucose for cell proliferation for both type of cells, indeed both UF and LF exploit glucose and aerobic glycolysis for biosynthesis. To further understand how cells adapt to the lack of glucose, we measured the expression of OXPHOS components under conditions of glucose deprivation. Cells were seeded at day 1, the experimental medium was added at day 4 and cells let grow until day 19. The protein expression profile of the respiratory chain complexes was evaluated by western blotting analysis and the results normalised to β -tubulin, grp75 and gapdh. To better visualise the deprivation effect, we normalised the obtained data to the control condition (glucose 25mM and glutamine 2mM). The two forks differently adopted to glucose deprivation, we found UF with increased Complex I (CI) expression, but looking at the normalisation to mitochondria content and gapdh there was no increase or a slight increase. On the other hand, LF increased the CI expression looking at all the normalisation done (Figure 6.4.3A). Similar results were obtained in presence and absence of pyruvate (Figure 6.4.3A-B). Summarising, upper fork co-regulates respiratory complexes and GAPDH, lower fork induces the expression selectively of the respiratory complexes.

Since the glucose deprivation treatment have induced the adaptive expression of respiratory chain complexes in both cell types, suggesting that mitochondrial biogenesis process was turn on, we decided to monitor the mitochondrial DNA (mtDNA) content in condition of nutrients deprivation. For this purpose we applied the same culturing protocol used for the mitochondrial membrane potential measurement. We observed an increase of the mtDNA content consequent to the deprivation treatment in both UF and LF in every deprivation conditions tested, indicating the activation of the mitochondrial biogenesis process (Figure 6.4.3B). Moreover, looking at the results normalised to the control condition (HH+), we observed that the response to the reduction of

nutrients availability was higher in LF cells (Figure 6.4.3C), suggesting that these cells need to increase more the mitochondrial content then UF, which we observed to already have an higher mitochondrial content in basal condition (Figure 6.2B, 6.3.1B).



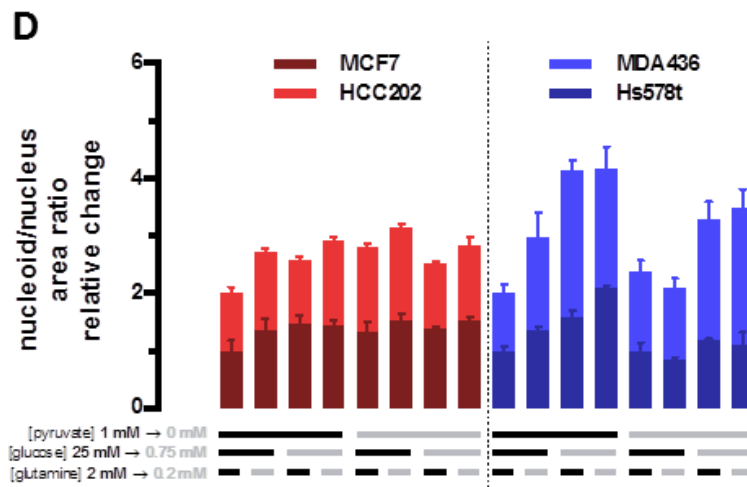


Figure 6.4.3 Adaptive mitochondrial biogenesis **A:** Complex I induction after glucose deprivation, in presence of pyruvate. **B:** Complex I induction after glucose deprivation, in absence of pyruvate. **C:** Increased mitochondrial DNA content: deprivation treatment induced mitochondrial biogenesis. **D:** Mitochondrial DNA content relative changes: the increase of mtDNA content is higher in LF.

Plots in C and D were produced by Gyorgy Szabadkai.

7. DISCUSSION

Tumours are currently defined by the hallmarks of cancer, which comprise the capabilities that enable tumour growth and metastatic dissemination (Hanahan D et al., 2000). It is of note that virtually all the hallmarks are linked to tumour metabolism, indeed the conceptual progress in the last decades suggests that two new hallmarks are involved in the pathogenesis of the majority of cancers: reprogramming of energy metabolism (Kroemer G et al., 2008; Hanahan D et al., 2011) and evading immune destruction (Hanahan D et al., 2011). We decided to address the reprogramming of cellular metabolism and bioenergetics in breast cancer model.

Breast cancer is one of the most common forms of cancer in women worldwide. It is a heterogeneous disease with various molecular alterations, cellular composition, and clinical outcome. It is defined by clinical and pathological features, histochemical markers (ER, PR, Her2) and diverse oncogenic transcriptional programs that led to a definition of the subtypes currently used in clinical studies to define the best treatment options: basal-like, Her2-enriched, Luminal A, Luminal B and normal-like. Importantly, so far none of the classification methods have taken into consideration the metabolic phenotype and gene expression pattern of these tumours.

