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Doctoral Thesis in

"The Role of ABCC6 Transporter in the Purinergic System: from Oncology to Regenerative Medicine"

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To Valentina
And to my family

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List of abbreviations

ABC - ATP binding cassette

ACDC - Arterial calcification due to deficiency of CD73

AECs - Amniotic epithelial cells

ALPs - Alkaline phosphatases

AMP - Adenosine monophosphate

ASC - Adult stem cells

ATP - Adenosine triphosphate

BSA - Bovine serum albumin

CALHM - Calcium homeostasis modulator

CD39 - Ecto-nucleoside triphosphate diphosphohydrolases

CD73 - Ecto-5-nucleotidase

cGMP - Current good manufacturing practices

CNS - Central nervous system

CNV - Copy number variation

CPPs - Calciprotein particles

CRC - Colorectal cancer

CSCs - Cancer Stem Cells

DCF - Fluorescent dichlorofluorescein

DCFH - Dichlorodihydrofluorescein

DCFH-DA - 2',7'-dichlorofluorescein diacetate

DCs - Dendritic cells

dECM - Decellularized Extracellular Matrix

DMSO - Dimethyl sulfoxide

ECM - Extracellular Matrix

EGF - Epidermal growth factor

EMT - Epithelial-to-Mesenchymal Transition

ENPP - Ectonucleotide pyrophosphatase-phosphodiesterase

ER - Endoplasmic reticulum

ERK - Extracellular signal-regulated kinase

EROD - ethoxyresorufin-O-deethylase

ESCs - Embryonic stem cells

FasL - Fas ligand

FBS – Fetal bovine serum

GACI - Generalized arterial calcification of infancy

GGCX - Gamma-glutamyl-carboxylase

GMP - Guanosine monophosphate

GVHD - Graft-versus-host disease

HA - Hydroxyapatite

HBSS - Hank's Balanced Salt Solution

HGF - Hepatocytes growth factor

HIFs - Hypoxia-inducible factors

hiPSC-CM – iPSC–derived cardiomyocytes

HLA - Human leukocyte antigen

HLCs - Hepatocytes-like cells

HLCs - Hepatocytes-like cells

HNF4a - Hepatocyte nuclear factor 4 alpha

HSCs - Hematopoietic stem cells

HTx - Hepatocytes transplantation

ICM - Inner cell mass

iPP - Inorganic Pyrophosphate

iPSCs - Induced pluripotent stem cells

ITS - Insulin Transferrin Selenium

L-ECM - liver-derived extracellular matrix

LTC4 – Leukotriene C4

MACs - Maxi-anion channels

MAPK - Mitogen-activated protein kinase

MDR - Multidrug resistance

MDSCs - Myeloid-derived suppressor cells

MG - Matrigel

MGP - Matrix gla protein

MHC - Major Histocompatibility Complex

MIF - Migration-inhibitory factor

MOI - Multiplicity of infection

MRP - Multidrug resistance protein

MSCs - Mesenchymal stem cells

MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolim bromide

NBDs - Nucleotide binding domains

NEAA - Nonessential amino acids

NFDM - Nonfat dried milk

NF-κB - Nuclear factor kappa-light-chain-enhancer of activated B cells

OLT - Orthotopic liver transplantion

PBS – Phosphate buffer saline

PD - Parkinson disease

PI3K - PhosphatidylInositol 3-Kinase

PLAG - Pleomorphic adenoma gene

PSCs - Pluripotent stem cells

PTCs - Premature termination codons

PXE - Pseudoxantoma elasticum

RFU - Relative Fluorescence Units

RIPA - Radioimmunoprecipitation assay

ROS - reactive oxygen species

SCI - Spinal cord injury

SCNT - Somatic cell nuclear transfer

SCs - Stem cells

SDS - Sodium dodecyl sulfate

SDS-PAGE - Sodium dodecyl sulfate - PolyAcrylamide Gel Electrophoresis

SNV - Single nucleotide variation

TAMs - Tumor-associated macrophages

TAP - Transporters associated with antigen processing

TFs - Transcription factors

TGF-β - Transforming Growth Factor-β

TKIs - Tyrosine Kinase Inhibitors

TMDs - Transmembrane domains

TME - Tumor microenvironment

TNAP - Tissue-nonspecific alkaline phosphatase

TNF-α - Tumor necrosis factor-α

TRAIL - Tumor necrosis factor-related apoptosis-inducing ligand

TRPCs - Transient Receptor Potential Channels

UGT - UDPglucuronosyltransferase

Abstract

ABCC6 is a member of ATP-binding cassette family of transporters, a class of membrane transporters sharing a common structural organization. Mutations in ABCC6 are associated with Pseudoxantoma elasticum (PXE) and with a minority of cases of Generalized Arterial Calcification of Infancy (GACI), two metabolic diseases characterized by an abnormal ectopic calcification of soft tissues. It is now recognized that ABCC6-mediated efflux of ATP from the liver is the main source of inorganic pyrophosphate, which is a strong anti-mineralization factor and is lacking in serum of patients with PXE or GACI. Extracellular ATP is not only important in controlling mineralization processes, as it is also a signaling molecule and is the main source of extracellular adenosine. Both ATP and adenosine can act as autocrine/paracrine signals, participating to the regulation of a variety of pathophysiological processes, through specific receptors. Purinergic signaling has a profound impact on tumor progression, affecting cancer cells proliferation, apoptosis, invasiveness and interaction with host immune response.

Most of studies on ABCC6 focused on the identification of substrates in relation to its role in mineralization processes, but little information is available in regard of the consequences of transport activity in cells expressing ABCC6 protein. In this thesis we studied the effects of ABCC6 gene silencing and pharmacological inhibition on migration and cytoskeleton rearrangement of cancer cells, focusing at first on liver cancer cell line HepG2. Encouraged by results obtained in these cells, we tried to extend the relevance of our findings to extra hepatic tumors as well. We investigated the effects of pharmacological inhibition on two colorectal cancer cell lines, Caco2 and HT29, which significantly differ in the levels of expression of ABCC6. Finally, we evaluated the feasibility of using hepatocytes-like cells (HLCs) derived from amniotic epithelial stem cells as a substitute of primary hepatocytes to assess the potential toxic effects arising from ABCC6 inhibition in normal cells.

Results reported here clearly show that in tumors where ABCC6 is highly expressed, it could have an important role in controlling cancer cells migration, through the modulation of extracellular purinergic signaling, thus representing a potential target for anti-cancer treatments aimed to reduce tumor invasiveness. HLCs derived from pluripotent stem cells represent a promising tool not only for regenerative medicine but also for disease modeling and toxicological studies. However, improvements in current protocols of in vitro

differentiation are required to broaden the scope of this technology to routine toxicological studies.

List of publications

I. Ostuni, A., Carmosino, M., Miglionico, R., Abruzzese, V., Martinelli, F., Russo, D., Laurenzana, I., Petillo, A., & Bisaccia, F. (2020). Inhibition of ABCC6 Transporter Modifies Cytoskeleton and Reduces Motility of HepG2 Cells via Purinergic Pathway. *Cells*, *9*(6), 1410.

