Università degli Studi di Ferrara

DOCTORAL COURSE IN
"MOLECULAR MEDICINE AND PHARMACOLOGY"

CYCLE XXXII

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P2X7R:role in the generation of an ATP-rich TME driven by nutrient deprivation

Scientific/Disciplinary Sector MED/05

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2017/2019

“Non andare dove il sentiero ti può portare;
vai invece dove il sentiero non c’è ancora e
lascia dietro di te una traccia”

(Ralph Waldo Emerson)

CONTENTS

ABBREVIATIONS

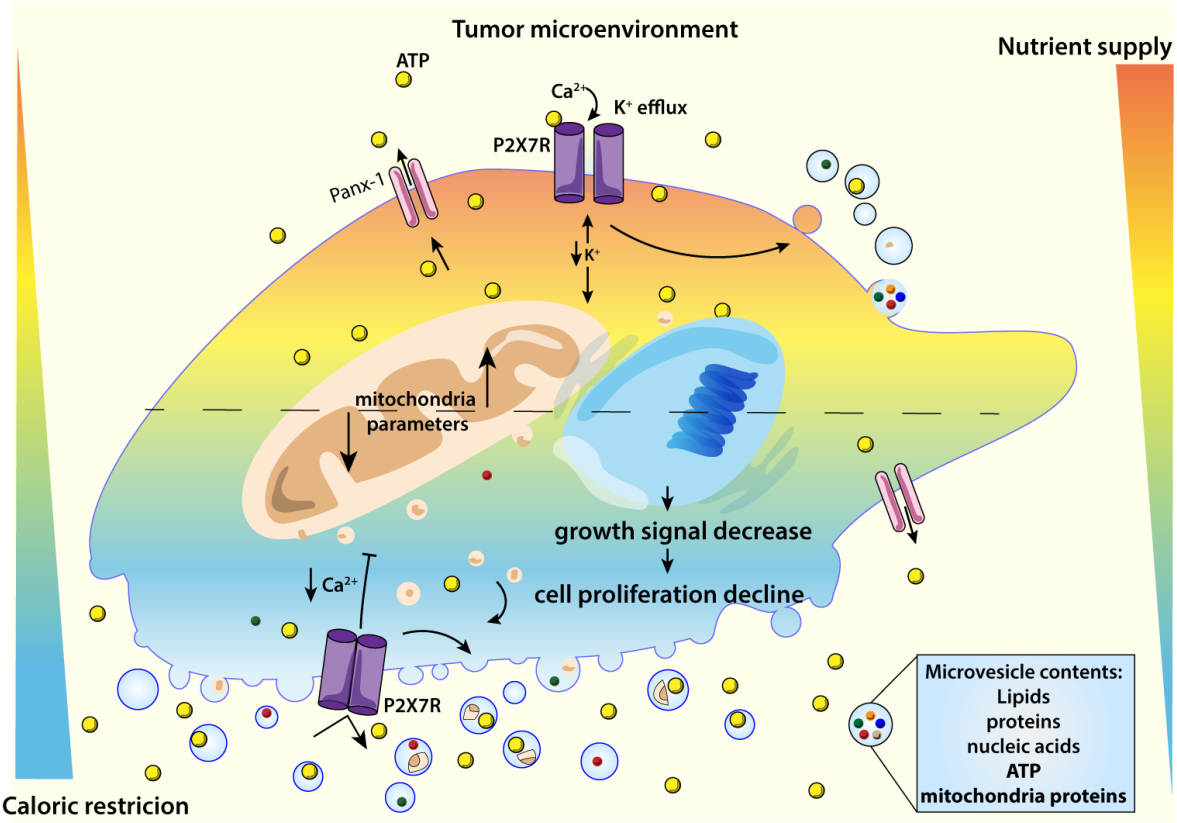
TME	Tumor microenvironment
CRMs	Caloric restriction mimetics
HC	Hydroxycitrate
ATP	Adenosine 5’-triphosphate
eATP	Extracellular adenosine 5’-triphosphate
iATP	Intracellular adenosine 5’-triphosphate
BzATP	2’,3’-(4-benzoil)-benzoil-ATP
pmeLUC	Plasma membrane luciferase
B16-shRNA	P2X7R-silenced B16F10 cells clones
B16 pmeLUC	B16F10 transfected with a pmeLUC
LDH	Lactate dehydrogenase
OXPHOS	Oxidative phosphorylation
OCR	Oxygen consumption rate
ECAR	Extracellular acidification rate
TMRM	Tetramethyrhodamine methyl ester
LC3-I	1A/1B-Light Chain 3
TOM20	Translocase of Outer Mitochondrial Membrane 20
AMPK	AMP activated protein kinase
EVs	Extracellular vesicle
PSD	Particle size distribution

ABSTRACT

Extracellular ATP, one of the major constituent of the tumor microenvironment (TME), promotes immunosuppression or anti-tumor immunity. It has been shown that caloric restriction improves the efficacy of anti-cancer therapy by increasing the ATP concentration in the TME. *In vivo* administration of the caloric restriction mimetic (CRM) hydroxycitrate reduced B16F10 melanoma tumor size and at the same time increased the ATP levels in the TME. We mimicked the *in vivo* CRM activity by *in vitro* exposing melanoma cells to serum starvation. Chronic incubation in the absence of serum inhibited melanoma cell growth and increased the extracellular ATP concentration in the absence of autophagy or cell death. The P2X7 receptor (P2X7R) is a main sensor of extracellular ATP and a promoter of cell proliferation or cell death depending on the level of activation. Serum starvation severely affected P2X7R-dependent responses, despite P2X7R expression was unchanged. Cytoplasmic Ca^{2+} levels and intracellular ATP concentration were strikingly reduced in serum-starved versus serum-supplemented cells. In the absence of serum, mitochondria were fragmented and depolarized, oxidative phosphorylation was dramatically impaired and Complex I and II of the mitochondrial respiratory chain were down-regulated. In parallel glycolysis and release of lactic acid were increased. Melanoma cells are known to release exosomes/microparticles, collectively referred to as extracellular vesicles (EVs). We show that serum-starved cells released a higher amount of EVs compared to serum-supplemented cells. EVs contained a high amount of ATP as well as mitochondria. Moreover P2X7R silencing reduced EV and extracellular ATP release, suggesting that an efficient ATP release mechanism required functional P2X7R expression.

Our findings suggest that serum starvation causes an impairment of cancer cell metabolism and support a role for the P2X7R in the mechanism by which nutrient deprivation drives the establishment of an ATP-rich tumor microenvironment.

GRAPHICAL ABSTRACT



In Brief: Caloric restriction inhibits tumor cell growth and increases the extracellular ATP concentration. Under serum starvation P2X7R-dependent responses are severely affected and cytoplasmic Ca^{2+} levels are strikingly reduced. Mitochondria are fragmented, oxidative phosphorylation is dramatically impaired and intracellular ATP concentration is reduced. Cells release a higher amount of exosomes/microvesicles, collectively referred to as extracellular vesicles (EVs), compared to nutrient-supplemented cells. EVs contain a high amount of ATP as well as mitochondria.

1. INTRODUCTION

1.1. Tumour microenvironment

The tumour microenvironment (TME) is a dynamic environment composed of neoplastic cells that coexists with a variety of host cells (including fibroblasts, vascular endothelial cells, immune cells) and extracellular matrix components (McAllister and Weinberg 2010), characterized by low glucose, hypoxia, low pH and low nutrient levels.

The cellular and biochemical composition of the TME is crucial for the regulation of proliferation, tumour cell metabolism, motility and dissemination (Quail and Joyce 2013). Endothelial cells are critical for the delivery of oxygen, nutrients and drugs, mesenchymal cells regulate carcinoma cell growth and motility, fibroblastic cells can become activated (CAFs) and remodel the extracellular matrix (Hirata and Sahai 2017). The tumour microenvironment is also infiltrated by tumour-associated macrophages (TAMs), MDSCs (myeloid-derived suppressor cells) and T lymphocytes with immunosuppressive activity (T_{reg} cells).

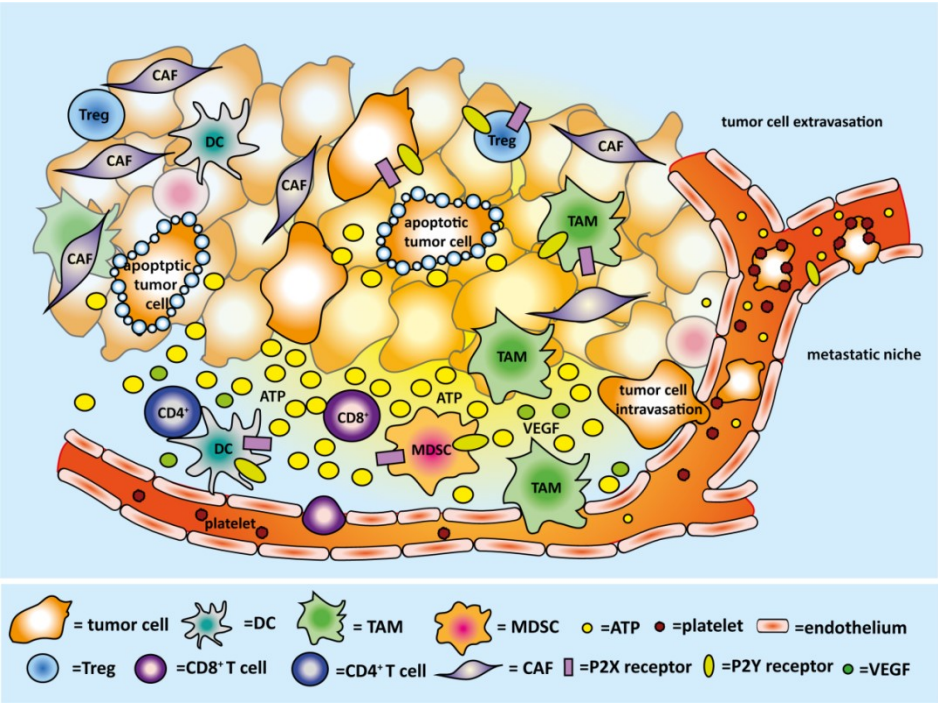


Fig.1: Schematic representation of the main TME components. Tumor-associated macrophages (TAMs) support growth, angiogenensis and invasion; immune-suppressor cells, including myeloid-derived suppressor cells (MDSCs) and T_{reg} cells, infiltrate the growing tumor; cancer-associated fibroblasts (CAFs) promote reorganization of extracellular matrix. Extracellular ATP stimulates tumor and immune cells to release further ATP and activates dendritic cells (DCs) to increase $CD4^+$ and $CD8^+$ T cell responses.

The TME can provide an immunosuppressive milieu or an antitumor immune response depending on the bidirectional communication between tumour and host cells (Munn and Bronte 2016).

Host cells release cytokines, growth factors, cytotoxic molecules, DAMPs (damage-associated molecular patterns) that affect tumour cell functions as well as the ability of host to fight the tumor. Similarly, cancer cells release metabolites that acidify the TME and molecules that signal injury and that recruit infiltrating inflammatory cells (Hanahan and Weinberg 2011, Di Virgilio, Sarti et al. 2018).

Nucleotides and nucleosides are also released into the TME by host and tumour cells (Di Virgilio and Adinolfi 2017). Among the nucleotides released, adenosine 5'-triphosphate (ATP) and adenosine (Ado) modulate several, different cellular responses, such as proliferation, cell death, differentiation, secretions of growth factors, and can drive immunostimulation or immunosuppression (Bours, Dagnelie et al. 2011, de Andrade Mello, Coutinho-Silva et al. 2017, Kepp, Loos et al. 2017).

Extracellular ATP: a major constituent of the TME

A role for extracellular nucleotides, namely adenosine 5'-triphosphate (ATP), has been identified in different physiological or pathological conditions (Dou, Chen et al. 2018): glial-neuron interaction (Butt 2011), hormone secretion (Burnstock 2014), sensory transmission (Nakatsuka and Gu 2006), inflammation (Bours, Dagnelie et al. 2011, Di Virgilio 2015), cardiovascular diseases (Erlinge and Burnstock 2008) and cancer (Di Virgilio 2012). Extracellular ATP is likely to be a neurotransmitter in the central and peripheral nervous system and also an inflammatory mediator in the immune system (Di Virgilio, Dal Ben et al. 2017).

ATP has all the requirements for an ideal extracellular messenger: (a) is virtually absent in the extracellular space (10-100 nmol/L) under physiological conditions; (b) is concentrated to high levels within the cells (from 5 to 10 mmol/L); (c) is water soluble and quickly hydrolysed by ubiquitous degrading systems; (d) ligates specific plasma membrane receptors, conferring specificity to its signalling (Di Virgilio and Adinolfi 2017). Furthermore, since ATP is the main intracellular high energy intermediate, its intracellular levels provide an indication of the cellular energy status. Extracellular ATP concentration may also give an indication of tissue metabolic activity, although oxidative phosphorylation and glycolysis may not correlate with extracellular ATP levels.

It might seem paradoxical, but changes in the extracellular ATP concentration in pathophysiological conditions have been demonstrated *in vivo* only few years ago.

Many sophisticated techniques have been proposed to measure ATP in the pericellular space: tandem enzyme reaction (Corriden, Insel et al. 2007), patch-clamp (Hayashi, Hazama et al. 2004), atomic force microscopy (Schneider, Egan et al. 1999) and a luciferase fused in frame with the IgG-binding domain of *Staphylococcus aureus* protein A, thus allows binding of this chimeric protein to IgG adsorbed onto the cell surface (Beigi, Kobatake et al. 1999). In 2005, a plasma membrane luciferase, named pmeLUC, was engineered in our laboratory, thus allowing ATP measurement close to the cell surface (Pellegatti, Falzoni et al. 2005). PmeLUC is a chimeric protein in which the *Photinus pyralis* luciferase cDNA was fused in frame between the N-terminal endoplasmic reticulum import sequence (leader sequence) and the C-terminal plasma membrane anchor sequence (glycophosphatidylinositol, GPI) of the human folate receptor.

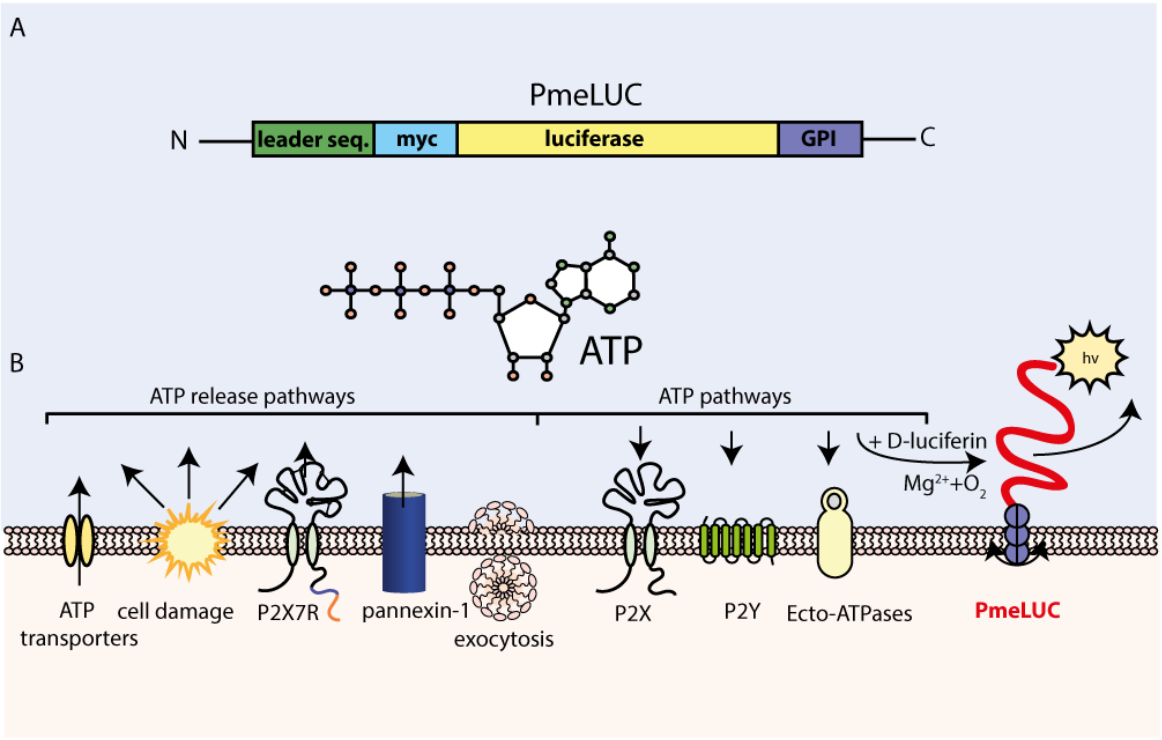


Fig.2: Schematic representation of pmeLUC and pathways for ATP release. A PmeLUC structure showing the full-length luciferase coding sequence inserted in-frame between the N-terminal leader sequence and the C-terminal GPI anchor of the folate receptor. **B** PmeLUC is targeted and localized to the outer side of the plasma membrane, thus allowing the measurement of extracellular ATP at discrete plasma membrane sites facing the pericellular environment. Cellular release of ATP is thought to occur via several mechanisms, including ATP transporters, cell damage, the P2X7 receptor, gap junctions and/or pannexin hemichannels and vesicular exocytosis (Morciano, Sarti et al. 2017)

Thanks to this modifications, the pmeLUC probe is targeted to the external side of the plasma membrane. This probe can be transfected into a variety of cell types for *in vitro* and *in vivo* experiments (Falzoni, Donvito et al. 2013). Cells engineered with pmeLUC can be either used to generate the primary tumour or can be inoculated into solid tumours or intraperitoneally to monitor ATP concentration within the TME (Morciano, Sarti et al. 2017). Use of the pmeLUC probe has demonstrated that at sites of tissue damage, inflammation and in the tumour microenvironment, extracellular ATP levels are in the range of 100-500μmol/L, whereas in the interstitium of healthy tissues ATP concentration is very low (10-100 nmol/L) (Pellegatti, Raffaghello et al. 2008, Lecciso, Ocadlikova et al. 2017).

Over the years the pmeLUC probe has been used to measure ATP levels in different experimental tumours (human melanoma, mouse CT26 colon carcinoma, human ovarian carcinoma OVCAR-3 cells), showing that the TME is rich in extracellular ATP (Michaud, Martins et al. 2011, Loo, Scherl et al. 2015, Lecciso, Ocadlikova et al. 2017).

In the TME extracellular ATP can promote immunosuppression or anti-tumour immunity, depending on its concentration and on the level of degradative enzymes and specific receptors expressed by immune and tumour cells.

ATP release in the TME

Virtually all living cells release variable amount of ATP into the extracellular space.

The release of extracellular ATP include both specific and non-specific pathways (Dosch, Gerber et al. 2018).

Intracellular ATP can be released in the tumor microenvironment in response to various cell stress- and cell death-inducing conditions, such as hypoxia, cytotoxic agents or plasma membrane damage (Forrester and Williams 1977, Giuliani, Sarti et al. 2019). In spite of the high extracellular ATP concentration in the TME, passive efflux of cytoplasmic ATP is facilitated by the very large intracellular/extracellular gradient (Giuliani, Sarti et al. 2019).