In this work we define for the first time Mitochondrial Tumour Subtypes (MTSs), moreover, we provide their characterisation looking at the link between specific mitochondrial biogenesis patterns and specific bioenergetics and metabolic phenotype.

The MTSs were found thanks to the development of a novel algorithm MCbiclust done by our collaborator Robert Bentham. Its application to breast cancer transcriptional data has provided a new classification system based on nuclear-encoded mitochondrial genes for breast cancer, where two Mitochondrial Tumour Subtypes (MTSs), upper fork (UF) and lower fork (LF) have been identified. Each MTS is characterised by two groups of mitochondrial genes co-

regulated in opposite ways. The classification we propose offers a new insight on the classical features which are commonly used by clinical scientists in the codification of breast cancer subtypes, thanks to the inclusion of the mitochondrial genes of which expression can underline different programs of mitochondrial biogenesis. Consequently, breast tumours can be classified according to their mitochondrial status. The new classification and the possible implications are promising. For this reason, breast cancer cell lines representative of the fork pattern were selected, by exploiting again the MCBiclust algorithm, and tested *in vitro*.

The upper fork cells MCF7, HCC202, MDA-MB-453 and the lower fork cells Hs578T, MDA-MB-436, characterised by a specific nuclear-encoded mitochondrial transcriptional signature, were used in this study.

Mitochondria are key metabolic organelles, important for ATP production, intermediary metabolic processes, controlling the redox state of the cell and other fundamental signalling pathways, revealing its crucial role in cell viability, hence it can control adaptive responses under limiting conditions. According to our hypothesis, mitochondria act as a hub of metabolic remodelling organelles favouring tumour adaptation and evolution. We provided the first experimental characterisation of mitochondrial tumour subtypes UF and LF in terms of bioenergetics and metabolism as the aim of the work.

The bioenergetics was evaluated at protein level and through functional assays. The western blotting analysis, focusing on mitochondrial respiratory chain proteins important for ATP production, revealed that UF subtype cells express more Complex I and Complex IV than cells belonging to the LF subtype. Moreover, UF cells generally have higher mitochondrial content compared to the LF, as highlighted by the ratio between the mitochondrial scaffold protein grp75 and housekeeping proteins β -tubulin and α -actin. Interestingly, this expression pattern reflects differences in mitochondrial function between the two subtypes, rendering the UF more oxidative, evidenced by the Oroboros assay showing

higher oxygen consumption rate in UF cells. These evidences gave information on the general status of the MTSs mitochondria at basal condition.

The metabolic profile of UF and LF was analysed in terms of expression of metabolic enzymes belonging to different pathways at both protein and mRNA level, respectively in breast cancer cell lines and breast cancer samples. Since the intermediary metabolism, in which mitochondria are involved, relies on the cross-talk between cytoplasmic and mitochondrial metabolites according to the well-established metabolic fluxes, the enzymes tested localise at both cytoplasm and mitochondria.

We found metabolic enzymes differently expressed at protein level between the forks in cell line models, while from transcriptomic analysis of breast cancer samples (TCGA) we observed mRNA levels of metabolic enzymes differently correlating with the fork pattern. Both approaches indicated that UF and LF display a different enzymatic arrangement. In particular, UF expresses more enzymes of which the substrates are glucose derived, as shown by the correlation pattern of glycolysis (Figure 6.3.2A) and the expression of IDH1 and IDH2 enzymes. Importantly, these results were confirmed also by metabolomics that highlighted a more efficient glucose-utilisation as mitochondrial substrate for the TCA cycle (Figures 6.3.3B-C), since the overall incorporation of glucose-derived labelled carbons was higher in UF. On the other hand, LF expresses more enzymes of which substrates are glutamine derived. Interestingly, detailed analysis showed significant differences for proteins involved in the glutamine/glutamate metabolism. After its transport into mitochondria, glutamine is subsequently converted by mitochondrial glutaminase (GLS1) to glutamate and ammonium ion, then glutamate is further catabolised to α -ketoglutarate through two different pathways involving glutamate dehydrogenases and the consequent production of ammonia (GLUD) and glutamate aminotransferases (GOT1, GOT2, PSAT1, GPT1 and GPT2), as recently reviewed by Altam et al.,2016. Interestingly, GLS1 was showed higher expression in the LF at both protein and transcriptional levels, indicating major glutamine

flux in the LF mitochondrial tumour subtype. Again, these results were further confirmed at the functional level by metabolomics flux analysis. Indeed, both the overall incorporation of labelled carbon from glutamine and specific glutamine-derived labelling of malate was higher in the LF indicating a more efficient glutamine utilisation in the TCA cycle by this fork (paragraph 6.3.3). The result for GLS1 are promising, indeed it has been reported that GLS1 upregulation is induced by c-Myc in cancer (Gao P et al., 2009) and its pharmacological inhibition suppresses tumour growth in various mouse models of cancer (Xiang Y et al., 2015). GLS1 might be a putative target for LF subtype.