II. Abruzzese, V., Matera, I., Martinelli, F., Carmosino, M., Koshal, P., Milella, L., Bisaccia, F., & Ostuni, A. (2021). Effect of Quercetin on ABCC6 Transporter: Implication in HepG2 Migration. *International journal of molecular sciences*, *22*(8), 3871.

III. Abruzzese, V., Sukowati, C. H., Tiribelli, C., Matera, I., Ostuni, A., & Bisaccia, F. (2022). The Expression Level of ABCC6 Transporter in Colon Cancer Cells Correlates with the Activation of Different Intracellular Signaling Pathways. *Pathophysiology*, 29(2), 173-186.

Additional publications not included in this thesys

IV. Petillo, A., Abruzzese, V., Koshal, P., Ostuni, A., & Bisaccia, F. (2020). Extracellular Citrate Is a Trojan Horse for Cancer Cells. *Frontiers in molecular biosciences*, 7, 593866.

V. Bisaccia, F., Koshal, P., Abruzzese, V., Castiglione Morelli, M. A., & Ostuni, A. (2021). Structural and Functional Characterization of the ABCC6 Transporter in Hepatic Cells: Role on PXE, Cancer Therapy and Drug Resistance. *International journal of molecular sciences*, 22(6), 2858.

Posters and oral presentations

- Abruzzese, V., Miglionico, R., Petillo, A., Bisaccia, F., Carmosino, M., Ostuni, A. (2019, September) *Synthetic and natural compounds regulate activity of ABCC6 transporter*. SIB 2019 60th CONGRESS, Lecce, Italy
- Abruzzese, V., Koshal, P., Matera, I., Carmosino, M., Milella, L., Ostuni, A., Bisaccia, F. (2021, July) *Role of ABCC6 in PXE and cancer therapy*. FEBS 2021 45th CONGRESS, Ljubljana, Slovenia
- Abruzzese, V., Koshal, P., Matera, I., Ostuni, A., Bisaccia, F. (2021, September) *The role of ABCC6 and NT5E inhibitors in colon cancer cells*. SIB 2021 61th VIRTUAL CONGRESS
- Abruzzese, V., *The role of ABCC6 and NT5E inhibitors in colon cancer cells*. (2021, September) Oral presentation at: SIB 2021 61th Congress, Poster Prizes Session. Virtual Congress

Introduction

ABC transporters

The transport activity of substrates across membranes is an essential task to maintain cell homeostasis. Since cells are delimited by a closed lipid membrane, there is need of very complex machinery to let exchange between the inner cytoplasmic side of this phospholipidic barrier and the external environment take place. This task is necessary because cells constantly need intake of small hydrophilic molecules and nutrients to supply their metabolic processes and at the same time have to get rid of toxic wastes or to secrete final product of a specific pathway, which ultimately fulfill diverse functions in another district of the organism.

The ATP binding cassette (ABC) transporters superfamily is one of the largest classes of membrane proteins which use energy from hydrolysis of ATP to move a wide variety of substrates across membranes, including ions, sugars, amino acids, polypeptides, toxic metabolites, xenobiotics, and even drugs and toxins. ABC transporters are ubiquitously widespread in all the domains of life, from the simplest prokaryotic archea to higher complex eukariota. A typical eukaryotic member of this group consists of two kinds of subunits: transmembrane domains (TMDs) and nucleotide binding domains (NBDs). These units can be mixed in different compositions: a configuration of two NBDs alternate to two TMDs on a single polypeptide in either of the two possible N-terminal/C-terminal orientations gives rise to a full transporter, while a half transporter is composed by just one TMD and one NBD. Some proteins are made of a single NBD or TMD and are defined as single domain structures; few others are encoded with two NBD and no TMD. The latter do not have any transport activity and are referred as ABC2 structure (Liu, 2019) (Figure 1).

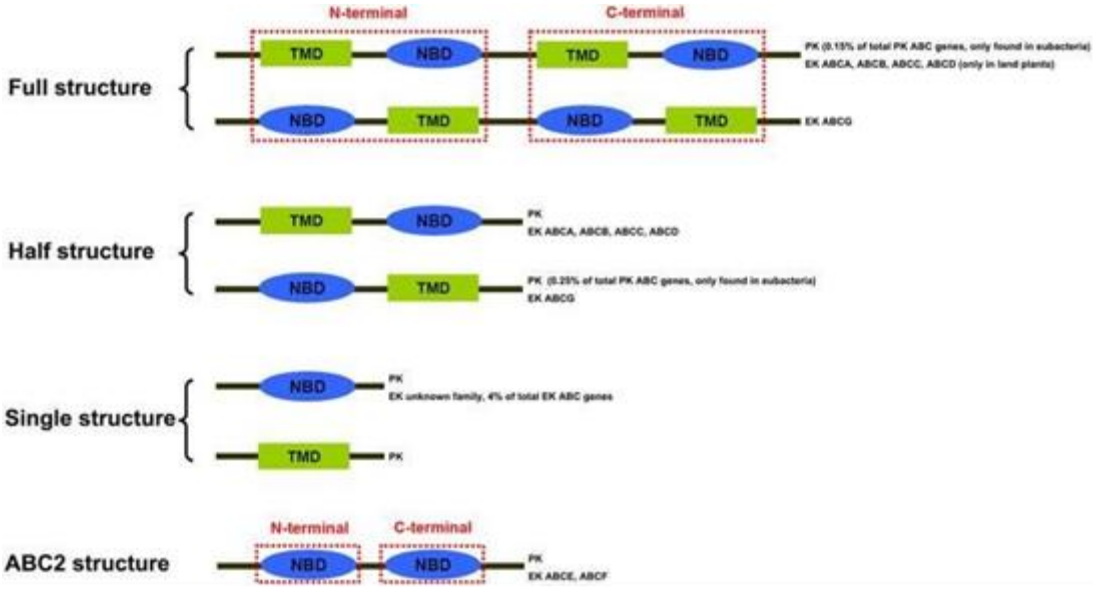


Figure 1: Illustration of the different structures in the ABC transporter superfamily (Xiong et al., 2015)**.**

As previously reported, all ABC transporters share a common modular structure consisting of two highly conserved NBDs, located in the cytoplasm, which are the unifying feature of ABC systems, and two variable TMDs, embedded in the membrane bilayer, that form the framework of the translocation pathway. Following the first determination of a crystal structure, that of the E. coli vitamin B12 importer BtuCD in 2002 (Locher et al., 2002), dozens of structural studies have been published in the past 20 years, which allowed to trace some details of the NBDs as well as the overall architecture of the active transporters and their conformational changes during substrate translocation. The NBDs consist of a RecA-type ATP-binding core in which a six-stranded β-sheet is surrounded by four α-helices (helices A–D), supplemented with an ABC-specific three-stranded antiparallel β-sheet (ABCβ) and an α-helical subdomain (ABCα). The ATP-binding cassette is formed of:

- the Walker A and Walker B motif, the D- and Q-loop, and the H-switch, positioned within the RecA-type ATP-binding core
- the A-loop, part of the antiparallel β-sheet region
- the signature motif (C-loop), located at the end of the ABCα subdomain

Nucleotide binding is primarily mediated by the phosphate-coordinating P-loop, formed by the consensus sequence (cs) GxxGxGK(S/T) (where x denotes any residue) within the Walker A motif and by the A-loop. The P-loop binds the β- and γ-phosphate of the nucleotide via its backbone amide-NH groups and the ε-amino group of the conserved lysine.