In addition to passive release, ATP is actively released via specific mechanisms such as vesicular exocytosis, different types of transporters and channels, and plasma membrane-derived microvesicles (Dosch, Gerber et al. 2018):

- **Vesicular exocytosis**

ATP accumulates in the lumen of cytosolic vesicles that can rapidly release their contents via stimulated or constitutive exocytosis (Lazarowski 2012).

A vesicular nucleotide transporter (VNUT or SLC17A9) mediates ATP storage within secretory granules. VNUT is localized to intracellular vesicles where it accumulates ATP at the expense of the electrochemical gradient (positive inside) established by the vacuolar ATPase (V-ATPase) proton pump (Sawada, Echigo et al. 2008, Miyaji, Sawada et al. 2011). Exocytosis of ATP-containing vesicles than occurs via the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-mediated route (Sudhof and Rothman 2009).

- **Connexins and pannexin channels**

Connexin and pannexin family members share similar structural features, with N- and C-terminal cytoplasmic domains, four membrane-spanning segments and intracellular and extracellular loop domains (Scemes, Spray et al. 2009). The main functional difference is that connexins can form gap junctions and hemichannels, while pannexins only form hemichannels (Sosinsky, Boassa et al. 2011). Connexins and pannexins can form hexameric membrane structures, called connexons and pannexons respectively, that mediate extracellular release of ATP, glutamate and small molecules.

Connexin-43, -37, -36 and -26 are the isoforms suggested to mediate ATP release. Activation of Connexin-43 may be induced by changes in the calcium concentration, reactive oxygen species (ROS), nitric oxide (NO) and cell membrane polarization (Kang, Kang et al. 2008).

Pannexin-1 is probably the major pathway for ATP release in different cell types. Activation of pannexin-1 channels is mediated by mechanical stress (Bao, Locovei et al. 2004), intracellular calcium increase, P2Rs activation (namely P2X7R) (Locovei, Wang et al. 2006) or during chemotherapeutic drug-induced apoptosis via caspase 3 (Boyd-Tressler, Penuela et al. 2014). Furthermore, released ATP can inhibit Pannexin-1 by binding to a low affinity site on the channel extracellular domain, thus preventing excessive ATP accumulation in the pericellular space (Qiu and Dahl 2009).

- **P2X7 Receptor** (See paragraph 1.3)

The P2X7 receptor (P2X7R) is a receptor/channel that is able to form a large, non-selective pore allowing passage of ATP and other molecules up to 900 Da.

It has been also demonstrated that gating of P2X7 is a stimulus that induces active ATP release paralleled by depletion of intracellular ATP (Pellegatti, Falzoni et al. 2005, Johnsen, Kaschubowski et al. 2019).

- **Calcium homeostasis modulators (CALHM)**

Recently six members of the calcium homeostasis modulators (CALHM) family have been identified as important contributors to ATP release.
CALHM-1 is expressed in different tissues and it is a pore forming plasma membrane voltage-gated ion channel whose size is compatible with ATP passage (Siebert, Ma et al. 2013, Taruno, Vingtdeux et al. 2013).

In addition, the amount of ATP in the TME can increase thanks to further mechanisms:

- **Adenylate kinase (AK) and Nucleoside diphosphate kinase (NDPK)**

Adenylate kinase (AK) and Nucleoside diphosphate kinase (NDPK) families are responsible for the conversion of extracellular AMP and ADP to ATP (Yegutkin 2014).
AK isoforms are present in the cytosol (AK1) and in the mitochondrial intermembrane space (AK2) or mitochondrial matrix (AK3). Recently, it has been discovered an ecto-AK activity on human hepatocytes (Fabre, Vantourout et al. 2006), leukemic cell lines (Yegutkin, Henttinen et al. 2002) and other different cell types, suggesting a role for this enzyme as a regulator of extracellular ATP.
NDPK enzymes are expressed on the plasma membrane and they are responsible for the outside-in and inside-out nucleotide transfer. Extracellular ATP synthesis by an ecto-NDPK has been found in different cell types, including glioma cells (Agren, Ponten et al. 1974), lymphocytes (Yegutkin, Henttinen et al. 2002) and hepatocytes (Fabre, Vantourout et al. 2006).

- **ATP synthase (F1F0 ATP synthase)**

ATP synthase is the enzyme found in the inner mitochondrial membrane that couples ATP synthesis to a transmembrane electrochemical protons gradient.
Ecto-ATP synthase has been identified on human keratinocytes (Burrell, Wlodarski et al. 2005), adipocytes (Kita and Arakaki 2015) and tumour cell lines (Chi and Pizzo 2006), although the exact contribution of F1F0 ATP synthase to extracellular ATP is still unknown.

In the TME cancer and immune cells express some of these mechanisms, suggesting that the non-lytic release is a main pathway for extracellular ATP accumulation.

ATP degradation in the TME

In the extracellular space, messenger molecules must be degraded to avoid receptor desensitization or overstimulation of the targets.

Four major families of ectonucleotidases are expressed in the TME: (I) ectonucleoside triphosphate diphosphohydrolases CD39 (NTPDase1), CD39L (NTPDase2) and CD39L3 (NTPDase3); (II) ecto-5'-nucleotidase (5'-NT also known as CD73); (III) ectonucleotide pyrophosphatase/phosphodiesterase family member CD203a (NPP1); (IV) alkaline phosphatase (ALP) (Zimmermann, Zebisch et al. 2012).

ATP is sequentially hydrolysed to ADP and AMP by CD39, or directly hydrolysed to AMP by NPPs. AMP hydrolysis by CD73 leads accumulation of adenosine, which is further degraded to inosine by adenosine deaminase (ADA). CD39 and CD73 are the two main enzymes involved in the modulation of the nucleotide and nucleoside composition of the TME. It is generally assumed that CD39 and CD73 can affect tumour growth depending on their ability to increase ATP degradation and adenosine accumulation, thus promoting an immunosuppressive TME (Synnestvedt, Furuta et al. 2002).

The hypoxic TME promotes tumour progression by facilitating selection of more aggressive tumour cells and by impairing the anti-tumour immune response. Sitkovsky and co-workers demonstrated that respiratory hyperoxia inhibits MCA205 lung fibrosarcoma growth, and reduces B16 melanoma pulmonary metastasis (Hatfield, Kjaergaard et al. 2015). CD39 and CD73 are upregulated in the hypoxic TME via activation of the transcription factors SP1 and hypoxia-inducible factor 1α (HIF 1α) (Synnestvedt, Furuta et al. 2002). CD39 is expressed by regulatory T cell (T_{reg} cells), dendritic cells (DCs), M2 macrophages and tumour infiltrating Th_{17} lymphocytes (Dwyer, Deaglio et al. 2007, Montalban Del Barrio, Penski et al. 2016). Moreover, stimulation with the potent immunosuppressive factor transforming growth factor-β (TGFβ) increases CD39 and CD73 expression (Ryzhov, Pickup et al. 2014), and accordingly, genetic deletion or pharmacological blockade of either CD39 or CD73 results in improvement in survival by increasing anti-tumour response and decreasing metastasis dissemination (Stagg, Divisekera et al. 2010, Stagg, Divisekera et al. 2011). Moreover soluble CD203a and CD39-like activity have been found in human blood and human plasma (Yegutkin, Samburski et al. 2003), suggesting that soluble ATPases (or in association with microvesicles) may accumulate in the TME, where they collaborate with plasma membrane ectonucleotidases in ATP hydrolysis and adenosine accumulation.

In the TME, the CD39/CD73 axis is important for the pro-inflammatory activity of ATP as suggested by the studies showing that *cd39*$^{-/-}$ or *cd73*$^{-/-}$ mice develop spontaneous inflammatory bowel or lung injury (Friedman, Kunzli et al. 2009).
Removal of extracellular ATP from the tumour microenvironment can follow alternative routes and might be a source of energy for cancer cells. Recent studies show that some cancer cell lines can internalize extracellular ATP by different mechanisms, such as micropinocytosis, thus increasing intracellular ATP level and improving cancer cells drug resistance or promoting tumour progression (Qian, Wang et al. 2014, Loo, Scherl et al. 2015, Qian, Wang et al. 2016, Wang, Li et al. 2017).

1.2. Purinergic receptors

The first evidence that nucleotides and nucleosides are among the mediators of cell-to-cell communication was provided by Burnstock in 1970 (Burnstock, Campbell et al. 1970), who was the first to coin the expression "purinergic signalling".
Purinergic signalling is mediated by two different types of receptors: adenosine receptors, named P1, and nucleotide receptors, named P2.
Virtually all mammalian cell types express P1 and P2 receptors that represent plastic mechanisms for controlling a wide range of cellular functions such as cell differentiation, cell adhesion and migration, cell death, cell proliferation, secretory exocytosis and membrane excitability (Verkhratsky and Burnstock 2014).
P1 receptors comprise four seven-transmembrane spanning (7TM) (A1, A2A, A2B and A3) G-protein coupled members (Gessi, Merighi et al. 2011).
P2 receptors are divided into two subfamilies, metabotropic P2Y and ionotropic P2X, depending on their different pharmacology and associated signalling pathways (Burnstock 2006).

P2Y receptors

P2YR are G-protein coupled metabotropic receptors that comprise eight members (P2Y1R, P2Y2R, P2Y4R, P2Y6R, P2Y11R, P2Y12R, P2Y13R and P2Y14R), identified in mammals with tissue and cell specific distribution. They contain seven transmembrane-

spanning motif, an intracellular C-terminus and an extracellular N-terminus, and are divided into two subgroups depending on their sequence divergence and phylogenetic.
P2Y1R, P2Y2R, P2Y4R and P2Y6R are coupled to inositol 1,4,5-triphosphate (IP3) – dependent signalling via Gq and phospholipase C. P2Y11R activates both Gq and Gs, thus triggering both increase of intracellular Ca^{2+} and cAMP (Thimm, Knospe et al. 2013).
P2Y12R, P2Y13R and P2Y14R activate Gi, thus promoting inhibition of adenylyl cyclase (AC) and reduction of intracellular cAMP concentration.
Different nucleotides, such as ATP, ADP, UTP, UDP or UDP-glucose, are P2YRs agonists. For example, P2Y12R and P2Y13R are preferentially activated by ADP, UTP is a better agonist at P2Y4R, while UDP is a preferential agonist for P2Y6R (Burnstock and Di Virgilio 2013) .
ATP is the best characterized endogenous ligand for P2YRs. P2Y11R is the only ATP-selective receptor at low ATP concentrations, but at higher concentrations ATP is an agonist for P2Y1R and P2Y13R or an antagonist for human P2Y4R and P2Y12R (Burnstock 2006). P2YRs are ubiquitously distributed in many different cells types participating in a wealth of different cellular responses, such as proliferation, cell death and differentiation (Di Virgilio, Falzoni et al. 2016). In the TME P2YRs are expressed by virtually all cancer and host cells binding ATP with different affinity (Burnstock and Di Virgilio 2013). P2Y1R and P2Y2R stimulate proliferation of healthy and cancer cells, thus the increased ATP content of the TME might promote tumour growth and support invasiveness and metastatic spreading via these receptors (Chadet, Jelassi et al. 2014). Recently it has been shown that P2Y12R blockade reduces B16 melanoma growth and metastatization (Gebremeskel, LeVatte et al. 2015), and that P2Y1R, P2Y2R and P2Y6R are responsible for driving resistance to ALK (Anaplastic Lymphoma Kinase) inhibitors in lung cancer (Wilson, Johannessen et al. 2015).

P2X receptors

The P2X ionotropic receptor family includes seven members (P2X1, P2X2, P2X3, P2X4, P2X5, P2X6 and P2X7) that assemble to form hetero- or homo- trimeric cation-selective channels. Each member has two transmembrane segments (TM1 and TM2), cytoplasmic N- and C-terminal and a large ectodomain including ATP binding sites. P2XRs physiological agonist is ATP and the functional receptors show three ATP-binding sites in

the extracellular loop, which all need to be occupied to trigger channel opening (North 2002).

P2XRs are widely expressed in excitable and non-excitable cells and they have a high permeability to Ca^{2+}, Na^{+} and K^{+}. Their activation mediates a large influx of Ca^{2+} and Na^{+} into the cells and a parallel efflux of K^{+}, thus resulting in a depolarizing inward current that may cause the opening of the voltage gated Ca^{2+} channels and the activation of the intracellular Ca^{2+}-dependent signalling as a result of a large increase in the intracellular Ca^{2+} levels (Surprenant and North 2009). In addition, a drop in intracellular potassium, following K^{+} efflux, can generate other signalling pathways, such as NLRP3 inflammasome activation (Martinon, Burns et al. 2002, Abderrazak, Syrovets et al. 2015, Amores-Iniesta, Barbera-Cremades et al. 2017).

In the same way as P2YRs, P2XRs are ubiquitously distributed in many different cells types and expressed by virtually all cancer cell types. It has been reported that P2X3R overexpression is involved in HCC (hepatocellular carcinoma) cell proliferation and survival in response to changes in ATP content in the TME (Maynard, Lee et al. 2015). P2X5 receptor overexpression is also crucial for human basal cell and squamous carcinomas proliferation (Greig, Linge et al. 2003).

1.3. P2X7 receptor

Among the P2X receptors, P2X7R is characterized by unique molecular structure and proprieties, and it is also widely distributed and highly conserved in the animal kingdom, from zebrafish to humans.

The P2X7 receptor is an ATP-gated ion channel with low affinity for ATP. Its activation is associated with two membrane permeability states: a small cation conductance, triggered by rapid exposure to ATP (North and Surprenant 2000), and the opening of a large non-selective pore. It was originally thought that the P2X7R-associated large conductance pore (macropore) opened only after prolonged stimulation, but it is now clear that large increases in conductance occur with no delay after P2X7R gating, thus allowing at the same time small and large cation fluxes (Di Virgilio, Schmalzing et al. 2018).

It was thought that under physiological conditions this receptor should be mainly inactive because of an ATP EC_{50} (the concentration evoking 50% of the response) in the submillimolar range, despite clear evidence of P2X7R association to many responses such as proliferation, cytokine release, differentiation, antigen presentation (Di Virgilio, Dal

Ben et al. 2017). It has been suggested that the low affinity for ATP is a mechanism to prevent P2X7R activation in improper situations that can trigger a strong cytotoxic effect (Di Virgilio, Chiozzi et al. 1998) or inflammatory mediators release. However, while in healthy tissues the ATP concentration is very low and the P2X7R is probably silent, at inflammatory and tumours sites ATP content can reach hundred μmol/L that are sufficient to trigger P2X7R stimulation.

Gene localization, splicing variants and single nucleotide polymorphysms (SNPs)

The human P2X7R is encoded by the *P2RX7* gene located on the long arm of chromosome 12, at 12q24.31 (Buell, Talabot et al. 1998), close to the *P2RX4* gene suggesting an origin by gene duplication. The mammalian *P2RX7* gene includes 13 exons, which in human generate ten (or nine according to the recent re-evaluation) splice variants (P2X7A-J) (Sluyter 2017).

P2X7A is the canonical full-length variant. P2X7B, P2X7C, P2X7E and P2X7G are C-terminally truncated. The first transmembrane region (TM1) is deleted in the P2X7G and P2X7H variants, due to the additional exon N3. P2X7C lacks exon 4, P2X7D exon 5, P2X7E lacks exons 7 and 8, and P2X7F lacks exons 4 and 8. P2X7I variant results from a 5-intronic splice site that generates a null allele (Sluyter and Stokes 2011). P2X7J is truncated after exon 7, in particular, compared to the P2X7A, it lacks the distal 337 amino acids corresponding to the intracellular C-terminal tail, the TM2 and the distal part of the extracellular loop. P2X7J variant is non-functional, but it has been shown to be expressed in cervical cancer cells where it might antagonize the full-length P2X7 receptor through oligomerization (Feng, Li et al. 2006). P2X7B is the predominant transcript in many tissues and it has been related to tumour progression and mesenchymal cell differentiation (Giuliani, Colognesi et al. 2014, Carluccio, Zuccarini et al. 2019). It lacks the last 249 COOH terminal amino acids and has an insertion of 18 extra aa. The receptor made by P2X7B assembly is unable to trigger membrane permeabilization lacking the ability to generate the large, non-selective pore typical of P2X7R. On the other hand, this receptor retains ion channel functions, stimulates growth and it can heteromerize with P2X7A to generate a receptor with different functional proprieties (Adinolfi, Cirillo et al. 2010). Recently has been described a non-functional variant of the human P2X7R (nfP2X7), although its molecular identity is unknown so far (Gilbert, Oliphant et al. 2019).

Splice variants have also been identified in mouse. The P2X7k variant bears an alternative N-terminal and TM1. Receptors formed by P2X7k monomers are fully functional and show increased sensitivity to agonists and pore formation activity compared to the human variants (Nicke, Kuan et al. 2009). This variant may escape inactivation in the P2X7-ko mouse generated in the Glaxo laboratory.
P2X7 13B and P2X7 13C are two mouse variants that are C-terminally truncated and exhibit low channel activity, low plasma membrane expression and no pore function (Masin, Young et al. 2012).

In human *P2RX7* have been reported more than 150 non-synonimus SNPs identified in the extracellular loop and in the C-terminal tail. Some of these genetic variants are related to gain- or loss- of receptor function and they have been associated to a variety of diseases. Two of the best characterized SNPs are the loss-of-function E496A that causes replacement of Glu 496 with Ala and the gain-of-function H155Y, which causes replacement of histidine 155 with tyrosine (Caseley, Muench et al. 2014, De Marchi, Orioli et al. 2016).

P2X7R structure

P2X7R is generated by the assembly of three P2X7 monomers into a homo-trimeric complex. The P2X7 subunit is long 595 amino acids, with a short cytoplasmic N-terminal domain (26 aa), two hydrophobic transmembrane helices (TM1 and TM2), a large extracellular loop and a long intracellular C-terminal tail (239 aa) (Surprenant, Rassendren et al. 1996).
The carboxy-terminal tail (aa 356-595) is unique to P2X7R. It is important for the sub-cellular localization of the receptor, for the initiation of the intracellular signalling cascade, and it is an absolute requirement for the formation of a typical non-selective pore, permeant to cations, nucleotides and other molecules of MW up to 900Da (Di Virgilio, Jiang et al. 2019).