Next, we followed the fate of glutamate. Looking at the glutamate dehydrogenase GLUD, we found this enzyme more expressed in the UF at protein level, even if the transcriptional data revealed the contrary. This opposite pattern for GLUD can be explained because of the two different approaches we used was applied to different specimens, the transcriptional data came from breast cancer samples derived from patients, however the proteomic results was obtained from breast cancer cell lines. Next, we focused on the expression aminotransferases protein. The phosphoserine aminotransferase (PSAT1) as a part of serine biosynthesis, was more expressed at protein level in the LF, indicating the usage of glutamine for biosynthesis. The aspartate aminotransferase GOT1 (cytoplasmic isoform) we analysed didn't show differences between the forks. The mitochondrial counterpart will have to be evaluated in follow up studies together with the cytoplasmic (GPT) and mitochondrial (GPT2) alanine aminotransferases in order to better elucidate the biosynthetic role of glutamine in our models. In addition, glutamine can be used in the hexosamine biosynthetic pathway, which couples glucose and glutamine metabolism (Wellen, K et al., 2010). This pathway leads to the production of uridine diphosphate N-acetylglucosamine (UDP-GlcNAc), required for glycosylation processes. Interestingly, the rate-limiting enzyme of this pathway GFPT2 was strikingly more expressed in the LF. This is an intriguing result, indeed, the produced UDP-GlcNAc can serve as a nutrient signal reflecting

cellular metabolic state. Wellen, K et al., 2010 have shown that glucose flux through the hexosamine biosynthesis pathway regulates growth factor receptor glycosylation and enables glutamine consumption and hence defining a novel mechanism through which cellular metabolism regulates signal transduction through metabolic regulation of glycosylation.

Another enzyme, significantly more expressed in the LF is the glycolytic enzyme PKM1 which promotes oxidative phosphorylation, that paradoxically is the PK isoform usually expressed in normal tissue. Indeed, the isoform normally associated with cancer is PKM2. However, it has been reported in literature that PKM1 was commonly up-regulated in various chemoresistent cells, among which colorectal cancers (CCC) resistant to 5-FU treatment (Taniguchi K et al., 2016; Denise C et al., 2015), hence PKM1 provide an advantage in CCC. However we still do not have an explanation of which is its role in LF mitochondrial breast cancer subtype, but prospectively given the possible implications in clinic, further investigation focused on understanding of the PKM1 upregulation in LF have to be done.

Globally, the results we obtained so far suggest that LF better exploits glutamine thanks to a specific enzymatic arrangement, whereas the UF asset of enzymes confers a better glucose utilisation by this subtype. However, it is important to take into account that the expression of enzymes is not the only factor that determine the metabolic fluxes in the cell. Moreover, these results were obtained in basal conditions in which nutrients were not limiting, so further analysis to examine in depth the metabolic behaviour of MTSs should be done in deprivation system, evaluating how enzymatic expression can vary and what are the possible implications on nutrient utilisation.

In this study, the deprivation approach was applied to MTSs with the aim to understand how UF and LF adapt to variable nutrient availability in the light of the results obtained so far: UF and LF are associated with a different mitochondrial biogenesis pattern characterised by a different enzymatic arrangement and nutrient utilisation; then, cell growth, metabolic changes (ATP

production, mitochondrial membrane potential) and adaptive mitochondrial biogenesis (mitochondrial protein expression and mtDNA content) were evaluated.

The imaging of cell growth showed that the aerobic glycolysis, hence glucose substrate, supports biosynthesis (cell growth) in both MTSs (Figure 6.4.1A-B), no growth occurred in both groups under glucose deprivation. Moreover, the results highlighted again that LF cells rely more on glutamine; indeed, we found glutamine deprivation to be limiting specifically for this fork. Interestingly, glutamine dependence could also be observed in UF cells, but only in the absence of pyruvate. Indeed, the results showed that reduced glutamine slows down the growth in both subtypes indicating its importance for the cell growth, but if the effect could be rescued in the UF after pyruvate supplementation, it was not in the LF (Figure 6.4.1A-B). Altogether, we have found that LF cells exploit better glutamine in contrast to cells of the UF group, which are able to use glucose or pyruvate even in the absence of glutamine for maintaining cell growth.

Next, looking at the functional results from both ATP and mitochondrial membrane potential measurements, we observed again that two MTSs differently utilise glucose and glutamine substrates. Moreover, we gained more insight into the role of the primary TCA cycle substrate pyruvate, exerting diverse effects in the upper and lower fork.