The A-loop provides an aromatic residue that interacts with the nucleotide base through π-π-stacking. The Walker B motif (cs: ϕ_4 D, where ϕ denotes a hydrophobic residue) supplies an aspartate that helps to coordinate the crucial Mg2+ ion. The Walker B motif is directly followed by the catalytic glutamate, which polarizes the attacking water molecule during ATP hydrolysis. The histidine of the H-switch has a central role in stabilizing the catalytic transition state, while the glutamine of the Q-loop *cs: ϕ(ϕ/Q)Q+ acts a Mg^{2+} ligand, senses the presence of the γ-phosphate, and is a crucial point of interaction with the TMDs. The C-loop, formed by the signature motif (cs: LSGGQ) and sitting at the N terminus of helix B in the ATP-binding core, contributes to ATP-binding-induced NBD dimerization and functions as a transactivation element during ATP hydrolysis.

Unlike the NBDs, TMDs display no significant sequence conservation but share a similar topology within a transporter class, reflecting the diversity of transported substrates. They are usually formed of 6 to 10 transmembrane α-helices each, packed in such a way that they form a pore accessible either from the cytoplasm or from the outside of the cell (Figure 2).

Dimerization of the NBDs is induced by ATP and happens in a yin-yang fashion, with the Walker A/B motif and A-, H-, and Q-loops of one NBD facing the signature motif of the other NBD, and two molecules of ATP sandwiched in the middle. NBDs dimerization is a prerequisite of ATP hydrolysis, allowing the signature motifs to align the ATP molecules so that the positive charge of the electrostatic dipole of helix B is directed toward the phosphate groups of the nucleotides. At the end of the catalytic cycle, the release of inorganic phosphate results in separation of the dimer. These changes in NBDs dimerization state are transmitted by means of coupling helices to the TMDs, which toggle between inward-facing (IF) and outward-facing (OF) conformations, causing substrate translocation from the two sides of the membrane (Thomas et al., 2020; Wilkens, 2015; Dawson et al., 2007) (Figure 3).

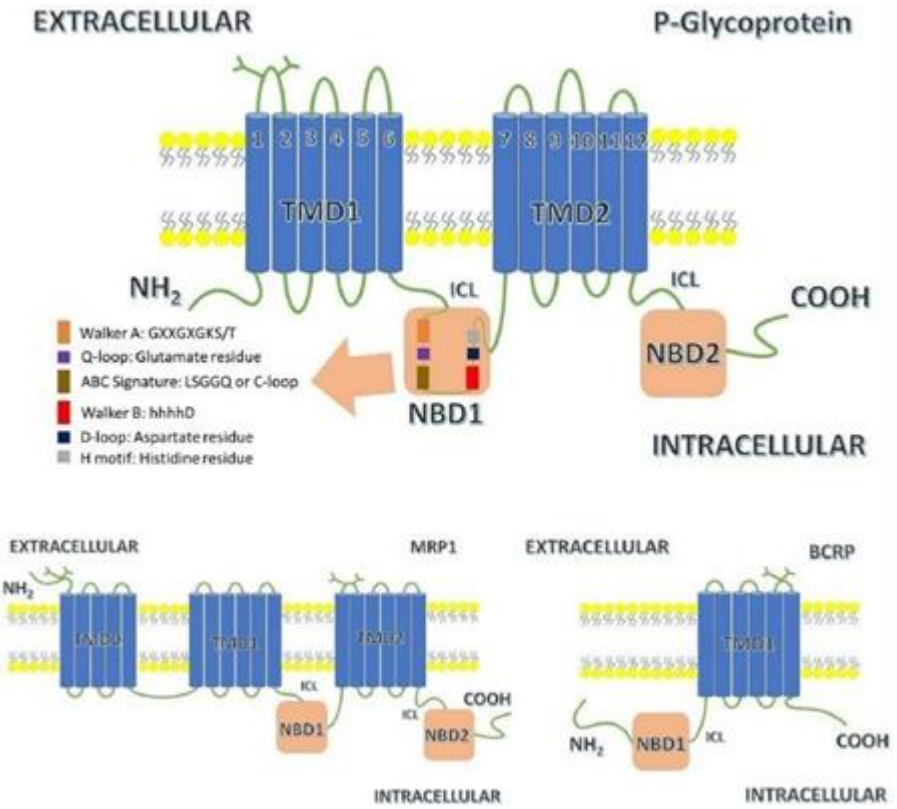


Figure 2: General structure of representative members of ABC transporters family (Juan-Carlos et al., 2021)

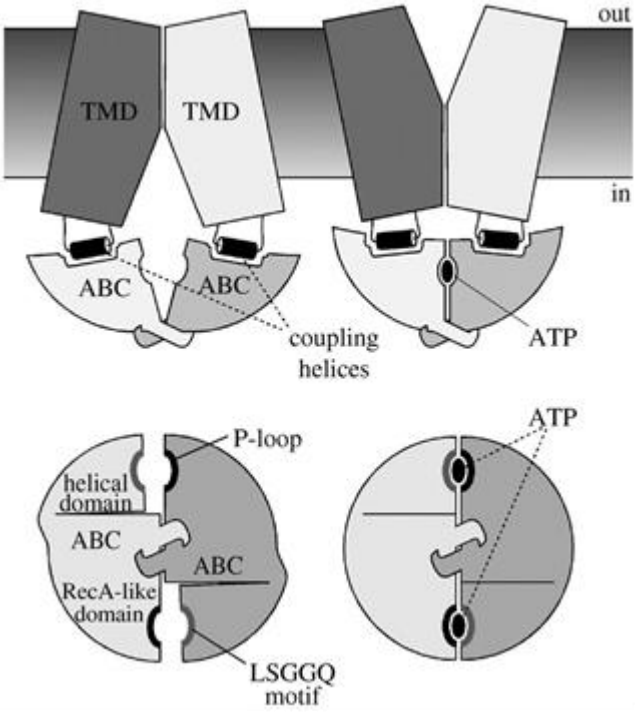


Figure 3: Conserved coupling mechanism of ABC transporters (Locher, 2009)

The enormous complexity of this group - more than 2000 known ABC ATPase domains or proteins - required a system of categorization. Since their most prominent characteristic is that they share a highly conserved ATPase domain, the ATP-binding cassette, which binds and hydrolyzes ATP, one of the first attempts of classification was based on functional and phylogenetic studies (Dassa et al., 2001; Bouige et al., 2002). On the basis of this system, the ABC members are divided into three categories: the first class, including all known exporters, has TMDs fused to NBDs; the second class is involved in cellular processes rather than transport activity since it does not contain any integral membrane domains, being composed of two NBDs fused together; the third one is mainly, but only, represented by bacterial importers, which are composed of four domains, two NBDs and two TMDs, located on different polypeptides, and depend on substrate binding domains (SBDs) to provide bacteria with essential nutrients from the external environment. However, recently, a new classification has been proposed, which is based on TMDs fold and groups ABC transporters in types I-VII (Thomas et al., 2020).