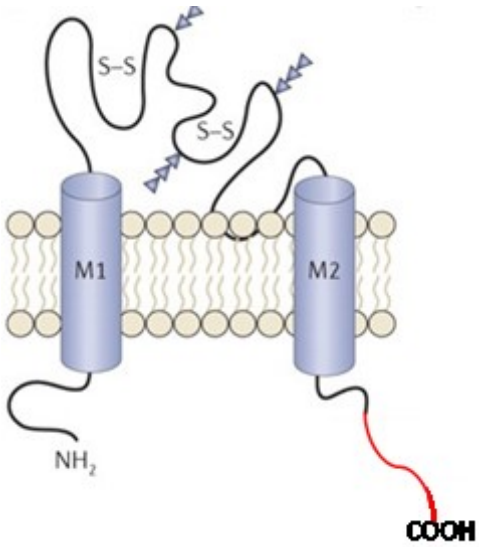


Fig.3: Schematic representation of the P2X7 subunit. The P2X7 protein is a 595 amino acid sequence consisting of an intracellular N-terminus, two hydrophobic transmembrane domains (M1 and M2), an extracellular loop (S-S=disulphide bond) and an intracellular C-terminus. Adapted from (Burnstock 2007)

Two main hypothesis have been put forward to explain the mechanism of pore formation: the first hypothesis states the pore is intrinsic to the receptor, and is either formed since the very early, initial phases of receptor activation (Di Virgilio, Schmalzing et al. 2018) or as a consequence of dilatation of the P2X7R channel; on the contrary, an alternative hypothesis suggests that the P2X7R itself is unable to form a pore, but when activated can recruit accessory molecules (such as pannexin-1) that mediate pore formation (Pelegrin and Surprenant 2006). Karasawa and Kawate also showed that the pore opening is crucially dependent on liposome membrane composition, and quite surprisingly independent of the presence of the C-terminal tail, as it occurs with a C-terminal-deleted P2X7R (Karasawa, Michalski et al. 2017). Furthermore, large pore opening by the full-length P2X7R is facilitated by palmitoylation of cysteine residues in the C-terminal tail (Karasawa, Michalski et al. 2017), and therefore by enhanced association of the C-terminal residue to plasma membrane cholesterol (Gonnord, Delarasse et al. 2009).

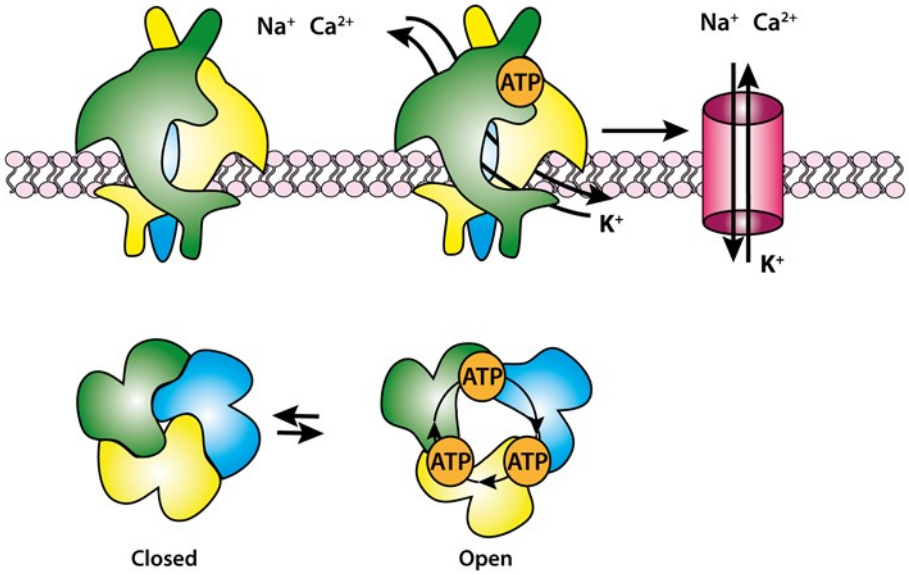


Fig.4 Hypotetical mechanism of channel/pore formation. Assembly of P2X7 subunits to form the functional homotrimeric P2X7 receptor (P2X7R) is shown. Binding of three ATP molecules gates a channel/pore that allows the influx of monovalent cations and other molecules. Adapted from (Di Virgilio, Schmalzing et al. 2018)

The first crystal structure of a mammalian P2X7R has been obtained from a truncated subunit of the *Ailuropoda melanoleuca* (giant panda) P2X7R, allowing the identification of the ATP-binding pocket and allosteric sites. The binding site of ATP is located at the interface of each pair of adjacent subunit and it has been suggested to be characterized by a restricted access, which might be the reason why several P2XRs agonists are inactive at the P2X7R (Karasawa and Kawate 2016).

P2X7R ligands and pharmacology

Agonists:

ATP is the only known physiological P2X7R agonist despite its low affinity, since ten- to hundred-fold higher ATP concentrations are needed compared to the other P2XR (North 2002).

The most widely used P2X7R agonist is the synthetic ATP analog 2',3'-(4-benzoil)-benzoil-ATP (BzATP), that is about ten times more potent than ATP, but is no highly selective for the P2X7R.

Over the last several years it has been proposed that other "non-ATP" agonists may trigger P2X7R activation (Di Virgilio, Giuliani et al. 2018). The murine P2X7k subunit undergoes ADP-ribosylation that causes a durable activation of the receptor. This modification is catalysed by the plasma membrane enzyme ADP-ribosyltransferase (ARTC2.2) that transfers an ADP-ribose moiety from NAD^+ to arginine 125 in the extracellular loop of the P2X7R, close to the ATP-binding pocket (Rissiek, Haag et al. 2015).

The amyloid β triggers several P2X7R-dependent responses such as Ca^{2+} influx, IL-1β release and cytotoxicity, acting as positive allosteric modulator that lowers the ATP threshold for P2X7R activation (Sanz, Chiozzi et al. 2009). Other positive allosteric modulators are the cathelicidin LL-37, an antimicrobial peptide presents in human granulocytes, lymphocytes, macrophages and epithelial cells, and polymyxin B, an antibiotic derived from the bacterium *Baciullus polymyxa*. These molecules potentiate the P2X7R-associated Ca^{2+} uptake, plasma membrane permeabilization and cytotoxicity. Furthermore, it has been suggested that serum amyloid A (SSA) directly activates P2X7 receptor and that LPS (lipopolysaccharide) and Alu-RNA intracellular accumulation can stimulate P2X7R acting on the cytoplasmic side (Di Virgilio, Giuliani et al. 2018).

Antagonists:

The most widely used P2X7R antagonist is oxidized ATP (oATP), however this reagent, albeit potent, can also inhibit other members of P2X family and unrelated plasma membrane receptors. oATP is a dialdehyde reagent that forms a Schiff base with unprotonated lysins close to the ATP-binding pocket, thus causing an irreversible inhibition of the receptor (Murgia, Hanau et al. 1993, Di Virgilio 2004).

KN-62 (1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine) is another potent human P2X7R antagonist with an IC_{50} of 40-100nM (Gargett and Wiley 1997). These two compounds belong to the first generation of P2X7R antagonists, e. g. Reactive Blu2, Brilliant Blu G and PPADS (pyridoxal phosphate-6-azophenyl-2-4-disulfonic acid, that exhibit generally low selectivity. Typical problems of the first generation compounds, such as predicted poor pharmacokinetics, were solved by the second generation of P2X7R antagonists, including *A-438079*, a tetrazolylmethylpyridine based compound, *AZ10606120*, an adamantly derivate, and *A740003*, a cyanoguanidine derivate (Donnelly-Roberts and Jarvis 2007).

Since 2013, the group at Janssen disclosed a series of brain penetrant P2X7R phenylpiperazine based antagonists (JNJ-47965567, JNJ-42253432) and several additional classes beginning with a series of fused 1,2,3-triazoles (JNJ-54140515, JNJ-54166060,

JNJ-54175446, JNJ-55308942). All these compounds demonstrated activity at rodent and human P2X7R, had good rat pharmacokinetic profiles and excellent brain penetration (Rech, Bhattacharya et al. 2016).

P2X7R: not only a cytotoxic receptor

P2X7 receptor is widely known as a prototypic cytotoxic receptor for its ability to trigger necrotic or apoptotic cell death. However, accumulating evidence shows that low level P2X7R stimulation promotes a trophic stimulation. Furthermore several cellular metabolic responses are dependent on P2X7R in quiescent cells, thus suggesting that this receptor has also a basal level of activation in resting cells. This tonic activity of P2X7R has been correlated with cell proliferation and differentiation, motility, intracellular ion balance and basal mitochondrial metabolism (Di Virgilio, Ferrari et al. 2009).

In 1999 Di Virgilio and co-workers provided the first demonstration of the trophic role of P2X7 receptor in K562 (human erythroleukemia) and LG14 (B-lymphoblastoid cell line) cells, both lacking endogenous P2X7R. In these cell types, transfection of P2X7 conferred a growth advantage in the absence of serum (Baricordi, Melchiorri et al. 1999), as shown later also in HEK-293 cells (Human Embryonic Kidney), another cell line lacking P2X7R (Adinolfi, Callegari et al. 2005). In addition, a proof to support the role of P2X7R in proliferation was indirectly generated from the finding that peripheral B lymphocytes from patients affected by the chronic lymphocytic leukemia (CLL) express higher P2X7R levels than B lymphocytes from healthy controls (Adinolfi, Melchiorri et al. 2002).

P2X7R activation induces different downstream signalling events, including the activation of apoptosis and NLRP3 inflammasome complex, the production and proinflammatory cytokine (IL-1β, IL-18, IL-2, ROS), the release of metalloproteases and the regulation of bone mineralization (Di Virgilio 2013).

Due to its dual role in cell death and cell proliferation, the P2X7 receptor is attracting interest in chronic inflammatory disorders and neurodegenerative diseases, in metabolic dysfunctions and in cancer (Adinolfi, Capece et al. 2015, Amadio, Parisi et al. 2017, Fabbrizio, Apolloni et al. 2019).

P2X7R in cancer progression

The TME is characterized by high ATP concentrations (hundred μmol/L), thus under these conditions the cytotoxic effect mediated by P2X7R should prevail, but surprisingly cancer cells seems to be resistant to such high extracellular ATP levels. It has been proposed that in tumour cells the P2X7R activation and the subsequent pore opening are uncoupled from the intracellular death pathways (Raffaghello, Chiozzi et al. 2006), or alternatively, the macropore opening is inhibit by the high plasma membrane cholesterol content (Robinson, Shridar et al. 2014).

Over the years several studies demonstrated the crucial role of P2X7R stimulation in tumour progression (Adinolfi, Melchiorri et al. 2002, Raffaghello, Chiozzi et al. 2006, Solini, Cuccato et al. 2008) and its association with a poor prognosis suggests that P2X7R overexpression might be a negative prognostic indicator in several cancers, such as neuroblastoma (Amoroso, Capece et al. 2015), prostate cancer (Ghalali, Wiklund et al. 2014), acute myeloid leukemia (Jelassi, Chantome et al. 2011), melanoma (Deli, Varga et al. 2007) and breast cancer (Tan, Han et al. 2015).

P2X7R promotes the activation of different pathways, including NFATc1, ERK, PI3K/Akt and HIF-1α, that are involved in tumor cell growth, invasiveness and metastasis (Jacques-Silva, Rodnight et al. 2004, Amoroso, Capece et al. 2015, Gomez-Villafuertes, Garcia-Huerta et al. 2015). Local invasion and metastatic spreading are complex processes facilitating by the release of proteins involved in extracellular matrix (ECM) degradation and reorganization, such as metalloproteinases (MMPs) and cathepsins. P2X7R stimulation triggers ECM invasion and the release of MMPs and active cathepsins (Jelassi, Chantome et al. 2011) in human melanoma MDA-MB-435s cells, human T47D breast cancer cells, human PC-3M prostate cancer cells and PC9 lung carcinoma (Takai, Tsukimoto et al. 2014, Xia, Yu et al. 2015). On the other hand, P2X7R silencing or pharmacological blockade affect invasion and metastatic dissemination by down-modulating expression of the epithelial/mesenchymal transition genes (Qiu, Li et al. 2014).

In the contest of TME, extracellular ATP and P2X7R may also be involved in immunogenic cell death (ICD), which is an atypical type of cell death associated with DAMP (damage-associated molecular pattern) release into the extracellular space (Galluzzi, Buque et al. 2017). ATP released by dying tumour cells activates P2X7 receptors in dendritic cells (DCs), thus triggering IL-1β release via NLRP3 inflammasome activation. IL-1β is a potent local stimulus for DC activation and presentation of tumor antigens to $CD4^+$ and $CD8^+$ T lymphocytes, that is crucial for the efficacy of chemotherapy

(Aymeric, Apetoh et al. 2010, Di Virgilio 2016). In addition, stimulation of host P2X7R triggers cytokine release and tumour infiltration by inflammatory cells, thus supporting an antitumor immune response. Recent studies have shown that genetic deletion of host P2X7 in mouse (P2X7-KO) affect immune response against melanoma cells (B16) and colon carcinoma cells (CT26) (Adinolfi, Capece et al. 2015), and tumours grow faster than P2X7-WT mice.

At the same time, the TME is heavily infiltrated by P2X7R expressing macrophages and other cells of the myeloid lineage with a strong immunosuppressive activity. It has been demonstrated that P2X7R activation contributes to the generation of an immunosuppressive TME by stimulating the release of ROS (reactive oxygen species), arginase-1 and TGF-β1 (transforming growth factor-β1) from MDSCs (myeloid-derived suppressor cells) (Bianchi, Vuerich et al. 2014).

However, all pre-clinical data show an anti-tumour effect of in vivo administration of P2X7R-targeting drugs, suggesting that impairment of P2X7R activity on the cancer cells prevails on the potential inhibition of the anti-tumour immune response on the host immune cells.

1.4. Cancer cell metabolism

In every cell, the main energy source for cellular metabolism is glucose, which is stepwise converted into ATP through three processes: glycolysis, tricarboxylic acid cycle (TCA or Krebs cycle) and finally oxidative phosphorylation. In the first step, one molecule of glucose is converted into two molecules of pyruvate, producing two molecules of ATP and NADH. Then pyruvate is converted to acetyl-Coenzyme A (acetyl-CoA) which enters the Krebs cycle, allowing the generation of the electron transporter molecules NADH and FADH2. Finally, the respiratory chain complexes (OXPHOS) use NADH molecules to generate a proton gradient across the inner mitochondrial membrane, enabling the mitochondrial ATP synthase to produce 36 molecules of ATP, plus CO_2 and H_2O.

In the absence of oxygen, mitochondrial respiration (oxidative phosphorylation) can't occur. On the contrary, glycolysis (anaerobic glycolysis) promotes the conversion of glucose to lactate by lactate dehydrogenise (LDH), producing only two molecules of ATP when oxygen is depleted.

In 1924 Otto Warburg reported that cancer cells exhibit an increase in glycolytic activity and produce large amount of lactate even under normal oxygen concentration (Warburg, Wind et al. 1927).

After these observation, Warburg hypothesized that cancer cells acquired a permanent respiratory defect, caused by an irreversible damage of the mitochondrial function, resulting in this "metabolic switch" knows as "Warburg effect". Since then, altered energy metabolism has been recognized as one of the hallmarks of cancer.

It has been proposed that the increase in glycolytic activity is a metabolic strategy that allows for ATP production with low oxygen concentration, ensuring survival and growth in hypoxic conditions. Other explanation suggests that glycolytic activity would be advantageous for cancer cell proliferation because glycolysis generates ATP at a higher rate than oxidative phosphorylation. Moreover, it has been proposed that metabolic intermediates of glycolysis confer selective advantage under limited nutrient conditions, playing a pivotal role in macromolecules biosynthesis.

However, in recent years it has become clear that many signalling pathways affected by genetic mutations and the tumour microenvironment contribute to altered metabolic phenotypes of cancer cells. Furthermore, it has been demonstrated that tumour cells do not necessarily exhibit mitochondrial defects and most cancers retain the capacity for oxidative phosphorylation (Cairns, Harris et al. 2011, Salem, Whitaker-Menezes et al. 2012).

P2X7R is a modulator of cancer energy metabolism

ATP might be considered not only the high-energy intracellular intermediate, but also an extracellular modulator of energy metabolism via P2X7R.

Activation of P2X7R stimulates aerobic glycolysis and increases lactic acid output. The stimulation of glycolysis is supported by increased expression of the key glycolytic enzymes and transporters involved in the "Warburg effect", such as GLUT-1, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphofructokinase (PFK), pyruvate kinase M2 (PKM2) and pyruvate dehydrogenase kinase 1 (PDK1). Furthermore, pyruvate dehydrogenase (PDH) and glycogen synthase kinase (GSK)-3β activity are inhibited, expression of phosphorylated Akt/PKB and hypoxia-inducible factor 1α (HIF1α) are increased, and intracellular glycogen stores are expanded after P2X7R activation (Amoroso, Falzoni et al. 2012, Amoroso, Capece et al. 2015). In addition, the P2X7R-dependent activation of the PI3K/Akt pathway is involved in tumour growth, invasiveness,

metastatization and angiogenesis, and this pathway triggers P2X7 expression via SP1 (Gomez-Villafuertes, Garcia-Huerta et al. 2015). A strong stimulation of glycolysis is also important to generate biosynthetic intermediates for the synthesis of phospholipids, non-essential amino acids and nucleic acids (anaplerosis). At the same time, basal activation of P2X7R induces a moderate elevation in intracellular and mitochondrial Ca^{2+} content. The elevated intra-mitochondrial Ca^{2+} stimulates NADH oxidation via oxidative phosphorylation, promoting ATP production and supporting cell growth (Adinolfi, Callegari et al. 2005). The potentiation of both oxidative phosphorylation and aerobic glycolysis, and the simultaneous stimulation of anaplerosis cause a large increase of the total intracellular ATP content, resulting in a consistent proliferative advantage of P2X7R-expressing cells.

Our laboratory showed that HEK-293 cells transfected with P2X7R exhibit an increased endoplasmic reticulum Ca^{2+} concentration and activate the nuclear factor NFATc1 to enhance cells survival (Adinolfi, Callegari et al. 2009). Additional studies about the effects of P2X7 expression on mitochondrial physiology revealed that mitochondria of P2X7-transfected cells display a membrane potential 20-30mV more negative than control cells, and this hyperpolarization is entirely dependent on P2X7R function.

These finding focused attention on the mitochondria, a crucial organelle for the energy metabolism and ion homeostasis.

Recently, we also showed that P2X7R expression is required for amyloid β-dependent mitochondrial toxicity in microglia (Chiozzi, Sarti et al. 2019) and Jameson and co-workers demonstrated that P2X7R activates $CD8^+$ T cells and supports their mitochondrial activity. In particular they found that P2X7R deficiency causes loss of mitochondrial mass and function in memory CD8 T cells (Borges da Silva, Beura et al. 2018). On the other hand, it has been found that P2X7 receptor overstimulation causes an uncontrolled increase of mitochondrial Ca^{2+} levels, resulting in mitochondrial network fragmentation and cell death (Adinolfi, Callegari et al. 2005).

These results highlight the dual role of P2X7 receptor: it can promote mitochondrial health and cell growth or causing mitochondrial damage and cell death, depending on the extracellular ATP concentration.

In conclusion, all these findings demonstrate the crucial role of extracellular ATP in the regulation of cancer cell metabolism and proliferation, and highlight the need to understand the mechanisms responsible for ATP accumulation in the TME.