In particular, we found the ATP production derives almost completely from glycolysis after both acute and adaptive response (Figure 6.4.2A-B), so the mitochondrial contribution to the energy production is limited (Figure 6.4.2C), indicating that both MTSs are primarily glycolytic. This suggesting that a major role of mitochondria in MTSs is to supply metabolic intermediates for biosynthesis but not for basal cellular energetic needs. In support of this observation, two papers have been published revealing that the despite of the best known role of mitochondrial ETC is the ATP synthesis, the essential function of the respiratory chain is making aspartate, claiming that proliferating cells

adjust their metabolism to support aspartate biosynthesis even at the expense of ATP synthesis (Van Vranken JG & Rutter J, 2015; Sullivan et al., 2015; Birsoy et al., 2015). In addition, our results evidenced different substrate usage between the two MTSs for ATP production; indeed, LF was sensitive to glutamine deprivation with a consequent decrease of the ATP production, independently from the presence of glucose (Figure 6.4.2A-B); on the other hand, the primary TCA cycle substrate pyruvate was able to increase the ATP synthesis only in the acute response of the UF (Figure 6.4.2A-B). From the mitochondrial membrane potential analysis, glucose deprivation caused depolarisation only in the UF, indicating a glucose dependence of this subtype and the utilisation of glutamine to sustain the membrane potential in the LF when glucose is limiting. On the other hand, glutamine deprivation induced depolarisation in both MTSs. Interestingly, during glutamine deprivation pyruvate was able to sustain $\Delta\psi_m$ in the LF but not in the UF. This suggest again that pyruvate acts in the MTSs at different level.

Finally, we observed that long term glucose deprivation induced the expression of OXPHOS components, conferring adaptive phenotype. Indeed, the expression of Complex I increased in both forks (Figure 6.4.3A-B), but in different way between upper and lower fork. UF upregulated both CI and GAPDH, indeed the normalisation of CI to the glycolytic enzyme revealed no changes, indicated a coordinate expression of both proteins; the CI content was unchanged referring to grp75, suggesting it goes up with the whole organelle. On the other hand, LF simply increased CI content considering all the normalisations done (β -tubulin, grp75, GAPDH) (Figure 6.4.3A-B). Overall, mitochondrial biogenesis was induced in both MTSs by substrate deprivation but with different features in the UF and LF groups. The activation of biogenesis was confirmed also by the increase of the mtDNA content, in all the deprivation condition tested (Figure 6.4.3C), and in particular the increase was higher in the LF (Figure 6.4.3D). This difference probably relies on the different mitochondrial content between the two forks at basal condition, in which UF has higher mitochondrial content compared to the

LF, rendering UF more prone to respond under deprivation; on the other hand, LF needs to increase more the mitochondria content to face disadvantageous conditions.

Overall, the data we presented in my thesis provide a first metabolic and bioenergetics characterisation of the newly identified Mitochondrial Tumour Subtypes in breast cancer, which have been identified according to their nuclear-encoded mitochondrial transcriptional pattern. Experimental analysis confirmed different mitochondrial status at both protein and functional level between UF and LF type breast cancer cells. Moreover, metabolic analysis and deprivation experiments revealed that UF and LF mitochondria are differentially coupled to cellular metabolic pathways. Mitochondria in UF cells better exploit glucose derived substrates, whereas LF mitochondria preferentially use glutamine, opening to the new concept of the *substrate preference*, a new insight in the study of cancer metabolism. In conclusion, we provide evidence that the mitochondrial biogenesis patterns associated with mitochondrial tumour subtypes drive nutrient choice in breast cancer model.

8. SIGNIFICANCE

Breast cancer is a heterogeneous disease involving many transcriptional alterations; however, so far it has never been assessed according to the mitochondrial profile of the tumours.

Reprogramming of cellular metabolism and bioenergetics is one of the leading-edge hallmarks of cancer, in which mitochondria play a central role driving tumour adaptation and evolution.

The identification of new breast cancer subtypes according to their nuclear-encoded mitochondrial transcriptional signature, called as Mitochondrial Tumour Subtypes (MTSs), upper fork and lower fork, provide a new classification system to exploit in the field of breast cancer.

The characterisation of the UF and LF subtypes, defined by a specific mitochondrial transcriptional signature, revealed an associated mitochondrial biogenesis pattern that drives nutrient choice, or substrate preference, with related metabolic program important for the adaptation, thus providing a new insight in the complex hallmark of cancer metabolic reprogramming.

Further characterisation of MTSs to understand how to exploit mitochondrial biogenesis for cancer diagnosis and therapy represents a new challenge.

Ultimately these studies are aimed lead to (i) development of novel strategies to target cancer by inhibiting mitochondrial biogenesis and (ii) the discovery of novel biomarkers to identify cancers where mitochondrial targeting is feasible will become future goals.

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