Mammalians ABC transporter have been divided in 7 subfamilies named ABCA-G based on similarity in gene structure (half vs. full transporters), order of the domains, and on sequence homology in the NB and TM domains. Human genome contains 49 of such genes (Dean et al., 2001; Vasiliou et al., 2009).

- The subfamily ABCA includes 12 full transporters divided into two subgroups based on phylogenetic analysis and intron structure. The first one is formed of seven genes dispersed on six different chromosomes (ABCA1-4, ABCA7 and ABCA12-13), whereas the second contains five genes (ABCA5-6, ABCA8-10) arranged in a cluster on chromosome 17q24. Members of this family are involved in lipids trafficking in various organs and cell types (Broccardo et al., 1999)
- The subfamily ABCB contains 4 full transporters and 7 half transporters. Among this group we find ABCB1/MDR1, the first human ABC transporter to be cloned and characterized for its ability to confer a multidrug resistent phenotype to cancer cells (Juliano et al., 1976), whose expression contributes to maintain a permeability barrier of sanctuary sites and to limit drug absorption. ABCB4 and ABCB11 are both localized in the liver, where they are involved in the secretion of bile acids (Van der Bliek eat. al., 1987; Strautnieks et al., 1998). ABCB2-3 are the transporters associated with antigen processing (TAP) which delivers cytosolic peptides into the endoplasmic reticulum (ER), where they bind to nascent MHC class I molecules (Suh et al.,1994).

ABCB6-8 and ABCB10 localize to the inner mitochondrial membrane, where they are involved in iron homeostasis (Seguin at al., 2018)

- The subfamily ABCC contains 13 members. Except from pseudogene ABCC13 all of them are full transporters. ABCC7 encodes a chloride ion channel which has a role in all the exocrine secretions (Rommens et al., 1989). ABCC8 and ABCC9 proteins are regulators of a particular class of potassium channels involved in modulation of insulin secretion and constitute the receptors of sulfonylureas (Bryan et al., 2007). The rest of the subfamily if represented by the multidrug resistance proteins (MRPs), which transport drug conjugates to glutathione and other organic anions. ABCC1-3, ABCC6-7 and ABCC10, differently the other MRPs, have an additional transmembrane domain (TMD0) at N-terminal (Toyoda et al., 2008)
- The subfamily ABCD contains 4 genes which encode half transporters located in the peroxisomes, where they function as homo- and/or heterodimers and are involved in the regulation of very long chain fatty acid transport (Shani et al., 1998)
- The subfamilies ABCE and ABCF members only contain ATP-binding domains, clearly derived from ABC transporters, but have no TM domain and are not known to be involved in any membrane transport functions. ABCE1, the sole member of its group and recognizes oligo-adenylate that is produced in response to viral infection and inhibits the interferon-regulated Ribonuclease L (Bisbal et al., 1995). The 3 ABCF genes encode for proteins which are associated with the ribosome (Tyzack et al., 2000)
- The subfamily ABCG is comprised of 5 reverse half transporters, which differ from other ABC for having a TMD at the C-terminal. ABCG1 is involved in cholesterol transport regulation (Klucken et al., 2000). ABCG2 is a multidrug resistance gene (Doyle et al.,1998) while ABCG5 and ABCG8, limit intestinal absorption and promote biliary excretion of sterols (Lu et al., 2001)

The ABC transporters not only move a variety of substrates into and out of the cell, but also are involved in intracellular compartmental transport, thus participating in important physiological processes. In fact, loss of functions of such genes is often associated with metabolic disorders. Currently, mutations in 24 of 49 ABC genes have been linked to human diseases, 12 of which involving abnormal lipid transport and/or homeostasis (Tarling et al., 2013). Among this group we can find Tangier disease, an inherited disorder characterized by significantly reduced levels of high-density lipoprotein (HDL) in the blood,

caused by mutations in the ABCA1 gene (Vedhachalam et al., 2007); X-linked adrenoleukodystrophy (X-ALD), which presents with progressive loss of myelin in the brain and spinal cord, due to a defective metabolism of very long-chain fatty acids (VLCFA), following mutations in the ABCD1 gene (Kemp et al., 2012); Dubin-Johnson syndrome, caused by mutations in ABCC2, associated with a failure to secrete conjugated bilirubin glucuronides into the bile, resulting in elevated levels of conjugated bilirubin in the plasma (Jemnitz et al., 2010); cystic fibrosis, an inherited disorder that affects lungs, digestive system and other organs in the body, which is unique among this list, since the member of ABC family being involved, the cystic fibrosis transmembrane conductance regulator (CFTR/ABCC7), is an ion channel, whose mutations cause a dysregulation in secretions, which are abnormally sticky and thick (De Boeck, 2020).

Transporter name	Function	Disease
ABCA1	Cholesterol and phospholipids transport	Tangier disease, Familial hypoapoproteinemia
ABCA2	Drug resistance	unknown
ABCA4	Rod photoreceptor retinoid transport	Stargardt/fundus flavimaculatis, Retinitis pigmentosa, Cone-rod dystrophy, Age-related macular degeneration
ABCB1	Drug resistance	unknown
ABCB2	Peptide transport	Immune deficiency
ABCB3	Peptide transport	Immune deficiency
ABCB4	Bile-acid transport	Progressive familial intrahepatic cholestasis-3
ABCB6	Iron transport	unknown
ABCB7	Iron transport	X-linked sideroblastosis and anemia
ABCB11	Bile-acid transport	Progressive familial intrahepatic cholestasis-2
ABCC1	Drug resistance	unknown
ABCC2	Bile-acid transport	Dubin-Johnson Syndrome
ABCC4	Nucleoside transport	
ABCC6	unknown	Pseudoxanthoma elasticum
ABCC7	Chloride ion channel	Cystic fibrosis
ABCC8	Sulfonylurea receptor	unknown
ABCD1	Very long chain fatty acids transport	Adrenoleukodystrophy
ABCE1	Oligoadenylate-binding protein	unknown
ABCG1	Cholesterol transport	unknown
ABCG2	Drug resistance	unknown
ABCG5	Sterol transport	Sitosterolemia
ABCG8	Sterol transport	Sitosterolemia

Table 1: List of human ABC transporters, their functions and diseases caused by ABC genes (Stefková et al., 2004)