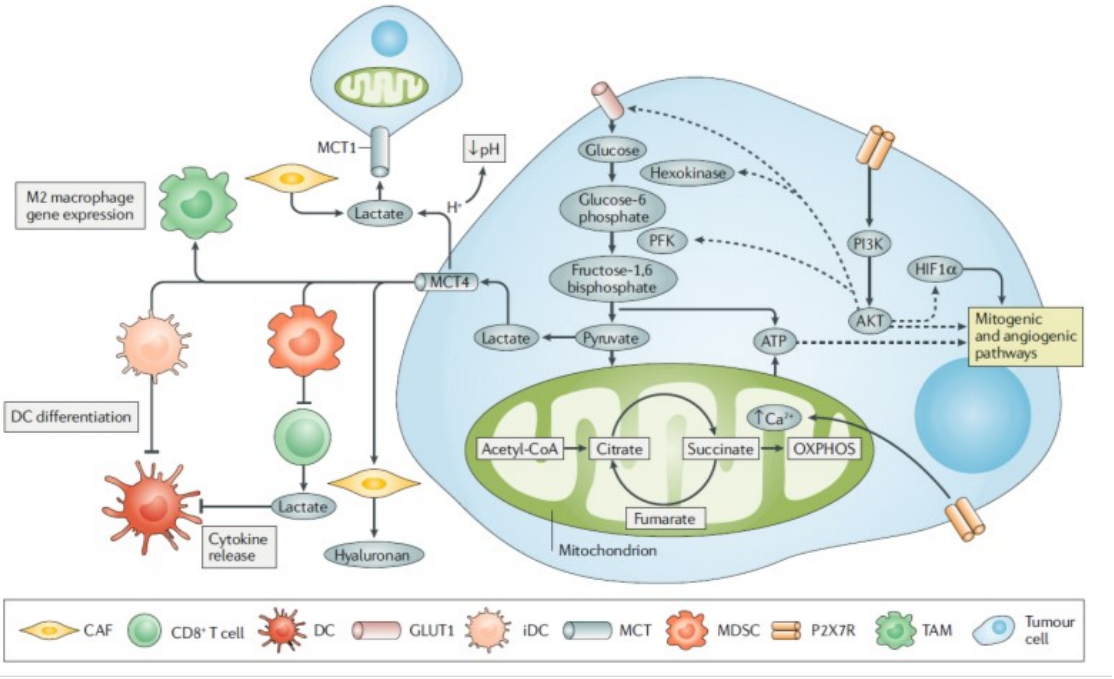


Fig.5: Stimulation of cancer cell metabolism by P2X7R. Tonic P2X7R activation causes moderate increase in the mitochondrial Ca^{2+} concentration, stimulation of oxidative phosphorylation (OXPHOS) and increased ATP generation. At the same time, P2X7R activation upregulates expression of the plasma membrane glucose transporter GLUT1, possibly via the PI3K-AKT pathway (denoted by the dotted arrow), and the expression of several enzyme of the glycolytic cascade. P2X7R activation leads to increased lactate generation. Lactate is extruded by monocarboxylate transporter4 (MCT4), causing acidification of the TME, and promotes M2 macrophage differentiation and myeloid-derived-suppressor cell (MDSC) activation. Lactate also inhibits differentiation of immature dendritic cells (iDC) into mature dendritic cells (DCs) and promotes secretion of extracellular matrix components (hyaluronan) by cancer-associated fibroblasts (CAFs), contributing to cancer cell proliferation and invasion. (Di Virgilio, Sarti et al. 2018)

2. AIMS AND MAIN FINDINGS

Caloric restriction has been shown to be effective in cancer-prevention in laboratory strains of rodents (Albanes 1987, Nencioni, Caffa et al. 2018) and treatment with caloric restriction mimetics (CRMs) inhibits tumor growth *in vivo*. A recent study has shown that this might be due to the ability of CRM to increase the ATP concentration in the tumor microenvironment (TME), modifying the TME composition and reducing the presence of immunosuppressive regulatory T cells (Pietrocola, Pol et al. 2016). The tumor microenvironment is rich in extracellular ATP, a nucleotide that acts at plasma membrane P2X7 receptor (P2X7R). P2X7R is a promoter of cell survival or of cell death, depending on the level of activation, and it establishes a special relationship with ATP because it is a main sensor for extracellular ATP, a conduit for ATP release and it also can increase the intracellular ATP concentration by stimulating glycolysis and oxidative phosphorylation.
In the TME, P2X7R is expressed on infiltrating immune cells and on tumor cells, inducing both tumor-reducing and tumor-promoting effects. Moreover, it has been proposed that extracellular ATP accumulation in the TME might be beneficial for tumors not only by stimulating P2X7R and promoting proliferation, but also because it could be a source of energy for cancer cells (Loo, Scherl et al. 2015), or because it might improve resistance of cancer cell to chemotherapics (Wang, Li et al. 2017).

My thesis aims at investigating the complex interrelationship between extracellular ATP, P2X7R and tumor cells energy metabolism. To mimic metabolic conditions found in the TME, we induced caloric restriction *in vivo,* by CRM administration to C57bl/6 WT mice, and *in vitro,* by exposing B16F10 mouse melanoma cells to serum starvation and CRM treatment. We correlated intracellular and extracellular ATP concentration using state-of-the-art techniques such as both soluble luciferase and plasma membrane-expressed luciferase (pmeLUC). ATP accumulation into the TME *in vivo* was explored using pmeLUC-expressing tumor cells as well as the pmeLUC-TG-mouse (a transgenic mouse model engineered with pmeLUC that ubiquitously expresses this probe) and mitochondrial metabolism was analysed with the SeaHorse apparatus in P2X7R proficient and deficient cells.

Main findings

In this work I show that caloric restriction causes ATP accumulation into the TME while at the same time paradoxically impairing intracellular ATP synthesis and accumulation. Inhibition of energy metabolism is likely due to the extensive fragmentation of the

mitochondrial network caused by serum starvation. ATP release under these conditions mainly occurs via release of ATP-loaded plasma membrane-derived microvesicles. The high ATP cargo is at least in part due to trapping of mitochondria (or mitochondrial fragments) within the microvesicles.

My findings show that caloric restriction dramatically upsets tumor cell metabolism but at the same time counterintuitively promotes extracellular ATP accumulation via a microvesicle-mediated pathway. These findings might help understand how the extracellular ATP concentration is regulated in the TME.

3. RESULTS

3.1. Hydroxycitrate increases extracellular ATP and reduces tumor growth *in vivo*

Caloric restriction reduces tumor incidence and short-term fasting improves anticancer chemotherapy. In different mouse models of tumors the efficacy of chemotherapy is increased by acute (48h) starvation (Lee, Raffaghello et al. 2012) and caloric restriction mimetics (CRMs) enhance the anticancer activity of chemiotherapics (Pietrocola, Pol et al. 2016).

Caloric restriction mimetics are non-toxic pharmacological agents or natural compounds that mimic the biochemical effects of nutrient deprivation by reducing cellular protein acetylation.

Hydroxycitrate (HC) is a CRM that acts as an inhibitor of the ATP citrate lyase (ACLY), an enzyme that generates cytosolic acetyl coenzyme A (AcCoA), impairing lipid biosynthesis (Onakpoya, Hung et al. 2011).

To investigate the effect of caloric restriction on extracellular ATP and tumor growth *in vivo,* we inoculated C57bl/6 WT mice with B16F10 mouse melanoma cells stably transfected with the plasma membrane luciferase construct (pmeLUC). These cells are hereafter referred to as B16F10-pmeLUC. After post-inoculum day 5, mice were treated with the CRM hydroxycitrate (i.p.). Tumor volume, assessed by caliper, and TME ATP levels were measured at post-inoculum (p.i.) days 5, 7 and 9.

As shown in Fig. 1B, hydroxycitrate reduced B16F10-pmeLUC tumor size. At the same time, the ATP concentration in the tumor microenvironment was statistically significantly increased ($p<0,001$) (Fig. 1A-C), confirming the data previously showed by Kroemer and coworkers (Pietrocola, Pol et al. 2016).

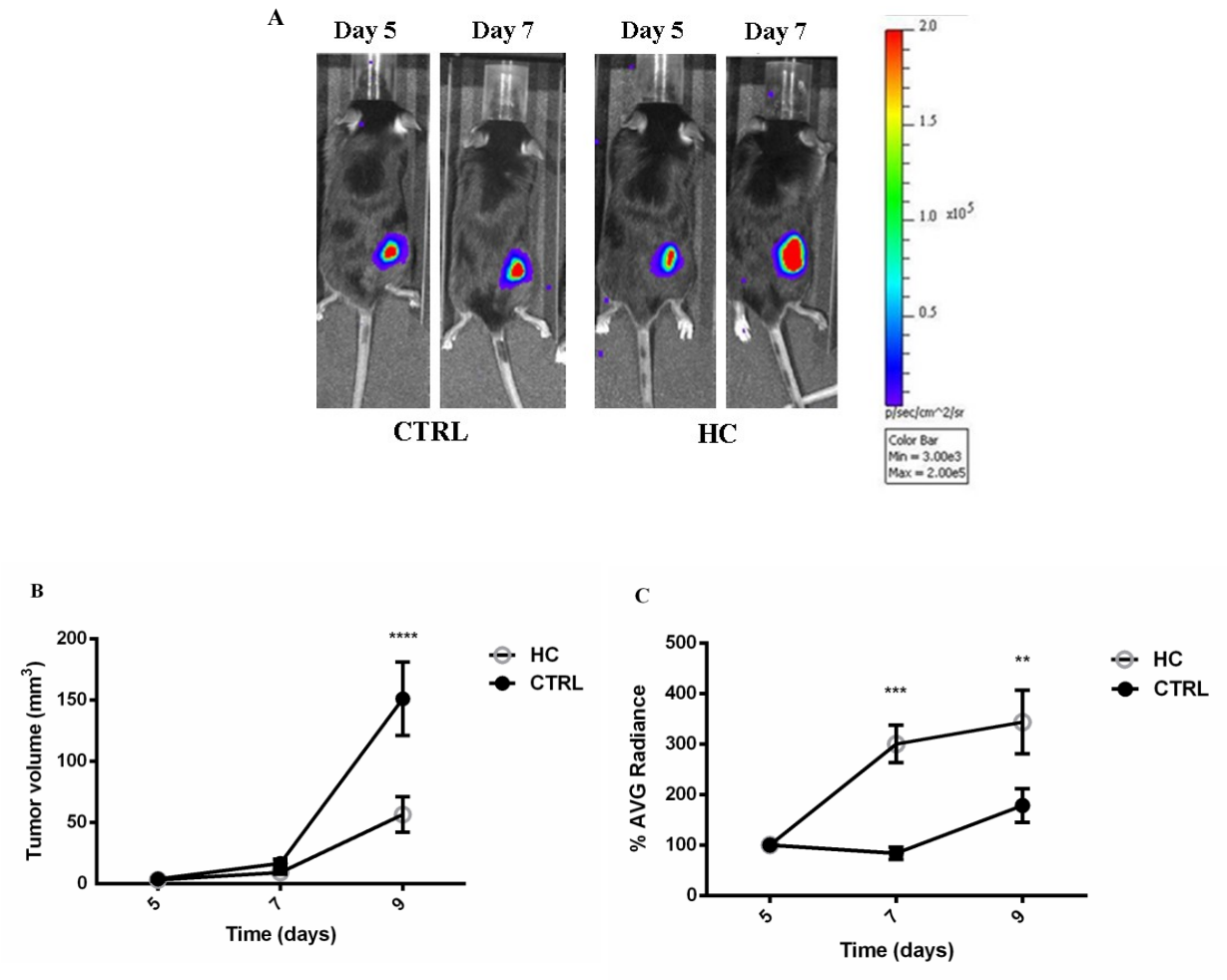


Fig.1: Hydroxycitrate increases extracellular ATP and reduces tumor volume. (**A-C**) C57bl/6 WT mice (n= 12) were inoculated into the right hind flank with B16-pmeLUC cells (2,5x10^5). **A** representative pictures of pmeLUC luminescence emission. At post-inoculum day 7, luminescence was much higher in the mouse treated with hydroxycitrate (300mg/kg) than control mouse. **B** Tumor volume was in vivo assessed by calliper at the indicated time points. **C** Measure of ATP levels in tumor-bearing mice estimated by pmeLUC luminesce emission (p/s/cm^2/sr). Data are reported as percentage increase in luminescence compared to day 5. Data are shown as mean ± SEM. **P<0.01; ***P<0.001; ****P<0.0001 Statistical analysis was determined by ANOVA.

The effects of hydroxycitrate were also investigated *in vitro* in B16F10 mouse melanoma cells. Extracellular ATP levels were measured in the pericellular space with soluble luciferase (Fig. 2A) as well as with the plasma membrane-expressed pmeLUC probe (Fig. 2B), 24 and 48 hours after treatment. Hydroxycitrate increased ATP release after 48 hours without serum. Moreover, it strongly impaired cell growth (Fig. 2C).

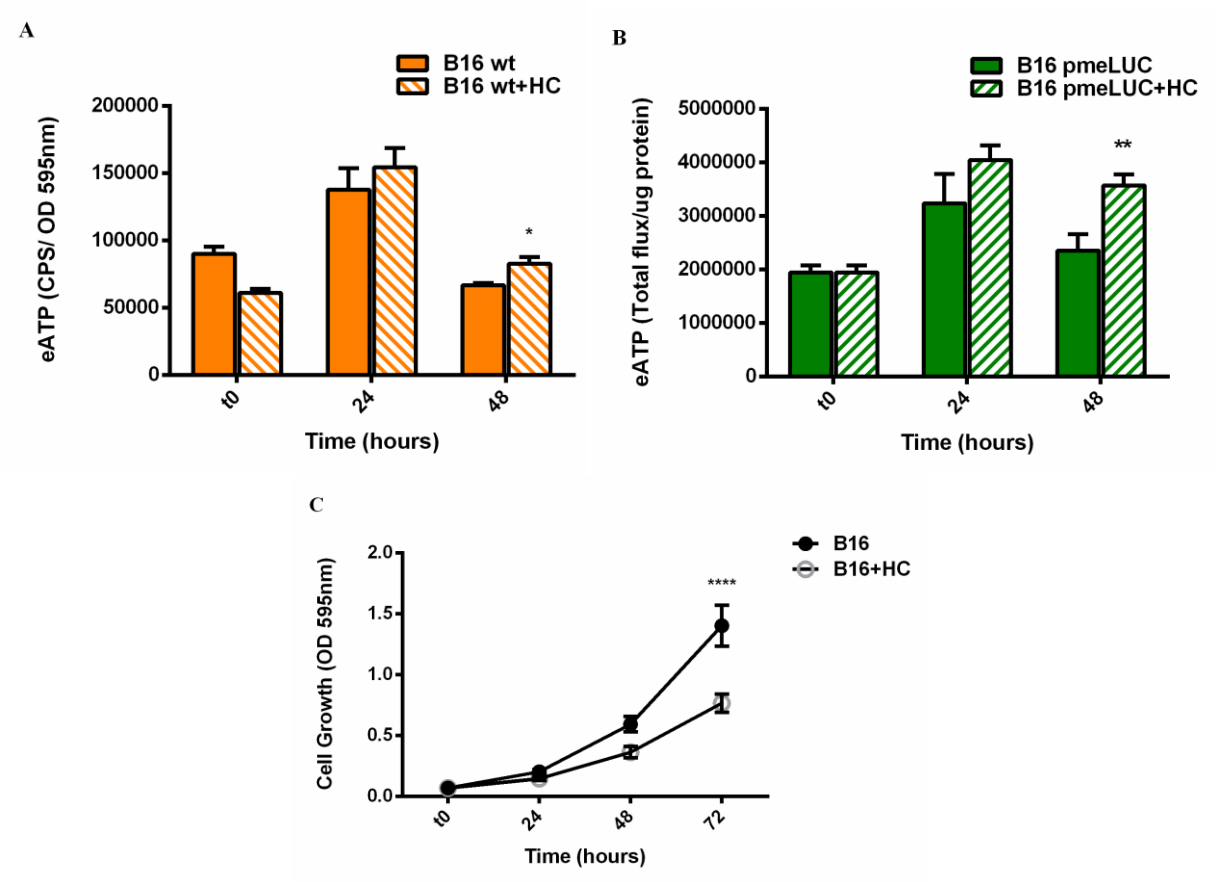


Fig.2: Hydroxycitrate reduces cell growth and increases eATP levels *in vitro*. A B16wt cells were treated with hydroxycitrate (1mM) at time zero and extracellular ATP levels were measured after 24 and 48 hours using soluble luciferase. CPS (counts per second) were normalized to cells content determined via crystal violet (OD 595nm) (n=4). **B** pmeLUC-transfected B16 cells were treated with hydroxycitrate (1mM) and extracellular ATP levels were measured using IVIS luminometer. Total flux was normalized to cell protein (µg) (n=6). **C** Cell proliferation assay of hydroxycitrate (1 mM)-treated B16 wt cells performed by crystal violet technique. (n=17) Data are shown as mean ± SEM, *P<0.05; **P<0.01; ****P<0.0001by ANOVA.

Extracellular ATP is sensed by host tumor-infiltrating inflammatory cells

In Figure 1A-C, the TME ATP was measured with pmeLUC expressed on the tumor cell plasma membrane, thus it cannot be excluded that this probe measures ATP within the tumor core, which might not be fully accessible to host immune cells. To verify whether the high ATP concentration detected by tumor-associated pmeLUC is also sensed by host inflammatory cells, we generated a melanoma tumor by inoculating B16F10-WT cells into the pmeLUC-TG-mouse. The pmeLUC-TG-mouse is a transgenic mouse generated in our laboratory which expresses the pmeLUC probe in all tissues, and therefore on the immune cells as well. Since the dark fur may interfere with light emission, the area over the right hip, where the tumor cells were inoculated, and the corresponding area on the control mouse were shaved. Eleven days post inoculum, tumor-associated luminescence was clearly visible and mainly concentrated at the periphery of the tumor mass (Fig. 3).
Overall luminescence intensity was lower than that of B16F10-pmeLUC tumors, likely due to the higher density of pmeLUC probe in the B16F10-pmeLUC compared to B16F10-WT tumors, in which pmeLUC is expressed only on the infiltrating immune cells, that are fewer than tumor cells. In any case, both experimental models suggest that most of the ATP detected in the TME derives from tumor cells.

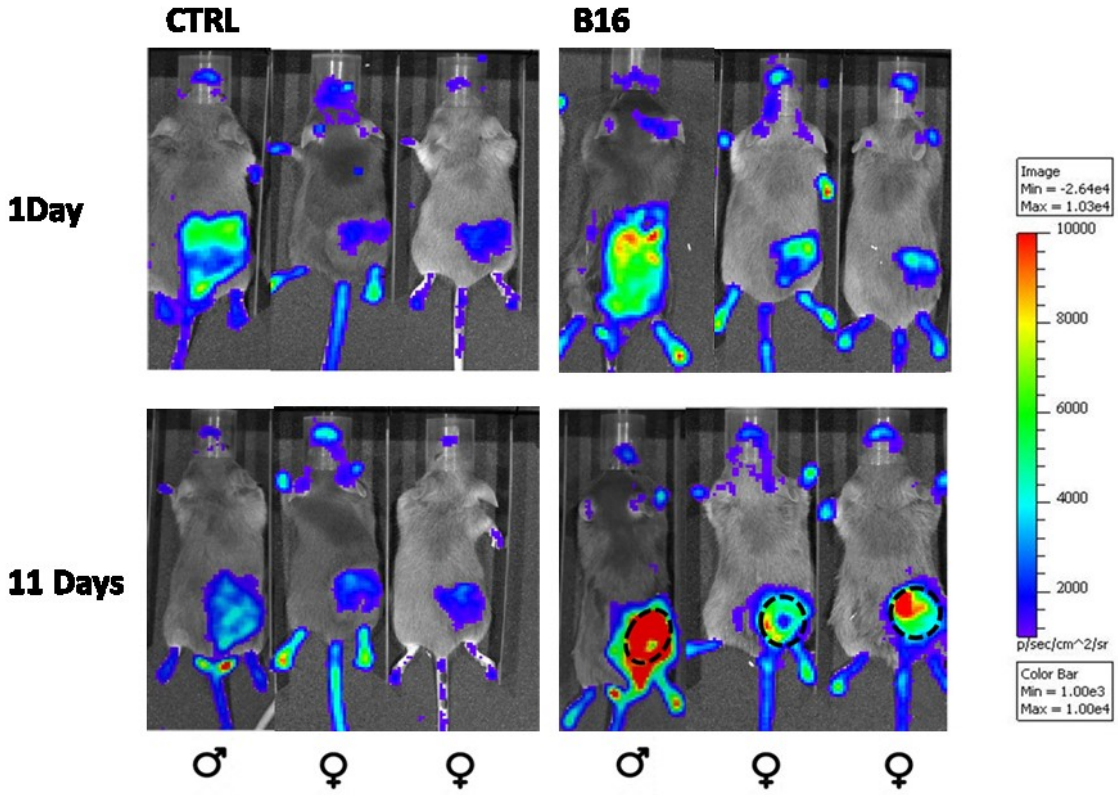


Fig.3: Tumor infiltrating immune cells detect TME ATP. Representative pictures of luminescence signal detected in pmeLUC transgenic mice after injection of D-luciferin. Mice on the right were inoculated into the right hind flank with B16 wt cells. Mice on the left (controls) were only shaved on the area corresponding to the inoculation area. Eleven days post-inoculum luminescence emission was higher in tumor-bearing mice (lower-right) than control mice (lower-left), and it was concentrated at the periphery of tumor mass. Dotted lines on tumor-bearing mice highlight the perimeter of the tumor mass.

3.2. Serum starvation mimics hydroxycitrate effects *in vitro*

Serum starvation increases extracellular ATP levels

We mimicked hydroxycitrate activity in vitro by exposing B16F10 cells to serum starvation (nutrient deprivation) for 24 and 48 hours. Serum starvation strongly increased extracellular ATP, whether measured with soluble luciferase (Fig. 4A) or with the pmeLUC probe (Fig. 4B). Since serum contains ecto-ATPases that might also degrade extracellular ATP, lower ATP levels in the supernatants from serum-supplemented versus serum-starved cells could be due to the ATP hydrolysis. To exclude this possibility, we measured the hydrolysis of ATP in B16F10 monolayers incubated in the presence and absence of serum. As shown in Fig. 4C, rate of hydrolysis of exogenous ATP was not significantly different between serum-supplemented or serum-free conditions, suggesting that in cell monolayers the main determinant of ATP hydrolysis was ectonucleotidase activity.

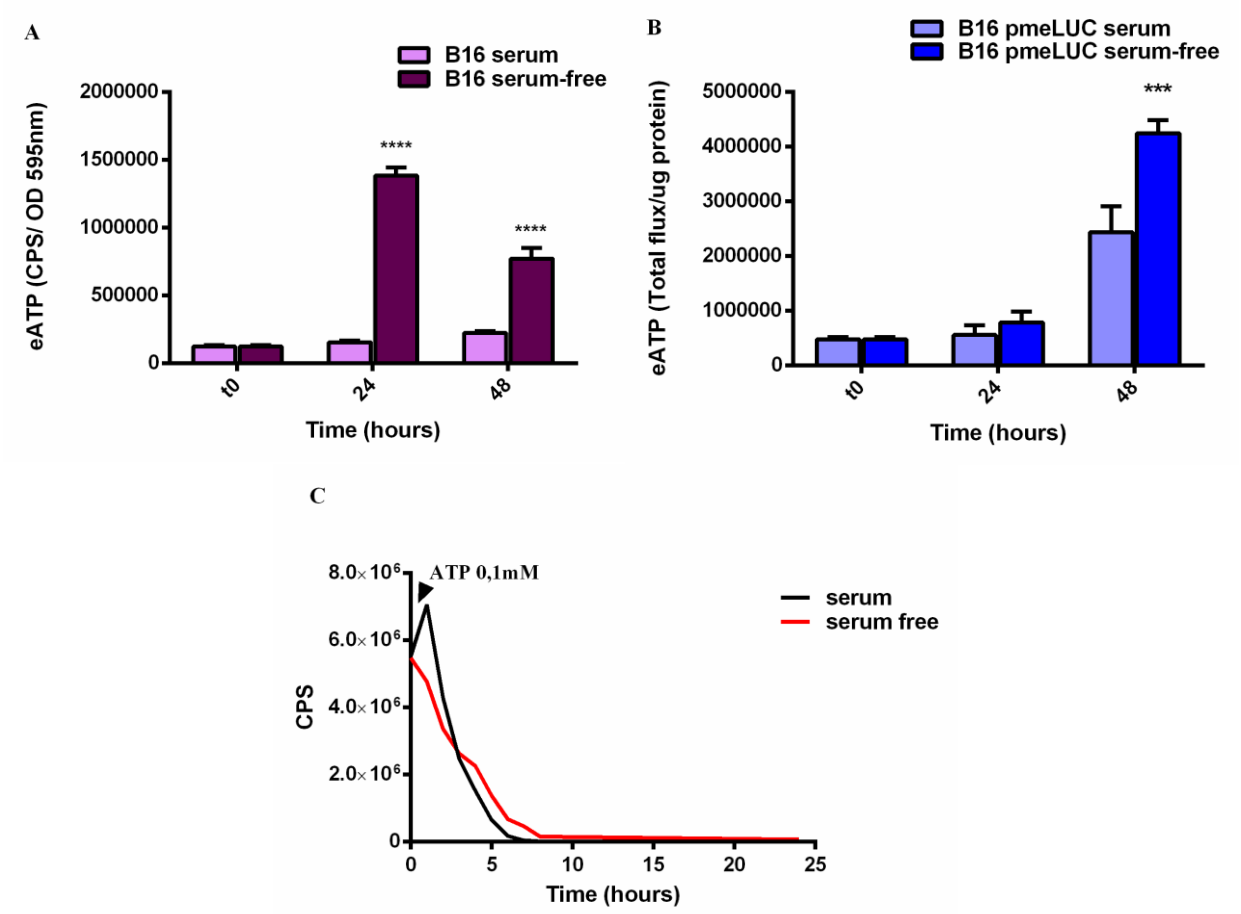


Fig.4: Serum starvation increases extracellular ATP. A-B B16wt and pmeLUC-transfected cells were cultured in the presence and absence of serum for 24 and 48 hours. **A** Extracellular ATP levels were detected using soluble luciferase. CPS (counts per second) were normalized to cells content determined via crystal violet (OD 595nm) (n=6). **B** ATP levels were measured in the

pericellular space of pmeLUC cells using IVIS luminometer. Total flux were normalized to cell protein (μg) (n=6), **C** B16 monolayers were maintained in the presence (black line) and absence of serum (red line) for 24 hours. After addition of ATP 0,1 mM, hydrolysis of ATP was measured in the supernatant at 1 h intervals. Serum extracellular vesicles may express P2X7R which confers susceptibility to ATP-dependent lysis causing the CPS increase during the first 1-2 hours (Pizzirani, Ferrari et al. 2007). Data are shown as mean ± SEM. ***P<0.001; ****P<0.0001. Statistical analysis was determined by ANOVA.

Serum starvation slows B16F10 cell growth but does not induces cell death or autophagy

Similarly to hydroxycitrate, chronic incubation in the absence of serum slowed down melanoma cell growth (Fig. 5A), without affecting cell viability, as shown by very low levels of LDH (lactate dehydrogenase) release from serum starved cells (Fig. 5B). It is known that serum/nutrient deprivation increases autophagy and mitophagy, which play a key role in several pathological processes, cancer included (Galluzzi, Pietrocola et al. 2015, White 2015). Autophagy is a self-degradative process that allows the turnover of cellular components and damaged organelles through a complex intracellular pathway involving formation of double-membrane vesicles called autophagosomes, which eventually fuse with lysosomes and leads to the final degradation of the autophagic cargo. During autophagy the microtubule-associated protein 1A/1B-Light Chain 3 (LC3-I) is processed to its phosphatidylethanolamine conjugate named LC3-II and recruited to autophagosomes. LC3-II quantification is a common way to quantify autophagy since it increases when autophagy is stimulated. On the contrary, degradation of TOM20 (Translocase of Outer Mitochondrial Membrane 20) is a commonly used marker of mitophagy. As shown in Fig. 5C, serum free conditions did not accumulate LC3-II protein and therefore did not inhibit the autophagic flux, as instead it is shown in the presence of the Bafilomycin A1, an inhibitor of autophagosome and lysosome fusion, that blocks LC3-II turnover and degradation. Moreover, TOM20 seems to be decreased at 48h, perhaps indicating increase in mitochondria degradation.

Since AMPK (AMP activated protein kinase) is a positive autophagy regulator (Mihaylova and Shaw 2011) and is activated during serum/nutrient deprivation, we also investigated AMPK activation. In line with autophagy, phosphorylation of AMPK isoform α in Thr172, which is involved in AMPK activation, was not increased (Fig. 5D).

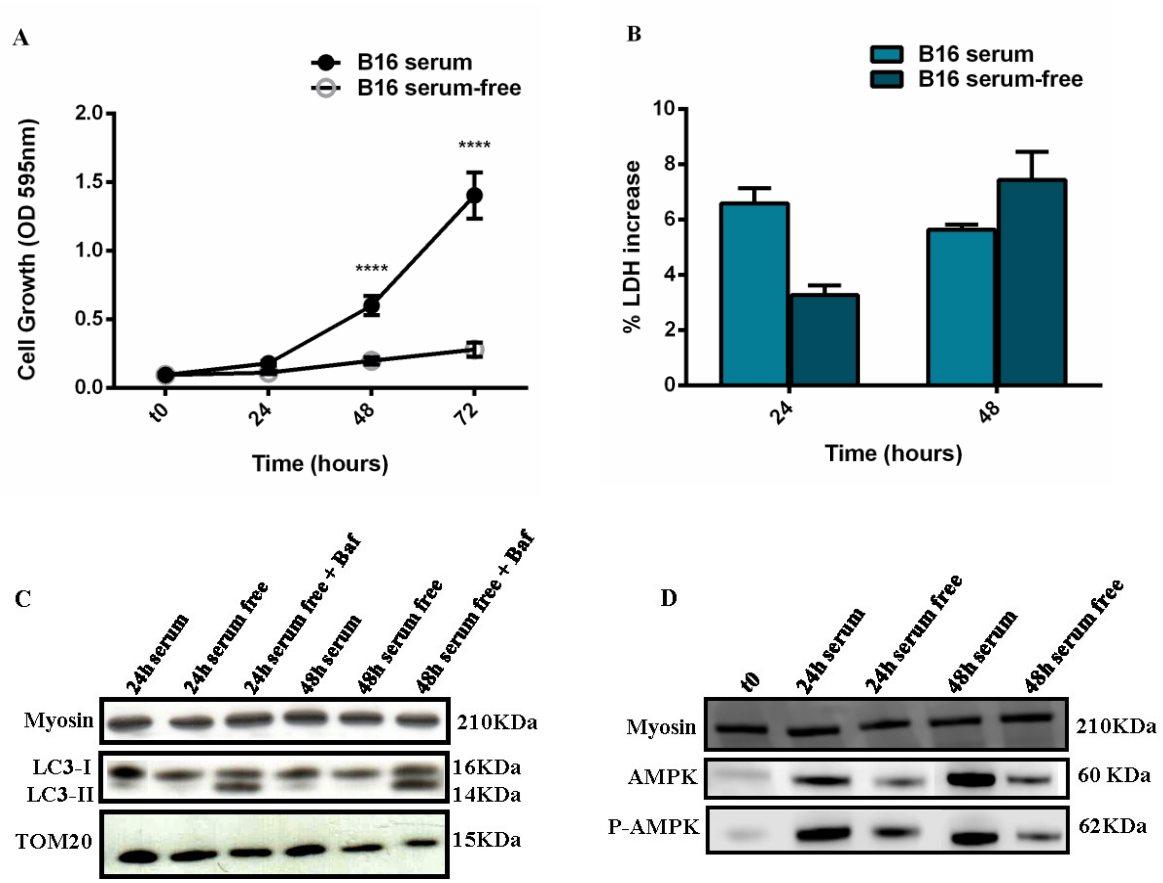


Fig.5: Serum starvation reduces cell growth without inducing cell death or autophagy. A Proliferation assay of B16 wt cells performed by crystal violet in cells incubated in the presence and absence of serum for 24, 48 and 72 hours. (n=17) Data are shown as mean ± SEM. ****P<0.0001 by ANOVA. **B** Lactate dehydrogenase (LDH) release. Graph shows LDH increase as percentage of total LDH cell content (n=3). **C** Representative blot of LC3 and TOM20 in B16 wt cells cultured in the presence and absence of serum, and after treatment with Bafilomycin A1. **D** Representative blot of AMPK and phosphorylated AMPK (P-AMPK) in serum-starved and serum-supplemented B16 wt cells.

3.3. Serum depletion impairs P2X7R functions

Since P2X7R is a major transducer of the signal conveyed by extracellular ATP as well as a bona fide growth-promoting receptor (Di Virgilio, Ferrari et al. 2009), we verified whether lower proliferation in serum-starved cells was associated with an impairment in P2X7R activity.

P2X7R is an ATP-gated cation channel which drives rapid inward cation currents. Thus, we measured intracellular Ca^{2+} levels in B16F10 cells cultured in the presence and absence of serum using fura-2/AM (acetoxymethyl ester) as a calcium indicator. Melanoma cells were stimulated with different concentration of the widely used P2X7 agonist BzATP (10-30-100-300-500 μM). The BzATP dose-dependency showed that P2X7R responses were substantially reduced in serum starved cells, especially at high BzATP concentrations (Fig. 6A). Moreover, Fig. 6B-C show that serum starvation significantly lowered also basal intracellular Ca^{2+} levels.

Nevertheless, immunoblot analysis showed that P2X7R expression was not decreased in serum starved cells (Fig. 6D).

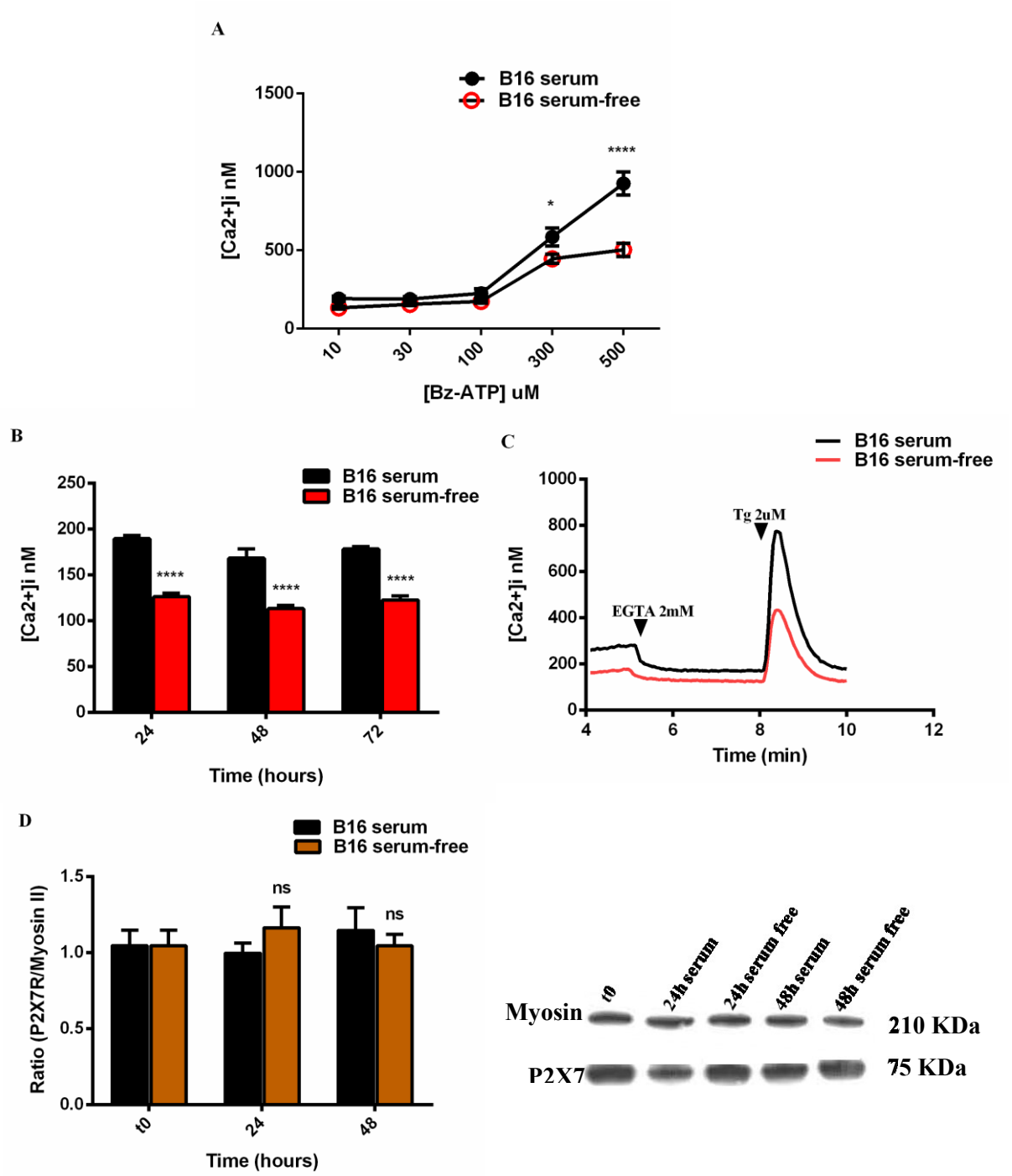


Fig.6: Serum deprivation impairs P2X7R-dependent responses. A BzATP dose-dependency. B16 cells cultured in the presence or absence of serum, were loaded with fura-2/AM and stimulated with increasing BzATP concentrations (10-30-100-300-500µM) (n=6), **B** Basal cytoplasmic Ca^{2+} level was measured in B16 serum-starved and B16 serum-supplemented cells at 24, 48 and 72 hours. Serum starvation significantly reduced intracellular calcium levels (n=10), **C** Representative trace showing basal intracellular Ca^{2+} levels after the addition of EGTA 2mM, **D** Western blot analysis of P2X7R protein in serum-starved and serum-supplemented B16 wt cells at 24 and 48 hours (n=6). The left graph shows no difference in the content of P2X7 protein in B16 starved-cells vs serum-repleted cells. Data are shown as mean ± SEM. ns= not significant *P<0.05; ****P<0.0001. Statistical analysis was determined by ANOVA.