Moreover, a growing number of evidence seems to suggest that ABCs proteins transport activity plays a critical role in human cancers, which is primarily due, but not

limited to, their nature of xenobiotics efflux transporters, which can limit the concentration of antitumor drugs achievable within cancer cells. Among the 49 members of this family, approximately 13 have been found to be clinically relevant for chemoresistance (Wang et al., 2021). ABCB1, also known as P-glycoprotein (P-gp) or multidrug resistance protein 1 (MDR1) is one of the most well-established ABC transporters that mediates MDR in cancer cells, being able to mediate the efflux of a great variety of anticancer drugs. Substrate recognition by the P-gp is facilitated by hydrophobic and aromatic contacts within the binding pocket and stabilized by Van der Waals forces (Mollazadeh et al., 2018). ABCG2, also known as breast cancer resistance protein (BCRP) is often overexpressed in many types of solid tumors and hematopoietic malignancies, resulting in cross-resistance to different chemotherapeutic drugs with unrelated structures and functions (Mo et al., 2012). Within subfamily C of ABC transporters, many members have been linked to drug resistance at various levels. ABCC1-6 and ABCC10-12 are also known as multidrug resistance proteins (MRP1-9) and, differently from ABCB1, which extrude mostly xenobiotics, they are also responsible for the extrusion of endogenous substrates, thus participating to several physiological functions, such as intracellular detoxification, oxidative stress, and inflammation. Apart from drugs, members of this group are able to transport leukotriene, glutathione and glucuronic acid conjugates. Some of them have an additional regulatory transmembrane domain (TMD0), which seems not to be necessary for transport activity (Wang et al., 2021).

But the role of ABC transporters in cancers is not restricted to drug resistance, since they can directly contribute to tumor progression. Cancer hallmarks include seven essential properties: (1) self-sufficiency in growth signals, (2) insensitive to anti-growth signals, (3) limitless replication, (4) evasion of apoptosis, (5) angiogenesis, (6) invasion and metastasis, and (7) inflammation (Fletcher et al., 2010). The expression and activity of ABC transporters can affect many, if not all, of these hallmarks, thus contributing to the early events of tumor formation, promoting cancer progression, and leading to poor outcomes.

Acquiring the ability of escaping apoptosis is a central event for cancer cell expansion. Several authors showed that inhibition of ABCB1 promotes cell cycle arrest and apoptosis in leukemia cells (Lopes et al., 2003; Lehne et al., 2002), while a marked increase of apoptosis was seen in both *in vitro* and *in vivo* models of neuroblastoma after knocking down ABCC1 (Peaston et al., 2001; Kuss et al., 2002). Although the exact mechanism of ABCB1 anti-apoptotic signaling is not known, several experiments have shown that it is

caspase-dependent and that overexpression of this transporter does not protect cells from caspase-independent cell death (Ruefli et al., 2002).

Besides the evasion of apoptosis, sustained proliferation due to the breakdown of cell-cycle control is also critical to cancer development. One of the main ABC transporters implicated in such control of proliferation is ABCG2. In fact, ABCG2 silencing or pharmacological inhibition in a model of breast cancer cell line selected for mitoxantrone resistance, showed a block of proliferation and arrest of cell cycle at G0/G1 phase through down-regulation of cyclin D3 and up-regulation of P21 (Chen et al., 2010), while overexpression of ABCG2 in retinal progenitor cells promoted proliferation through the increase in expression of cyclin D1 and reduction of the cycle-static p27 (Bhattacharya et al., 2007). Although the exact mechanism of ABCG2-mediated control of proliferation is unclear, it was proposed that it could participate to the efflux of endogenous or exogenous cytotoxins as physiological substrates (Chen et al., 2010). Another ABC transporter shown to regulate proliferation in tumor cells is ABCB1. In both in vitro and in vivo experiments, the knockdown of ABCB1 in a colon-cancer cell line was shown to suppress proliferation by causing a G0/G1 phase cell cycle arrest (Katoh et al., 2008).

Inflammation is closely related to cancer, although it can be considered a tumor-promoting condition, rather than a hallmark, and this is more evident in the earliest stages of neoplastic progression (Hanahan et al., 2011). In this case, the relationship with ABC transporters is almost intuitive, since many eicosanoid mediators of inflammation, such as prostaglandins (PGs), prostacyclin (PGI12) and thromboxanes are substrates of specific ABC transporters. Another mediator of inflammation derived from arachidonic acid, the leukotriene C4 (LTC4), is transported by many MRPs, and sphingosine-1-phosphate (S1P), a pleiotropic lipid mediator generated inside cancer cells by sphingosine kinases, is exported into the tumor microenvironment by ABCC1 (Muriithi et al., 2020). The association among cancer, inflammation and ABC transporters is particularly evident for colorectal cancer (CRC), where colitis and inflammatory bowel syndrome are predisposing conditions, and low levels of ABCB1 in the colonic tissue biopsies were associated with mild/moderate dysplasia, an early event that preceded malignancy (Andersen et al., 2013).

Acquiring the ability to form metastasis is an important milestone in clinical history of solid tumors, when they become more aggressive, and it is associated with poor prognosis. The transporter ABCB1 has been linked to migration, invasiveness, and metastatic ability of several cancers. This is particularly noticeable in the case of melanoma, which very often

evolves in metastatic dissemination. In fact, in uvea melanoma cells, the expression of ABCB1 identifies a subpopulation of cells which are highly metastatic and more tumorigenic, compared to bulk ABCB1(-) cells (Landreville et al., 2011). Different authors reported a more invasive phenotype in human multidrug resistant Pgp-positive melanoma cells, compared to parental ones (Colone et al., 2008). The evidence of a relationship between ABCB1 overexpression and invasiveness was also found in multiresistant breast and laryngeal cancers and in both cases ABCB1 knockdown and transport inhibition was able to reduce invasive capacity (Zhang et al., 2014; Li et al., 2009). These results have been also confirmed in clinical studies, which found a statistically significant increase in the risk of liymph node metastases in breast and colon tumors bearing expression of ABCB1, compared to the group which did not express it (Schneider et al., 2001; Weinstein et al., 1991), while another group found a significant association between MRP1 and Pgp expression and the risk of metastasis in melanoma (Walsh et al., 2010).