3.4. Serum starvation affects mitochondrial metabolism

Serum deprivation inhibits intracellular energy synthesis

The dramatic effect of serum/nutrient deprivation on extracellular and intracellular ATP levels prompted us to investigate in more detail cellular energy synthesis. Mitochondrial metabolism was investigated by measuring oxygen consumption rate (OCR) in the presence and absence of serum using the Seahorse XFe96 Analyzer. Sequential addition of specific mitochondrial inhibitors allowed investigation of different sites of the respiratory chain. The addition of oligomycin, a natural antibiotic that inhibits F0/F1 ATPase (complex V), allowed measurement of ATP-coupled respiration, and discrimination from oxygen consumption due to the proton leak. Next FCCP, an uncoupler that maximally stimulates OCR, was added to collapse the inner membrane gradient and to determine the maximal respiration rate. Finally, antimycin A and rotenone addition inhibited the electron flux at complex III and I, allowing to measure residual OCR from non-mitochondrial sources. As shown in Fig. 7A all the mitochondrial respiratory parameters were severely inhibited in melanoma cells starved for 24 and 48 hours.

Impairment of oxidative phosphorylation (OXPHOS) was likely due to decreased expression of Complex I and II of the mitochondrial respiratory chain, as shown by immunoblot analysis (Fig. 7B), and it was paralleled by reduction of intracellular ATP levels (Fig. 7C).

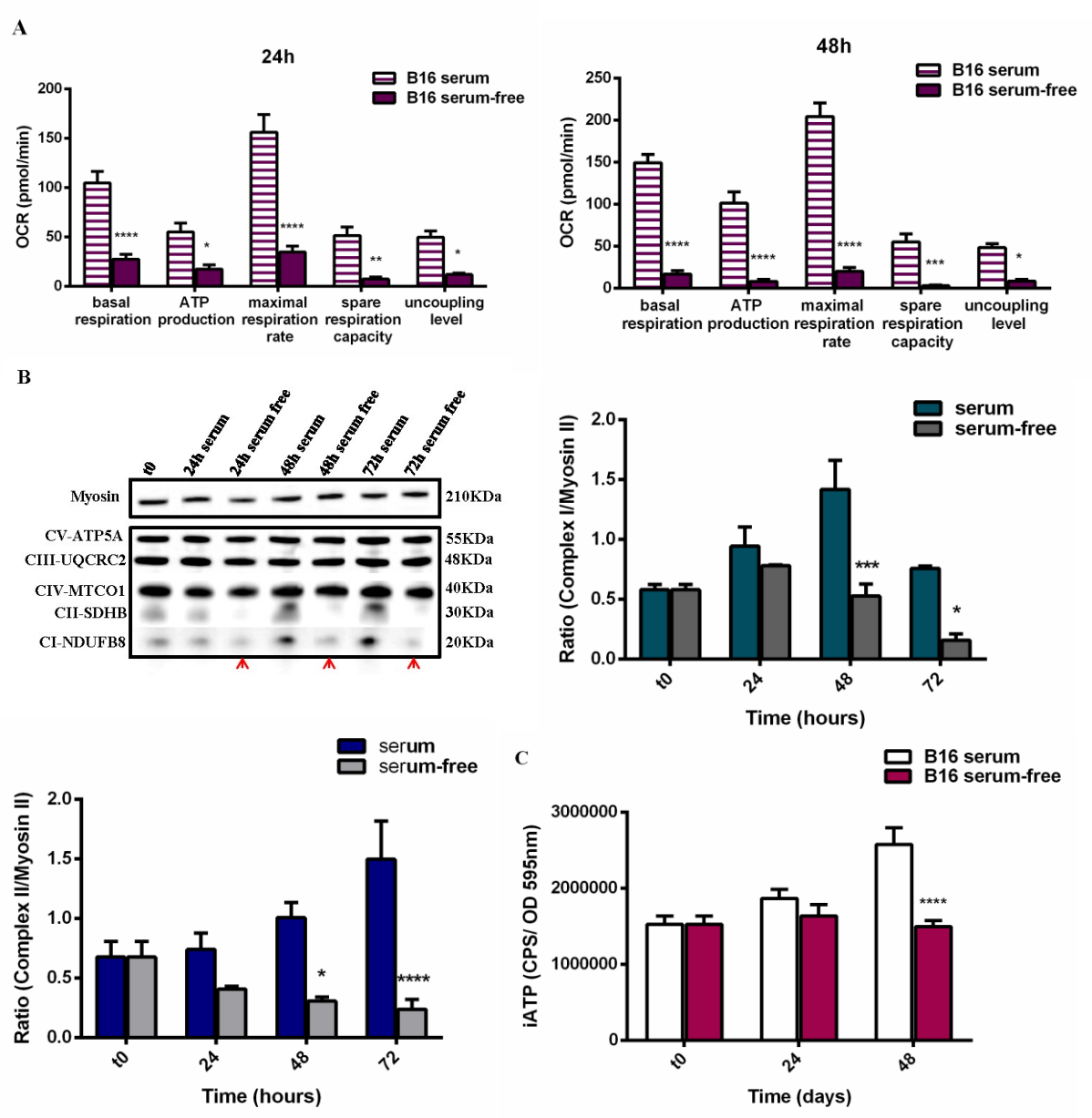


Fig.7: Serum deprivation reduces intracellular energy synthesis. A Oxygen Consumption Rates (OCR) and respiration parameters in B16 cells cultured in the presence and absence of serum. Oxygen consumption rate traces were determined at 24 and 48 hours using a Seahorse XF24 Analyzer. Respiration parameters were all decreased in serum starved cells (n=10). **B** Western blot analysis of mitochondrial respiratory chain complexes (OXPHOS) in serum-starved and serum-supplemented cells at 24, 48 and 72 hours (n=4). The graphs show that Complexes I and II were down-regulated in serum starved conditions (red arrows). **C** Intracellular ATP levels were measured in serum-depleted and serum-supplemented B16 cells. Intracellular ATP was measured in lysed cells using soluble luciferase. CPS (counts per second) were normalized to cells content determined via crystal violet (OD 595nm) (n=13). Data are shown as the mean ± SEM. *P<0.05; **P<0.01; ***p<0.001; ****P<0.0001 by ANOVA.

Serum depletion causes mitochondria depolarization and fragmentation

Mitochondrial membrane potential (Ψm) provides the proton motive force needed for ATP synthesis at mitochondrial complex V. Tetramethyrhodamine methyl ester (TMRM) is a fluorescent lipophilic dye currently used to monitor the membrane potential of mitochondria. We used TMRM to analyse the effects of serum starvation on mitochondrial membrane potential. As shown in Figure 8A, Ψm was to collapsed after 24 hours of serum starvation, and even more so after 48 hours. In parallel, staining with mitotracker green, a potential-insensitive, mitochondrial-selective fluorescent label, revealed that mitochondria were extensively fragmented under serum free conditions (Fig. 8B).

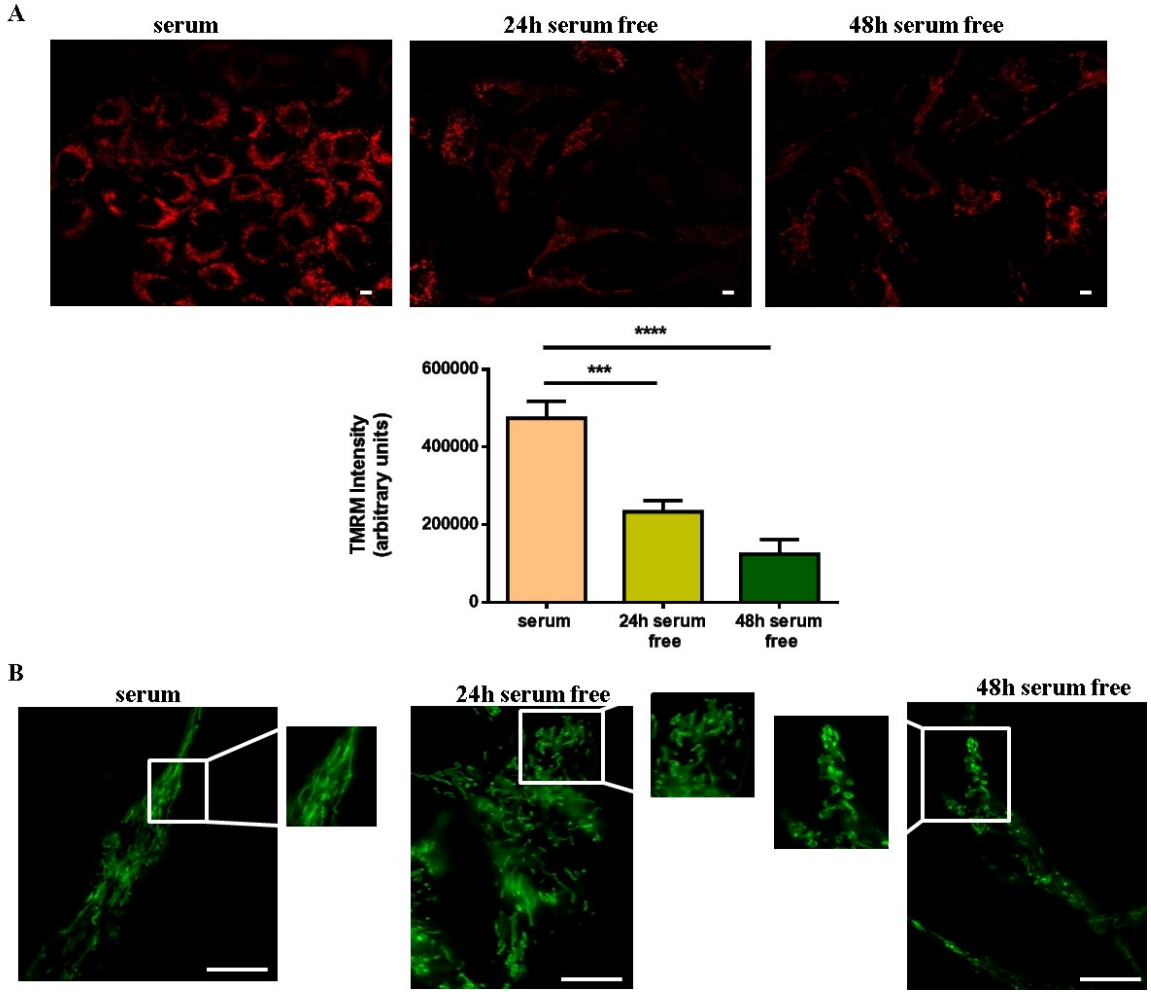


Fig.8: Nutrient deprivation causes mitochondria depolarization and fragmentation. A B16 cells were stained with TMRM (50nM) after incubation in the presence and absence of serum for 24 and 48 hours. The intensity of TMRM reflects the level of $\Delta\Psi_{m.}$. Bars 10µm. The graph below shows the TMRM intensity. The signal was significantly reduced in serum-starved cells relative to serum-supplemented cells (n=10). Data are shown as mean ± SEM. ***P<0.001; ****P<0.0001by ANOVA. **B** B16 cells were loaded with Mitotracker green (200nM) after incubation in the presence and absence of serum for 24 or 48h. Bars 10µm.

Glycolysis is increased in serum starved cells

Mitochondrial OCR and ECAR allow measurement of the effect of nutrient deprivation on oxidative phosphorylation and glycolysis, respectively.

To measure the acidification rate (ECAR) cells were incubated in the glycolysis stress medium without glucose and pyruvate. First, the addition of a saturating concentration of glucose caused a rapid increase in ECAR, indicated as the rate of glycolysis under basal conditions. Next, addition of oligomycin shifted energy production to glycolysis inhibiting mitochondrial ATP production. Finally, 2-deoxy-glucose (2-DG), a non-hydrolysable glucose analogue, inhibited glycolysis through competitive binding to glucose hexokinase, confirming that ECAR was due to glycolysis. In serum free conditions there was a significant increase in glycolytic activity after 24 hours without serum (Fig. 9A), as well as of lactate release, which was significantly increased after 48 hours without serum (Fig. 9B).

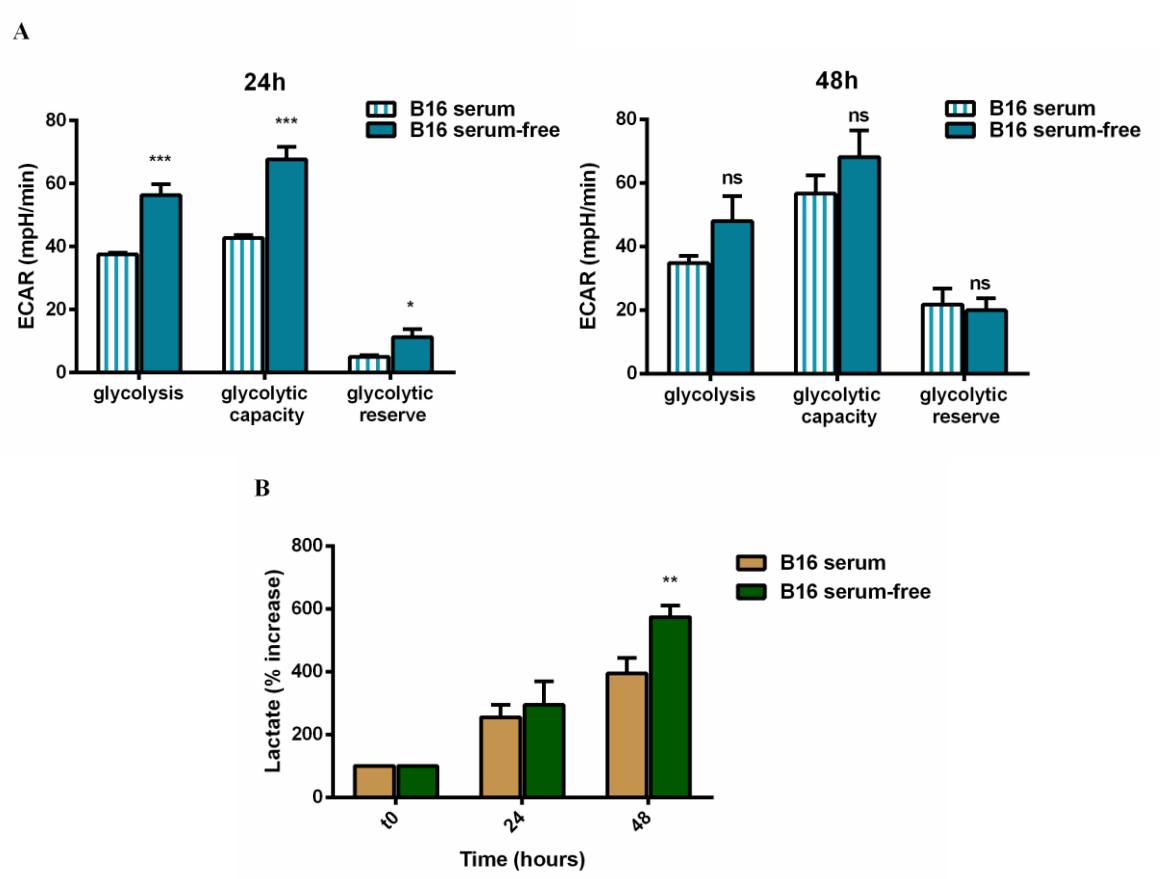


Fig.9: In serum starved cells glycolysis is slightly increased. A Acidification rates (ECAR) in B16 cells cultured in the presence and absence of serum. Acidification rates were measured at 24 and 48 hours with the Seahorse XF24 Analyzer. (n=7) mpH=milli pH units. **B** Lactate release was measured in the cell supernatant of B16 serum-starved and serum-supplemented cells at 24 and 48 hours. The graph shows the percentage increase in relation to the control (t0) (n=10). Data are shown as mean ± SEM. *P<0.05; **P<0.01; ***P<0.001 by ANOVA.

3.5. P2X7R silencing decreases intracellular and extracellular ATP levels

Since P2X7R is a conduit for ATP release (Pellegatti, Falzoni et al. 2005) and its activity modulates mitochondrial metabolism and aerobic glycolysis (Adinolfi, Callegari et al. 2005, Amoroso, Falzoni et al. 2012), we investigated the effects of P2X7 inhibition on intracellular and extracellular ATP levels.

We generated stable P2X7R-silenced B16F10 cells clones by shRNA transfection (B16-shRNA) and measured extracellular and intracellular ATP levels using soluble luciferase. As shown in Fig. 10 A-B, P2X7R silencing reduced intracellular ATP levels and all respiratory parameters, in particular after 48h as analysed by Seahorse. In parallel, extracellular ATP release was also decreased compared to wild type cells (Fig. 10 C).

Moreover, we measured intracellular and extracellular ATP levels after incubation in the absence of serum. Although serum starvation promoted a significant increase in ATP release in P2X7R-silenced cells (Fig. 10D), intracellular and extracellular ATP levels were lower in B16-shRNA cells than in wild type in serum starved conditions (Fig. 10E).

Fractional ATP release was much larger in wild type cells than in P2X7R-silenced cells, both in the presence and absence of serum, confirming the role of the P2X7 receptor to support full ATP release.

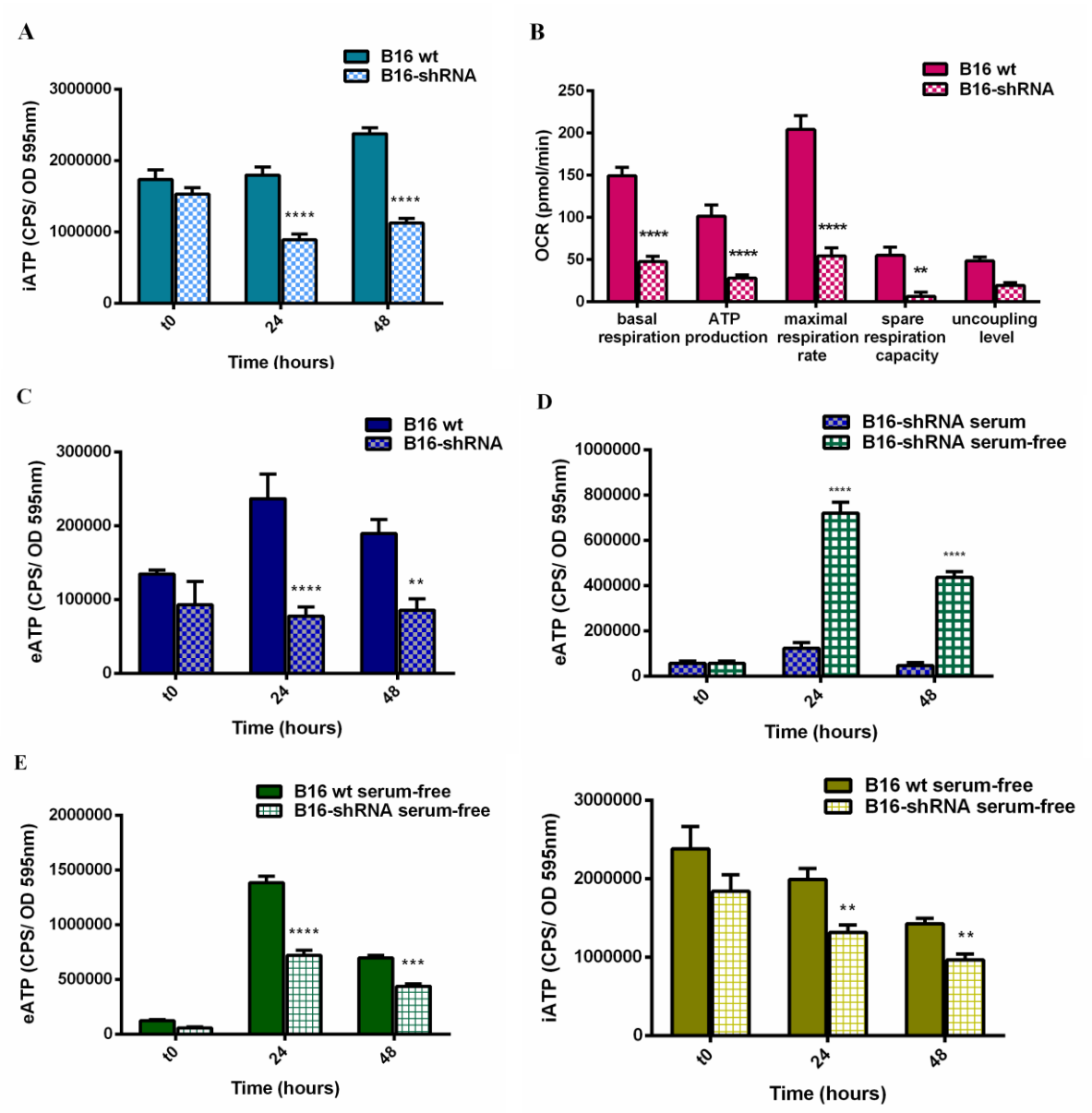


Fig.10: P2X7R silencing decreases intracellular and extracellular ATP levels. A Intracellular ATP levels measured in B16 wild type and B16 P2X7R-silenced cells. Intracellular ATP was measured in lysed cells using soluble luciferase. P2X7R silencing lowers cytoplasmic ATP levels. (n=5) **B** Oxygen Consumption Rates (OCR) and respiration parameters in B16 wild type cells and B16 silenced cells. Oxygen consumption rates were determined at 48 hours using a Seahorse XF24 Analyzer. P2X7R silencing strongly reduces all respiratory parameters (n=9). **C-D** Extracellular ATP levels measured using soluble luciferase. **C** extracellular ATP levels are lower in P2X7R-silenced cells than wild type cells. (n=10) **D** serum starvation increases extracellular ATP levels in B16 P2X7R-silenced cells. (n=6) **E** Extracellular ATP levels (left graph) and intracellular ATP content (right graph) measured in B16 wild type and P2X7R-silenced cells cultured in the absence of serum. P2X7R silencing reduces eATP and iATP levels. (n=10) CPS (counts per second) were normalized to cells content determined via crystal violet (OD 595nm). Data are shown as mean ± SEM. **P<0.01; ***P<0.001; ****P<0.0001 by ANOVA.

3.6. B16F10 cells release ATP- and mitochondria-containing microvesicles

B16F10-starved cells release a large amount of ATP-loaded microvesicles

Since in serum starved cells the extracellular ATP increase was not paralleled by an increase in cell death, we hypothesized a release mechanism involving a non-lytic pathway.

B16F10 tumor cells are known to release exosome/microvesicles (extracellular vesicles, EVs) (Hood, San et al. 2011, Muhsin-Sharafaldine, Saunderson et al. 2016). EVs are nano-sized vesicles generally comprising microvesicles or microparticles (100-1000 nm in diameter) and exosomes (50-150 nm in diameter). Several studies have shown that extracellular vesicles contain proteins, lipids, mRNAs, microRNAs, and they promote tumor growth and metastasis mediating the intercellular communication and the interaction between cancer cells and the TME (Soung, Nguyen et al. 2016, Whiteside 2016, Shu, Yang et al. 2018). Recently, it has also been shown that exosomes convey the stress-induced responses of melanoma cells (Harmati, Gyukity-Sebestyen et al. 2019).

Thus we set to investigate whether microparticles released from B16F10 cells contained ATP.

For EVs production we cultured B16F10 cells in the absence of serum and in EV-depleted serum-containing medium. B16F10 cell-derived extracellular vesicles were isolated by ultracentrifugation as previously described (Pizzirani, Ferrari et al. 2007) and the particle size distributions (PSDs) was determined by the NTA devices NanoSight LM10. Nanoparticle Tracking Analysis (NTA) is commonly used to determine EV concentration and diameter.

As shown in Fig. 11A, B16F10 melanoma cells, whether cultures in the presence or absence of serum, spontaneously release a large amount of EVs of size in the range of 100-200 nanometres (nm). However, amount of EVs release per cell was much larger in the absence of serum (Table I). Interestingly, P2X7R silencing strongly reduced EVs release (Fig. 11B). Amount of EVs released from B16F10-silenced cells was one order of magnitude lower than that released from B16F10 wild type cells (Table I). Moreover, we found a striking difference in the amount of ATP content as EVs from B16F10 cells incubated in the absence of serum contained about two/three times as much ATP as those incubated in its presence (Fig. 11C).

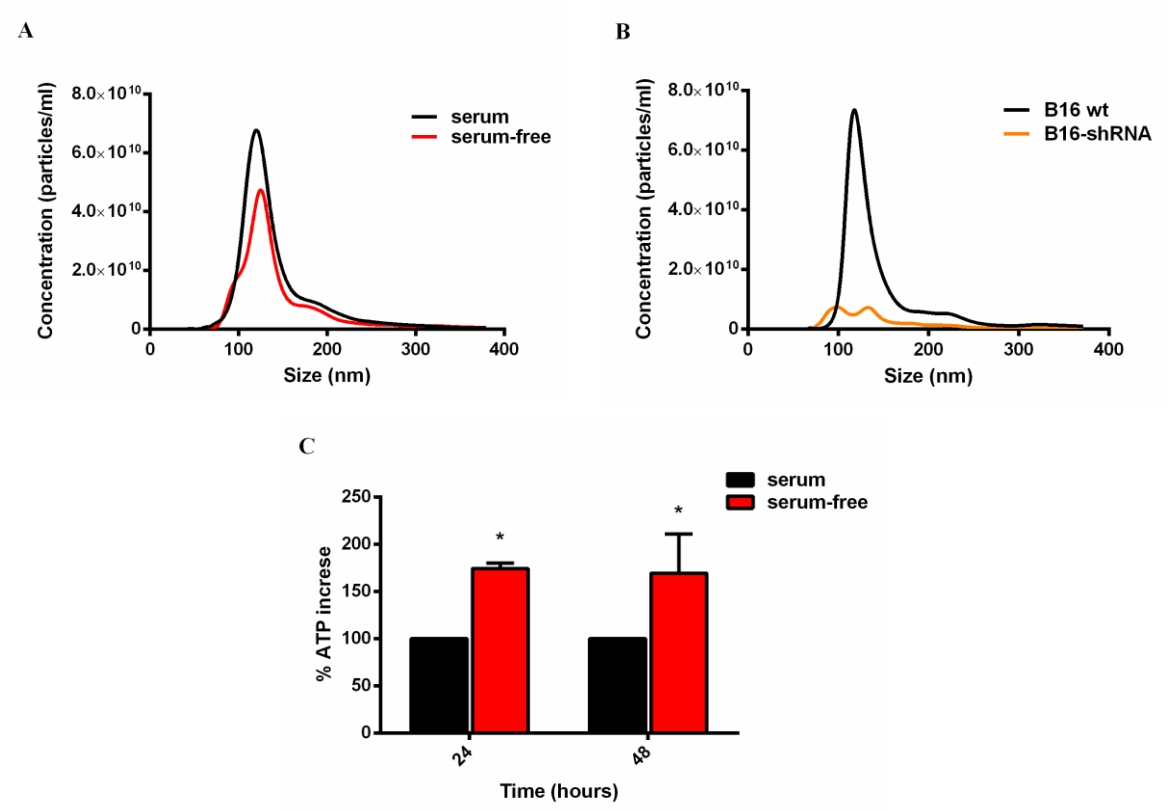


Fig.11: B16-starved cells release large amount of microvesicles rich in ATP. A-B Representative trace of microparticles size distribution obtained by NanoSight LM10 instrument. This technology utilizes the properties of both light scattering and Brownian motion in order to obtain the size distribution and concentration measurement of particles in PBS suspension (1ml). **A** Serum-starved and serum-supplemented cells released a large amount of microparticles between 100 and 200 nm in size. **B** Microparticles concentration was one order of magnitude less in B16 P2X7R-silenced cells than B16 wild type cells. **C** ATP content within the microvesicles obtained from B16 cells cultured in the presence and absence of serum. Microvesicles were isolated by ultracentrifugation and ATP was measured using soluble luciferase. Data are reported as ATP percentage increase compared to ATP concentration within microvesicles obtained in serum conditions. (n=3) Data are shown as mean ± SEM. *P<0.05 by ANOVA.

Table I

vesicles concentration: (vesicles/cell) ± SD			
B16 wt		**Serum**	**Serum free**
	24h	$5x10^3$ ± 110	$6,4x10^3$ ± 150
	48h	$3,4x10^3$ ± 110	$6x10^3$ ± 140
B16-shRNA	**48h**	$17x10^2$ ± 50	$22x10^2$ ± 16

Table I: number of microvesicles released from B16 wt and B16 P2X7R-silenced cells cultured in the presence and absence of serum. Number of microvesicles was obtained after normalization of the microparticles concentration (particles/ml) on the cell number.

Extracellular vesicles contain mitochondria

We were intrigued by the observation that EVs from serum-starved cells contained a higher ATP content despite intracellular ATP was lower than serum-supplemented cells. We hypothesized that cytosolic ATP or the ATP-generating machine was trapped within budding EVs. Thus we checked whether mitochondria were contained within the EVs.
B16F10 cells were labeled with Mitotracker green, a fluorescent mitochondrial stain, and cultured in the absence and presence of serum for 24 and 48 hours. Extracellular vesicles were isolated from cell culture supernatant by ultracentrifugation and mitochondrial fluorescence was measured using fluorescence spectrophotometer. As shown in Fig. 12A, fluorescent mitochondria were identified within the microparticle population released from B16F10 cells, and fluorescence intensity was much higher in EVs released from 24 hours serum-starved cells than serum-repleted cells. Moreover, immunoblot analysis showed that EVs contained complexes of the mitochondrial respiratory chain (OXPHOS) (Fig. 12B), suggesting that extracellular vesicles may contain active mitochondria.

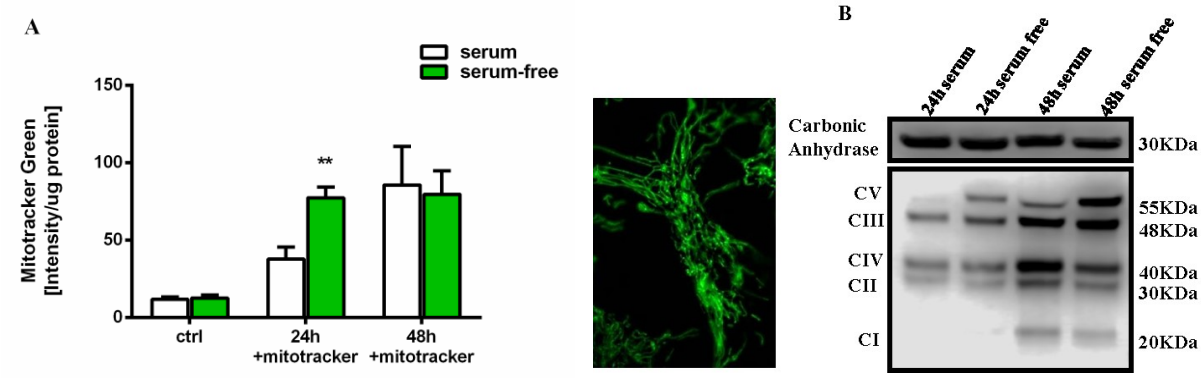


Fig.12: Extracellular vesicles contain mitochondria. A Extracellular vesicles were isolated from cell culture supernatants and fluorescence intensity of mitochondria contained within microvesicles was measured using fluorescence spectrophotometer. The graph shows the intensity normalized to microvesicles protein (μg). Microvesicles released from not labeled cells were used as control. (n=3) Data are shown as mean ± SEM **P<0.01 by ANOVA. The picture on the right shows microscopy image of stained mitochondria in B16 cell. **B** Representative blot of mitochondrial respiratory chain complexes (OXPHOS) in microvesicles isolated from serum-starved and serum-supplemented cells at 24 and 48 hours. Carbonic anhydrase was added in all the microvesicles suspension. The same volume of microvesicles was loaded and carbonic anhydrase was used as loading control.

4. DISCUSSION

ATP is one of the major biochemical constituent of the tumor microenvironment (TME). While in the interstitium of healthy tissues the concentration of this nucleotide is in the low nanomolar range, at sites of tissue damage, inflammation and tumours ATP can reach the high micromolar level (Pellegatti, Raffaghello et al. 2008). In the TME, ATP can promote immunosuppression or anti-tumour immunity (Di Virgilio and Adinolfi 2017), whether directly or as a precursor of adenosine, depending on its concentration and on the level of degradative enzymes and specific receptors expressed by immune and tumour cells (Di Virgilio, Ferrari et al. 2009, Vijayan, Young et al. 2017). Extracellular ATP acts at plasma membrane P2 receptors, among which P2X7R is attracting increasing interest in chronic inflammatory disorders and cancers due to its dual role in cell death and cell proliferation. In the TME, P2X7R is expressed on both infiltrating immune cells and on tumor cells. On the immune cells, the P2X7R is coupled to generation of pro-inflammatory factors (i.e. IL-1β, IL-8, TNFα, ROS) or immunosuppressive agents (i.e. TGFβ, arginase), to activation of dendritic cells (DCs) and differentiation of cytotoxic $CD8^+$ T lymphocytes (Di Virgilio 2013, Bianchi, Vuerich et al. 2014). In cancer, the P2X7R stimulates proliferation and promotes tumor invasion and metastasis (Qiu, Li et al. 2014, Amoroso, Capece et al. 2015).

It has been proposed that ATP accumulation in the TME might be beneficial for tumors not only by stimulating cancer cell P2X7R, but also because it might be converted to high energy compunds (phosphocreatine), taken-up by the tumor cells themselves and used as energy source (Loo, Scherl et al. 2015). It has also been suggested that TME ATP might contribute to immune cell recruitment and that some cancer cell lines may internalize extracellular ATP by different mechanisms, such as micropinocytosis, increasing intracellular ATP level and improving cancer cells drug resistance or promoting tumour progression (Pietrocola, Pol et al. 2016, Qian, Wang et al. 2016, Wang, Li et al. 2017). These observations make it difficult to understand the pathophysiological significance of ATP accumulation into the tumor microenvironment and the possible effects of its manipulations.

It is understood that both tumor cells and tumor-infiltrating inflammatory cells release ATP in response to locally acting stimulatory factors (hypoxia, nutrient deprivation) or to the activation of specific intracellular pathways (autophagy). Recent studies have shown that caloric restriction and caloric restriction mimetics (CRMs) enhance the anticancer activity

of known chemiotherapics and reduce tumor incidence by increasing the ATP concentration in the TME and modifying TME composition (Lee, Raffaghello et al. 2012, Pietrocola, Pol et al. 2016).

To clarify the relationship between extracellular ATP, P2X7R and tumor cell responses under conditions where energy metabolism may be drastically modified, we induced nutrient deprivation *in vivo* by i.p. administrating the CRM hydroxycitrate and by *in vitro* exposing melanoma cells to serum starvation.

Our results show that *in vivo* CRM administration and *in vitro* serum starvation strongly inhibit tumor proliferation and largely increase extracellular ATP levels, which seems paradoxical because it would be anticipated that nutrient deprivation, that impairs ATP generation by reducing substrate availability, should also decrease the extracellular ATP content.

It has been suggested that the increase in the TME ATP supports anticancer immune response via the activation of autophagy and the potentiation of tumor antigen presentation by tumor-associated dendritic cells (DCs) (Pietrocola, Pol et al. 2016). However, we were unable to detect any increase in autophagy or cell death in serum-starved cells. Nevertheless, we measured tumor-associated luminescence into transgenic mice ubiquitously expressing the pmeLUC probe showing that extracellular ATP in the TME can be also detected by host infiltrating cells, which are therefore likely affected.

Since extracellular ATP might promote cell growth by stimulating P2X7R, we verified whether lower proliferation in serum-starved melanoma cells was associated with reduction in P2X7R-dependent responses. Our findings show that chronic incubation in the absence of serum severely impairs P2X7R functions and decreases cytoplasmic Ca^{2+}, although P2X7 receptor expression is not reduced. It has been demonstrated that transfection of P2X7R increases resting mitochondrial potential, basal mitochondrial Ca^{2+} and intracellular ATP content (Adinolfi, Callegari et al. 2005), and that P2X7R modulates aerobic glycolysis (Amoroso, Falzoni et al. 2012). Our results confirm the correlation between P2X7R functions and energy metabolism. B16F10 starved cells show a decrease of all the mitochondrial key parameters, e.g. basal respiration, ATP production and maximal respiratory capacity, compared to serum-repleted cells. In serum starved cells mitochondria are fragmented and depolarized, complex I and complex II of the respiratory chain are down-modulated, intracellular ATP levels are decreased and glycolytic activity is increased. These data fit with the impaired mitochondria metabolism. Moreover, cells silenced for P2X7 receptor show lower levels of respiration compared to wild type cells. P2X7R silencing affect not only intracellular ATP, but also extracellular ATP levels, both

in the presence and absence of serum, suggesting that lack of P2X7R hinders the activation of an efficient ATP release mechanism. In any case, serum deprivation promote a significant increase in ATP release both in B16F10 wild type and B16F10-silenced cells.