These were just few examples but, to better understand the nature underlying the relationship between ABC transporters and cancers, it is important to get more inside the changes that occur while tumor cells become able to disseminate. During this process, tumor cells co-opt an embryonic morphogenesis mechanism: epithelial cells lose their polarity, decrease cells adhesion, and become more mesenchymal-like. This is accompanied by the expression of Epithelial-mesenchymal transition (EMT) transcription factors (TFs), which regulate the expression of several genes involved in normal developmental morphogenesis, but which can contribute to tumor cells invasiveness. This peculiar transcriptional program includes, but is not limited to, reduction of E-cadherin and increase in the expression ofmesenchymal markers N-cadherin and vimentin (Jiang et a., 2017). Many authors reported that these EMT-ralated TFs can control the expression of ABC transporters as well. A positive correlation between the expression of EMT transcription factor Twist1 and ABCB1 was found in cervical cancer and breast cancer cells, whereas silencing of Twist1 sensitized cells to chemoterapic treatment and suppressed proliferation (Zhu et al., 2012; Tsou et al., 2015). Studies aimed to find a relationship between EMT transcriptional factors Snail and MSX2 and multidrug resistance conducted in breast and pancreatic cancer cells respectively showed that they positively correlate with ABCG2 (Chen et al., 2010; Hamada et al., 2012), while different authors found a significant upregulation of TFs ZEB1 and Twist1 in a subpopulation of thyroid papillary carcinoma cells expressing ABCG2 (Mato et al., 2014). In a study conducted on liver cancer cells, the authors disclosed that the establishment of resistance to

the first-line drug in this kind of tumor 5-fluorouracil was accompanied with the appearance of typical EMT features, together with increased expression of Twist1 and ABCC5 (Uchibori et al., 2012). A number of signaling pathways have been identified to participate in regulation of MDR and EMT, such as PI3K/AKT, TGF-β, HIF-1, NF-κB, EGF, which can be directly modulated through the interaction with the tumor microenvironment (TME). One of the most important features of the TME in regulating EMT is hypoxia. In fact, under hypoxic conditions, the activation of hypoxia-inducible factors (HIFs) can induce the expression of EMT-related genes and MDR proteins, thus giving cancer cells a more aggressive and drug resistant phenotype (Liu et al., 2014; Xie et al., 2013). But hypoxia can also cooperate with other oncogenic pathways to generate the chemoresistance in tumor cells. Different authors have observed that the ubiquitous NF-kB, when activated, can migrate into the nucleus of cancer cells, where it plays a critical role for the induction and maintenance of EMT and for expression of MDR1 (Bentires-Alj et al., 2003; Huber et al., 2004). The cytokine TGF-β, the most common EMT inducer, which is also found in the tumor microenvironment, can increase ABC transporters expression, thus suggesting that tumor cells can produce autocrine and paracrine factors leading to the development of tumor metastasis and drug resistance (Panousis et al., 2001) (Figure 4).

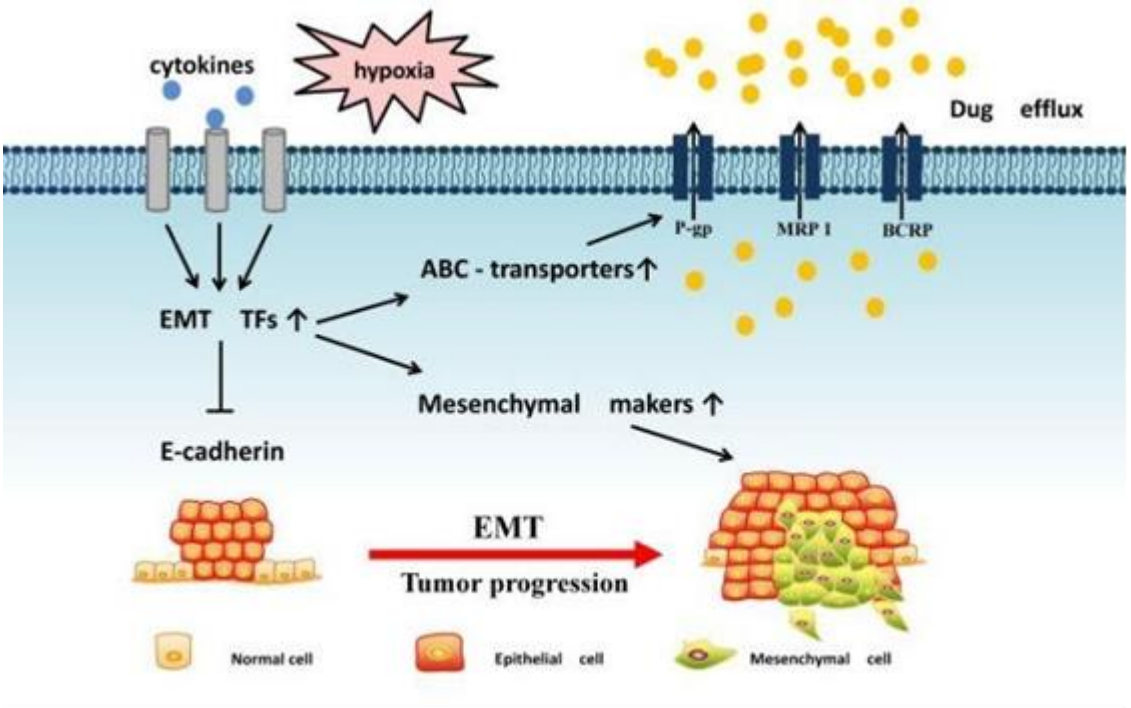


Figure 4: The potential linkages between EMT and ABC transporters (Jiang et al., 2017)

According to the current model, cancers are a heterogeneous mixture of genetically distinct subclones that contribute to the functional and phenotypic heterogeneity of the tumour. At the apex of this hierarchical organization, there is a small population of slow-cycling cells, endowed with enhanced tumorigenic potential, self-renewal capabilities and an intrinsic resistance to targeted and conventional therapies (Begicevic & Falasca, 2017). The first discover of these "dormant cells" dates back to several decades ago (Clarkson et al., 1970), but the paradigm of Cancer Stem Cells (CSCs) was fully validated with the work by Dick and Clark, who first described in leukemia and solid tumors a subset of highly tumorigenic cells, distinguishable for the expression of particular surface markers, similar to those of normal progenitor cells (Bonnet & Dick, 1997; Al-Hajj et al., 2003). Research of such markers has been complicated by the high variability among tumors and within the same tumor and by the fact that they are not tumor specific, so it is likely that finding the right combination would help to identify CSCs. Some of these markers, as NANOG, octamer-binding transcription factor 4 (OCT4) and sex determining region y-box 2 (SOX2) are required for the maintenance of stem cell pluripotency. But another important property of CSCs is their enhanced resistance to conventional and targeted therapies. In fact, as shown in many papers, they express high levels of specific ABC transporters. One of such members of ABC family is ABCG2: indeed the presence of ABCG2 was demonstrated in stem cells from a variety of sources, as well as the ability to impart a side population phenotype and reduced maturation in progeny by its enforced expression (Zhou et al., 2001). Further studies confirmed that ABCG2 overexpression is necessary for maintaining the stemness of embryonic cells and that its role in not limited to acting as a transporter of xenobiotics (Susanto et al., 2008). Among the other ABC transporters, ABCB5 has been proposed as a marker of skin progenitor cells and malignant melanoma-initiating cells (MMIC), regulating tumor initiation and progression in nude mice (Schatton et al., 2008). It has been shown that, in MMIC, ABCB5 controls IL1β secretion, which serves to maintain slow cycling, chemoresistant cells through an IL1β/IL8/CXCR1 cytokine signaling circuit (Wilson et al., 2014). But this list is even longer than that and is likely to grow as more and more studies accumulate.

ABCC6

The human ABCC6 gene is located on the short arm of chromosome 16 in position 13.11 and encompasses 31 exons spread over nearly 75 kb and is flanked by two pseudogenes that share ~99% sequence identity with the 5' end of the functional gene (Pulkkinen et al., 2001). The first one, called ABCC6-Ψ1, in centromeric position, shows homology with the first nine exons ofABCC6, while the second, ABCC6-Ψ2, from the telomeric side, is nearly identical to the first four exons. Interestingly, both pseudogenes are transcriptionally active and may play a role in regulation of ABCC6 expression (Piehler et al., 2008) (Figure 5).

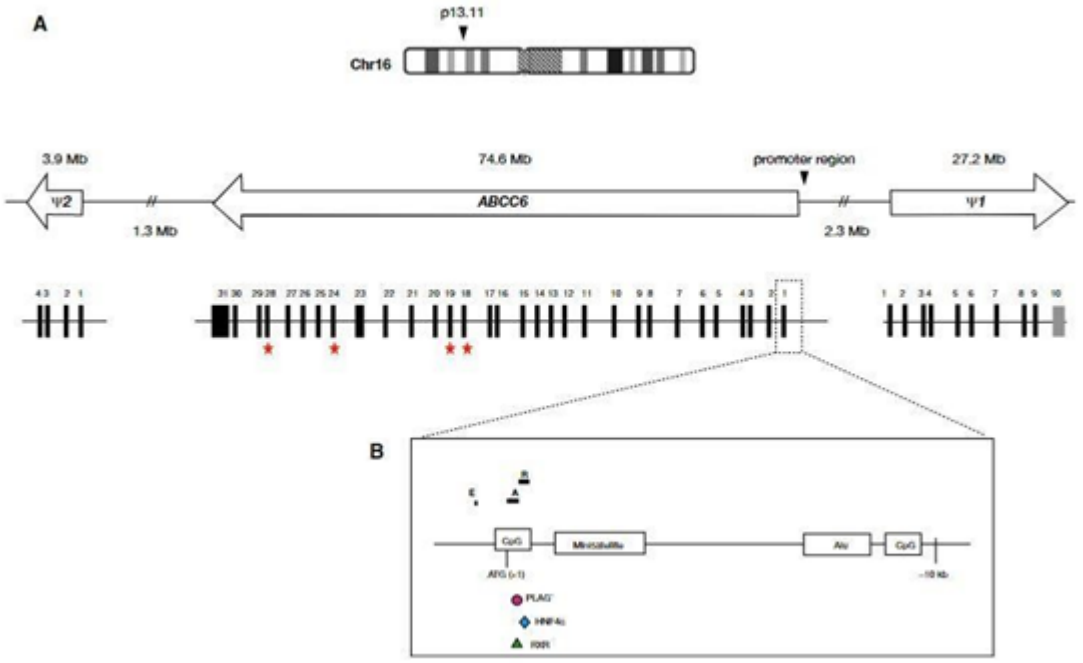


Figure 5: Genomic architecture of the ABCC6 locus and the ABCC6 promoter. (**A**) Schematic representation of the genomic organization ofABCC6and its pseudogenes. (**B**) Organization of the ABCC6 promoter region (Verschuere et al., 2020)

ABCC6 mRNA encodes a polypeptide of 1503 aminoacids. As with many members of subfamily C of ABC transporters, besides the canonical four domain-structure, ABCC6 also contains an additional trasmembrane domain called TMD0, linked to the rest of the protein through a cytoplasmic L0 loop (Moitra et al., 2017). A number of functional and topological studies were studies conducted in the lab of Professor Bisaccia in order to elucidate the nature and role of this N-terminal region. These studies showed that the TMD0 is formed of five transmembrane hydrophobic helices, similarly to the relative domain which can be found in other MRPs (Cuviello et al., 2015), and that the L0 loop, which is also present in

remaining ABCC proteins lacking TMD0, is well structured, being made of three α helical regions separated by more flexible ones (Ostuni et al., 2019). This cytoplasmic loop seems to be necessary for correct membrane localization, as a construct lacking both TMD0 and L0 (ΔTMD0L0), not only was inactive but also failed to target the membrane, unlike the one lacking TMD0 only (ΔTMD0), which showed transport activity comparable to the full-length ABCC6 protein. Moreover, the L0 loop seems also to be involved in regulation of Ca^{2+} influx through the interaction with some ion channels belonging to the family of Transient Receptor Potential Channels (TRPCs) (Miglionico et al., 2016).

Studies performed of the NBDs of MRP1, which share a high level of homology with the homologous in MRP6, proved that the two domains are not functionally identical as the NBD1 has higher affinity for ATP, while the Km for ATP in support of LTC4 transport is mainly determined by ATP hydrolysis at NBD2 (Yang et al., 2003). These findings were also confirmed for the NBDs of ABCC6 by experiment conducted in our lab, which showed that the NBD2 of ABCC6 has a lower ATPase activity compared to NBD1 and that the rate of ATP hydrolysis of the heterodimer NBD1-NBD2, which is the only physiological possible configuration, since ABCC6 is a full transporter, is limited by the NBD2 itself (Ostuni et al., 2011).

At the C-terminal of ABCC6, a PDZ-like sequence, relatively conserved in ABCC transporters, was identified. This sequence can interact with PDZ-domain-containing proteins as localized scaffolds and is essential for steady-state expression of ABCC6 in membrane structures. Removal of this PDZ-like sequence resulted in decreased steady-state ABCC6 levels, decreased cell surface expression and stability, and mislocalization of the ABCC6 protein in polarized cells (Xue et al., 2014) (Figure 6).