It is peculiar that tumor cells are able to increase the extracellular ATP concentration despite a striking reduction in the intracellular levels. There is a large body of literature showing that tumors release large amount of ATP into the TME (Michaud, Martins et al. 2011, Di Virgilio 2012, Loo, Scherl et al. 2015, Lecciso, Ocadlikova et al. 2017), but the overall pathophysiological meaning is still rather obscure. Several mechanisms have been suggested over the years for non-lytic ATP release, pannexin-1 (Penuela, Gyenis et al. 2012), connexion-43 (Ableser, Penuela et al. 2014) and P2X7R itself (Pellegatti, Falzoni et al. 2005). We show here that ATP can also be released by an extracellular vesicle (EVs) mediated mechanism. EVs have recently emerged as a mechanism to mediate intercellular communication in tumor microenvironment. Exosomes carry and transfer biomolecules such as proteins, lipids and nucleic acids and they are important players in the regulation of immune response and in the formation of metastatic niche for cancer progression (Barcellos-Hoff, Lyden et al. 2013), therefore contributing also to cancer cell migration and invasion (Peinado, Aleckovic et al. 2012, Mu, Rana et al. 2013). Secretion of exosomes in cancer cells is increased under conditions of cellular stress such as oxidative stress (Atienzar-Aroca, Flores-Bellver et al. 2016), hypoxia (Wozniak, Peczek et al. 2017), and senescence (Kadota, Fujita et al. 2018, Jakhar and Crasta 2019). Our data show that a larger amount of EVs is released under nutrient deprivation conditions and that such EVs contain a higher ATP content compared to those released in the presence of serum. Interestingly, the amount of EVs released from B16F10-silenced cells is one order of magnitude less than that released from B16F10 wild type cells, confirming the need for P2X7R expression to support EV-mediated ATP release. In agreement with a recent study showing that melanoma tissue-derived microvesicles contain mitochondrial membrane proteins (Jang, Crescitelli et al. 2019), we provide evidence that B16F10-derived EVs contain complexes of the mitochondrial respiratory chain and mitochondria-derived vesicles, suggesting that EVs might contain functioning mitochondria. Mitochondrial content is also increased in serum-starved B16F10. These findings argue that the high ATP EV content might be due partly to trapping of cytosolic ATP and partly to trapping of mitochondria fragments generated in the absence of nutrients.

In conclusion our findings suggest that nutrient deprivation causes at the same time impairment of cancer cell metabolism and EV-mediated ATP release. The increased concentration of ATP in the TME is sensed by host inflammatory cells thus promoting a

more efficient anti-tumor response. These observations provide further rationale to anticancer therapies based on caloric restriction or on the administration of caloric restriction mimetics. The P2X7 receptor is a key player in these processes.

5. MATERIALS AND METHODS

Reagents

Potassium hydroxycitrate (HC), ATP, Benzoyl ATP (BzATP), Bafilomycin A1 and Ethidium bromide were purchased from Sigma-Aldrich (St. Louis, MO). TMRM and MitoTracker Green (Molecular Probes, Life Technologies) were dissolved in DMSO to obtain a 10mM and 1mM stock solution respectively.

Cells Culture and transfections

B16 melanoma cells were grown in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/mL streptomycin (Euroclone, Milan, Italy) (complete medium). For EVs production cells were cultured in RPMI medium containing 10% EV-depleted FBS (Gibco, Thermo Fisher Scientific, Waltham, Massachusetts, USA) In serum starved conditions B16 cells were grown in RPMI medium supplemented only with penicillin and streptomycin. To obtain B16 cell clones stably expressing plasma membrane luciferase, cells were transfected with the pmeLUC probe using Lipofectamine LTX (Thermo Fisher Scientific) as per the manufacturer's instructions. Stably transfected cell clones were obtained by selection with neomycin/G418 disulfate (0.2-0.8 mg/mL) (Sigma-Aldrich) followed by limiting dilution.
B16 P2X7R-silenced cells were obtained by transfection with the P2X7 short hairpin RNA (sh-RNA) in pSuper.neo.green fluorescent protein (GFP) vector, that was a kind gift of Dr Diaz-Hernandez (Universidad Complutense, Madrid, Spain). B16 transfection was performed with TransIT-2020 transfection reagent (Tema Ricerca, Bologna, Italy) following the manifacturer's instructions. Stably transfected cell lines were obtained by selection with G418 disulfate (0.2-0.8 mg/mL). Single cell-derived clones (B16 shRNA) were obtained by limiting dilution (Di Virgilio, Jiang et al. 2019).

Mice strains, tumor generation, *in vivo* imaging and drug administration

In vivo experiments were performed with C57bl/6 WT mice, a gift from GlaxoSmithKline to F. Di Virgilio, and pmeLUC transgenic mice.
A total of $2.5x10^5$ B16-pmeLUC cells were subcutaneously injected into C57bl/6 4–6 weeks old male mice (n=12). Tumor size was measured with a calliper, and volume calculated according to the following equation: volume = $\pi/6$ [w1 × $(w2)^2$], were w1 is the major diameter and w2 is the minor diameter. Luminescence emission was measured day 5, 7 and 9 from tumor cell inoculum with a total body luminometer for small animals (IVIS

Lumina, Caliper; Perkin Elmer, Hopkinton, Massachusetts, USA). Mice anesthetized with 2.5% isofluorane were intra-peritoneum injected with 150 mg/kg D-luciferin (Promega, Madison, Wisconsin, USA) and, after a 15-min interval allowing for biodistribution, luminescence was captured from dorsal view. Photon emission was quantified using the Living Image® software (Perkin Elmer) and averaged as photons/ seconds/cm2/steradian (abbreviated as p/s/cm2/s). Potassium hydroxycitrate (300mg/kg) or vehicle (sterile phosphate-buffered saline (PBS)) were intra-peritoneum injected (100µl) every 2 days after day 5, corresponding, when applicable, to first tumor mass detection.

pmeLUC transgenic mice (pmeLUC-TG-mice) were generated in collaboration with The Danish Center for Genetically Modified Mice (Aarhus University). A linearized pmeLUC DNA construct [Pellegatti 2005] was injected into the pronuclei of B6D2F1 zygotes. Transduced zygotes were then transferred to pseudopregnant foster mothers.
A total of $2.5x10^5$ B16WT cells were subcutaneously injected into pmeLUC transgenic 4–6 weeks old male and female mice (n=12). Mice anesthetized with 2.5% isofluorane were intra-peritoneum injected with D-luciferin (150 mg/kg) and luminescence was daily captured from dorsal view, day 1 to 11 from tumor cell inoculum.

In vitro measure of ATP levels

Extracellular and intracellular ATP levels were measured with ENLITEN rLuciferase/Luciferin reagent (Promega). ATP-driven chemoluminescence was recorded on a Perkin Elmer Wallac Victor3 1420 luminometer (Perkin Elmer, Wellesley, Massachusetts, USA).
Briefly, $2x10^3$ B16 cells per well were plated in ninety-six-well black microplates (Greiner Bio-One). Following adhesion cells were incubated for 48h in the presence and absence of serum, or treated with 1mM hydroxycitrate, corresponding to in vivo administered concentration. Luminescence was measured after removal of supernatant and following addition of 50µl of diluent buffer (FireZyme, San Diego, CA), to stabilize pericellular ATP, and 50µl of ENLITEN reagent per well. To measure total cellular ATP content, cells were lysed with 5µl of milli-Q water and supplemented with 45µl of diluent buffer (FireZyme) and 50µl of ENLITEN reagent.
Pericellular ATP levels were also measured, thanks to the pmeLUC probe and the use of an IVIS Lumina luminometer (Caliper). $50x10^3$ B16-pmeLUC cells per well were plated in twenty-four-well plate and cultured in the presence and absence of serum or treated with hydroxycitrate (1mM). D-Luciferin (Promega) was added to all wells at a concentration of

300 μg/ml. Luminescence data were expressed as total photons measured in a 5 min acquisition.

Cell proliferation assay

Cell proliferation assays were performed by crystal violet technique. Briefly, $3x10^3$ B16 cells per well were plated in forty-eight-well plate and cultured in the presence and absence of serum. At the 0h, 24h, 48h and 72h, cells were fixed with 4% paraformaldehyde and stained with 0,1% crystal violet. Cell proliferation was analysed by optical density (OD 595nm) in a microplate reader after dye extraction with acetic acid 10%. In parallel, to check the effect of CRM on cell growth, B16 cells were treated with 1mM hydroxycitrate.

Lactate dehydrogenase (LDH) measurement

Lactate dehydrogenase activity was determined by measuring the conversion of pyruvate to lactate in the presence of the NADH cofactor. This reaction can be quantified by measuring the oxidation of NADH to NAD^+, which causes an optical density reduction to 340nm. Briefly, $5x10^3$ B16 cells per well were plated in twenty-four-well plate and cultured in the presence and absence of serum in RPMI-1640 media without red phenol. At the 24h and 48h, 500µl of cell supernatants were put in a 2ml cuvettes in the presence of a pyruvate solution (14.4 mM K_2HPO_4, 16.8mM KH_2PO_4, 0.63 mM pyruvate, pH 7.5) (1450µl) and a NADH solution (11.3mM NADH) (50µl). Experiments were performed at 340nm wavelength using an Eppendorf BioSpectrometer kinetic. The data were normalized to the total LDH content within cells lysed by TRITON X-100.

Cytosolic calcium concentration measurement

The cytosolic Ca^{2+} concentration was measured using the fluorescent Ca^{2+} indicator Fura-2-acetoxymethyl ester (Fura-2/AM) (Thermo Fisher Scientific) (Falzoni, Munerati et al. 1995). Cells were loaded with 4µM Fura-2/AM at 37° C for 20 minutes in the presence of 250µM sulfinpyrazone (Sigma-Aldrich), rinsed, and resuspended in sodium solution (125 mM NaCl, 5 mM KCl, 1 mM MgSO4 , 1 mM NaH2 PO4 , 20 mM HEPES, 5.5 mM glucose, 1 mM CaCl2 , pH 7.4) at final concentration of $2x10^6$/ml. Cells were kept at 37° C in a thermostat-controlled and magnetically-stirred cuvette of a Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies, Milan, Italy) and $[Ca^{2+}]i$ was determined at the 340/380nm excitation ratio and at 505 nm emission wavelengths. Cells were stimulated with different BzATP concentrations (10-30-100-300-500 µM). In experiments performed in the absence of external Ca^{2+}, CaCl2 was omitted and 2mM EGTA was added when

indicated and Thapsigargin (Tg) (Sigma-Aldrich) 2μM was used as a control of intracellular calcium concentration.

Seahorse mitochondrial flux and glycolysis analysis

Oxygen consumption (OCR) and extracellular acidification rate (ECAR) in B16wt and B16 P2X7R-silenced cells were measured using the Seahorse XF96 Extracellular Flux Analyzer instrument. Cells were seeded at different time points in 100μl RPMI-1640 complete medium at adequate density in XF96 96-well cell culture plate (Seahorse Bioscience). On the first day we have seeded cells for the 48h conditions (serum and serum-free); the next day we changed these cells' media (RPMI-1640 with and without serum) and seeded the 24h conditions. The third day the media of 24h conditions were replaced and the XF96 sensor cartridge was hydrated with 200μl/well of XF Calibrant buffer and placed in incubator without CO_2 for all the night. The last day, before running the assay, we replaced cells media with Seahorse assay medium supplemented with Glucose 10mM (not for ECAR assay), Sodium Piruvate 2mM (not for ECAR assay) and L-glutamine 1mM, pH to 7.4 and equilibrated for 1h at 37°C without CO_2. Through different port in the cartridge we injected Oligomycin, FCCP, Rotenone/Antimycin A (final concentration 1μM), Glucose and 2-D-Glucose (final concentrations 10mM and 100mM respectively).
After the calibration the cell plate was placed in analyser and OCR and ECAR were measured before and after the injection of the different compounds. ECAR and OCR measurements were normalized to cells content in each well determined via crystal violet.

Lactate measurement

Lactate release was measured with a Lactate Colorimetric Assay Kit (Abcam, Cambridge, UK), according to the manufacturer' s indication. Briefly, a total of $10x10^3$ cells per well were plated in twenty-four-well plate in the presence and absence of serum. Supernatants were withdrawn at the various time points and centrifuged 10 minutes at 1100rpm to eliminate detached cells and debris.
Lactate was measured on a microplate reader at OD 450nm. The data were normalized to μg of protein.

Mitochondrial membrane potential measurement

Mitochondrial membrane potential ($\Delta\Psi_m$) was measured using 50nM tetramethyl rhodamine methyl ester (TMRM) on a confocal microscope (model LSM 510; Carl Zeiss

MicroImaging, Inc.) equipped with a plan-apochromat x63 oil immersion objective (Carl Zeiss, Oberkochen, Germany). Cells were seeded onto 24-mm glass coverslips at adequate density and maintained in serum and serum-starved conditions for 24h and 48h. Experiments were performed in a saline solution, referred to as standard saline solution, containing 125 mM NaCl, 5 mM KCl, 1 mM MgSO4, 1 mM NaH2PO4, 20 mM Hepes, 5.5 mM glucose, 5 mM NaHCO3, and 1 mM CaCl2 (pH 7.4). Cells were loaded with TMRM for 20 minutes at 37°C and mitochondrial potential was analysed by mounting coverslips in a thermostated chamber (set at 37°C; Medical Systems, Greenvale, NY) placed on the stage of the microscope. At the concentration used, TMRM is mainly localized in the mitochondria, emitting a level of fluorescence proportional to the absolute value of mitochondrial potential. FCCP (carbonyl cyanide p-trifluoromethoxyphenylhydrazone) (Sigma-Aldrich) was added to collapse mitochondrial $\Delta\Psi$. The signal was collected as total emission >570nm and analysed with ImageJ software.

Mitochondrial staining

Cells were plated onto 40-mm glass coverslips at adequate density, and culture in the presence and absence of serum for 24h and 48h. Mitochondrial localisation within cells was visualised with Mitotracker Green FM using an inverted Nikon Eclipse TE300 microscope equipped with a 100x/05.-1.3 NA oil Iris objective (Nikon, Tokyo, Japan)
Before images acquisition, cells were incubated with Mitotracker Green (200nM) in RPMI-1640 medium without red phenol and FBS for 15 minutes at 37°C. Coverslips have been mounted in a thermostated Leyden Chamber (set at 37°C; Medical Systems, Greenvale, NY) placed on the stage of the microscope. The images were captured by a back-illuminated chargecoupled device (CCD) camera (Princeton Instruments, Trenton, NJ) using the Metamorph software (Universal Imaging, West Chester, PA).

Extracellular vesicle (EVs) Purification

For characterization of cell-derived microvesicles, B16 cells were seeded in T175 flasks at a concentration of $2x10^6$/flask for the 24h conditions and 10^6/flask for the 48h conditions. Since fetal bovine serum was described to contain extracellular vesicles which might alter the results of the analysis (Eitan, Zhang et al. 2015), cells were cultured in the absence of serum and in microvesicle-free medium. At every time point, the supernatant was harvested and centrifuged at 3000rpm for 20 minutes at 4°C, to eliminate detached cells and debris. Microvesicles were then purified by centrifuging supernatants at 100,000g in a

Beckman L8-M Ultracentrifuge equipped with a 70Ti rotor (Beckman Coulter SpA, Milano, Italy) for 90 minutes at 4°C. Microvesicle pellets were collected and resuspended in 50-70µl of appropriate buffer.

Nanoparticle Tracking Analysis (NTA)

Nanoparticle tracking analysis was applied to determine particle size and concentration of all samples. Purified microvesicles were diluted in 70µl of 0.22 µm filtered PBS to a final volume of 1ml. Samples were loaded into the sample chamber of a NanoSight LM10 instrument (Malvern, UK) and ideal measurement concentrations were found by pre-testing the ideal particle per frame value (20–100 particles/frame). Camera level was increased until all particles were distinctly visible and the ideal detection threshold was determined to include as many particles as possible. Five videos of 60 seconds were recorded of each sample under the following conditions: cell temperature: 25°C; syringe speed: 40µl/s.
After capture, the videos have been analysed by the in-build NanoSight Software NTA 3.1 Build 3.1.46 with a detection threshold of 5.

Microvesicle mitochondrial intensity measurement

Cells were seeded and isolated as described for microvesicle purification. Cells were incubated with Mitotracker Green (200-300nM) for 20 minutes at 37°C before being cultured in the presence and absence of serum for 24 and 48 hours. Isolated microvesicles were resuspended in 50µl of sucrose saline with Ca^{2+} and mitochondrial fluorescence was measured using a Cary Eclipse Fluorescence Spectophotometer (Agilent Technologies, Milan, Italy) at the 480nm excitation and at 516 nm emission wavelengths.

Western blot analysis

Cell lysates and isolated microvesicles were and were solubilized in RIPA-buffer (150 mM NaCl/0.1% SDS/0.5% Na-deoxycholate/1% Triton X-100/50 mM Tris, pH 7.2). Protein concentration was measured with the Bradford assay. Fifteen µg of protein were loaded onto a 4–12% Bis-Tris NuPAGE gel (Invitrogen, Life Technologies, Carlsbad, CA, USA). Proteins were blotted for 3 h onto a nitrocellulose membrane (Amersham, GE Healthcare, Chicago, IL, USA). Non-specific binding sites were blocked with 5% skim milk in TBS buffer (10 mM Tris-HCl, 150 mM NaCl, pH 8.0), and nitrocellulose strips were then incubated overnight with primary antibodies at 4◦C either. The rabbit anti-P2X7R antibody (Merk-Millipore, cat. n. AB5246) was diluted 1:500 in TBS-t buffer (TBS plus 0.1%

Tween 20) and 2,5% milk. The rabbit anti-non-muscle Myosin IIA antibody (abcam, cat. n. ab75590) was diluted 1.1000 in TBS-t buffer and 2,5% BSA. Rabbit anti-P-AMPK (Thr172) (Cell Signaling, cat. n. 2535) and anti-AMPKα (Sigma-Aldrich, cat. n. SAB4502329) antibodies were diluted 1:1000 in TBS-t buffer and 5% BSA. The rabbit anti-LC3B antibody (Sigma-Aldrich, cat. n. L7543) was diluted 1:2000 in TBS-t buffer and 5% BSA. Rabbit anti-TOMM20 (Sigma-Aldrich, cat. n. C115995) and anti-carbonic anhydrase II (CA2) (Sigma-Aldrich, cat. n. SAB 2900749) antibodies were diluted 1:1000 in TBS-t buffer and 5% milk. The rodent anti-OXPHOS antibody (abcam, cat. n. ab110413) was diluted 1:1000 in PBS 1% milk.

Membranes were incubated with secondary goat anti-rabbit or anti-mouse HRP-conjugated antibodies (Invitrogen-Termo Fisher Scientifc, Monza, Italy, cat n. 31460) at a 1:3000 dilution for 1 h at room temperature. Finally, ECL reagent (Invitrogen, Life Technologies) was used for detection with a LI-COR blot scanner (LI-COR Biosciences, Lincoln, NE, USA). Densitometric analysis of the protein bands was performed with the freely available ImageJ sofware.

Statistical analysis

Data were analysed for statistical significance by two way ANOVA followed by a post-hoc test for multiple comparison (Tukey test). The data are expressed as mean±S.E.M. Differences were considered significant at $p<0.05$.

ACKNOWLEDGEMENTS

For this work I would like to thank

My tutor Prof. Francesco Di Virgilio and my colleagues Dr. Alba Clara Sarti, Dr. Simonetta Falzoni, Dr. Paola Chiozzi and Dr. Anna Lisa Giuliani.

Prof. Elena Adinolfi and her research group.

Prof. Paolo Pinton and Dr. Sonia Missiroli for helping me with Seahorse analysis.

Dr. Anna Tesei and Dr. Michele Zanoni from IRST of Meldola who performed Nanoparticle Tracking Analysis with NanoSight platform.

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