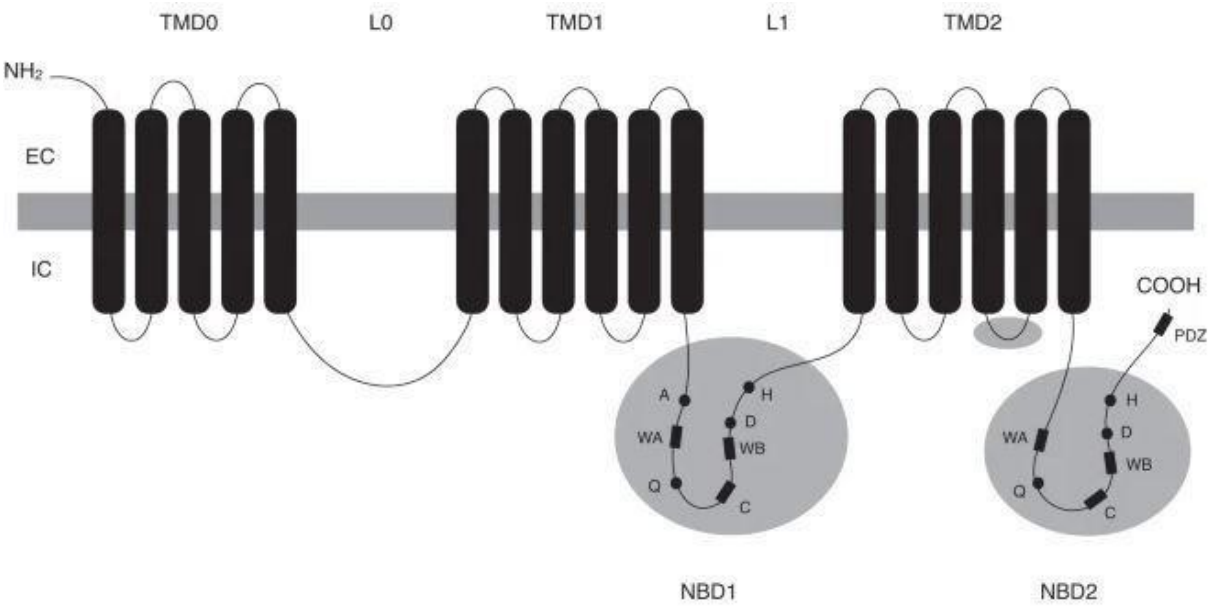


Figure 6: Structure of ABCC6 protein. The ABCC6 transporter contains the following structures: 3 transmembrane domains (TMD0-2), 2 linker domains (L0-1), and 2 nucleotide binding domains (NBD1–2). Conserved sequences within the NBD domains are: A-loop (A), Walker A (WA), Q-loop (Q), LSGGQ-signature (C), Walker B (WB), D-loop (D), H-loop (H) and PDZ-like sequence. Frequently mutated regions are indicated in gray circles (Verschuere et al., 2021)

mRNA of ABCC6 is highly expressed in liver and kidney, with numerous reports of low levels of expression in several other tissues, including epithelial cells of exocrine and endocrine tissues, mucosal cells of the intestine and follicular epithelial cells of the thyroid, neurons and lymphocytes (Kool et al., 1999; Beck et al., 2005; Taipalensuu et al., 2001), even if evidences are there that this low expression found in many tissues could be represented by products of aberrant splicing in the 3' end of the ABCC6 mRNA, resulting in a premature termination codon (Matsuzaki et al., 2005). However, immunostaining of ABCC6 protein was observed in several of these tissues (Beck et al., 2005; Scheffere et al., 2002). Nevertheless, it is currently accepted that the main sites of ABCC6 expression are hepatocytes and renal tubular kidney cells. Regarding the exact cellular localization of ABCC6 protein, the most convincing evidence supports the hypothesis that it is a basolateral membrane protein (Pomozi et al., 2013), even if some authors arguably reported that it could localize to the mitochondria-associated membrane (Martin et al., 2012).

Control of ABCC6 expression has been also studied and seems to be finely regulated. Analysis of the region 10 kb upstream of the translation start site of ABCC6 and coincidental with its promotor region revealed two evolutionary conserved sequence blocks, the first of

which is under epigenetic control, since DNA methylation of the CpG islands correlated inversely with ABCC6 expression in a cell-type-specific manner (Arányi et al., 2005). The promotor region also contains several transcriptional factor binding sites such as for agonists of the retinoid X receptor (RXR) (Ratajewski et al., 2006), for pleomorphic adenoma gene (PLAG) transcription factors (Ratajewski et al., 2008) and for the zinc fin-ger transcription factor specificity protein 1 (SP1), which can be the responsible for TGF-β1-induced expression of ABCC6 and other genes (Jiang et al., 2006). Finally, a crucial role in regulation of ABCC6 transcription is played by the hepatocyte nuclear factor 4 alpha (HNF4a). The activity of this transcription factor, which imprints a tissue-specific expression, can be also modulated through the interaction with other proteins or pathways, such as ERK cascade or the CCAAT/Enhancer binding protein ß (C/EBPß), which reduce or increase HNF4α binding to the promotor of ABCC6, thus ultimately regulating its expression (de Boussac et al., 2010; Ratajewski et al., 2012) (Figure 5).

Classical studies on transport activity in vesicles from ABCC6 expressing cells clearly showed that it is an efflux pump and that it is able to extrude the anionic cyclopentapeptide and endothelin receptor antagonist BQ-123 (Madon et al., 2000), as well as glutathione-conjugates of leukotriene C4, 2,4-dinitrophenol and N-ethylmaleimide (Belinsky et al., 2002), while transport was effectively inhibited by organic anions as probenecid, benzbromarone and indomethacin (Iliás et al., 2002). Since it belongs to the group of MRPs, a role of ABCC6 in drug resistance has been also proposed. In fact, in the same study from Belinsky et al., the authors reported that ABCC6-transfected cells showed low levels of resistance to several natural product agents, including etoposide, teniposide, doxorubicin, and daunorubicin. A recent work suggested that ABCC6 could be involved in resistance to Tyrosine Kinase Inhibitors (TKIs). In fact, a significant increase in ABCC6 mRNA was observed during in vitro development of nilotinib resistance in a BCR-ABL1-expressing cell line derived from chronic myeloid leukemia, while treatment with ABCC6 inhibitor indomethacin, probenecid and pantoprazole reduced IC50 of nilotinib and dasatinib (Eadie et al., 2018). One study on glioma suggests a link between the expression of ABCC6 and cancer stem cells. Treatment with inhibitor of differentiation 4 (ID4) dedifferentiates mouse astrocytes and human glioma cells to glioma stem-like cells (induced GSCs or iGSCs). These cells exhibit chemoresistant behavior to anticancer drugs through a SOX2-mediated induction of ABCC3 and ABCC6 (Jeon et al., 2011). However, the role of ABCC6 is not always straightforward, as there are many reports which seem to point in the opposite direction, and coamplification of ABCC1

adjacent gene may be also confounding. A downregulation of ABCC6 gene has been associated with cisplatin resistance development in ovarian cancer (Januchowski et al., 2017), with lower probability to respond to palliative chemotherapy of colorectal cancer (Hlavata et al., 2012) and with progression of pancreatic ductal adenocarcinoma (Mohelnikova-Duchonova et al., 2013).

Pseudoxantoma elasticum

Pseudoxantoma elasticum (PXE) (OMIM #264800) is a late-onset inborn genetic disorder characterized by a generalized calcification throughout the body, but primarily affecting the skin, the eye, and the blood vessels. The clinical course is extremely variable, with onset usually in young adults and slow progression, but with important exceptions of fulminant cases of calcification culminating in cardiac infarction of infants.

Typical cutaneous manifestations include yellowish papules localized tendentially on the nape, on sides of the neck and in flexural areas. Histological exams reveal mineral deposits that disrupt and break elastic fibers. Ophtalmological manifestations are angioid streaks in the retina that reflect lesions in Bruch's membrane and can lead to blindness over time. Vascular manifestations usually appear evident years after skin and ocular ones and reveal the calcification of connective tissue of the intima and media and stenosis of blood vessels, mainly small and medium-sized arteries. The first sign is usually claudication intermittens, patients are also at higher risk of aneurysms, strokes, and transient ischemic attack (Figure 7).

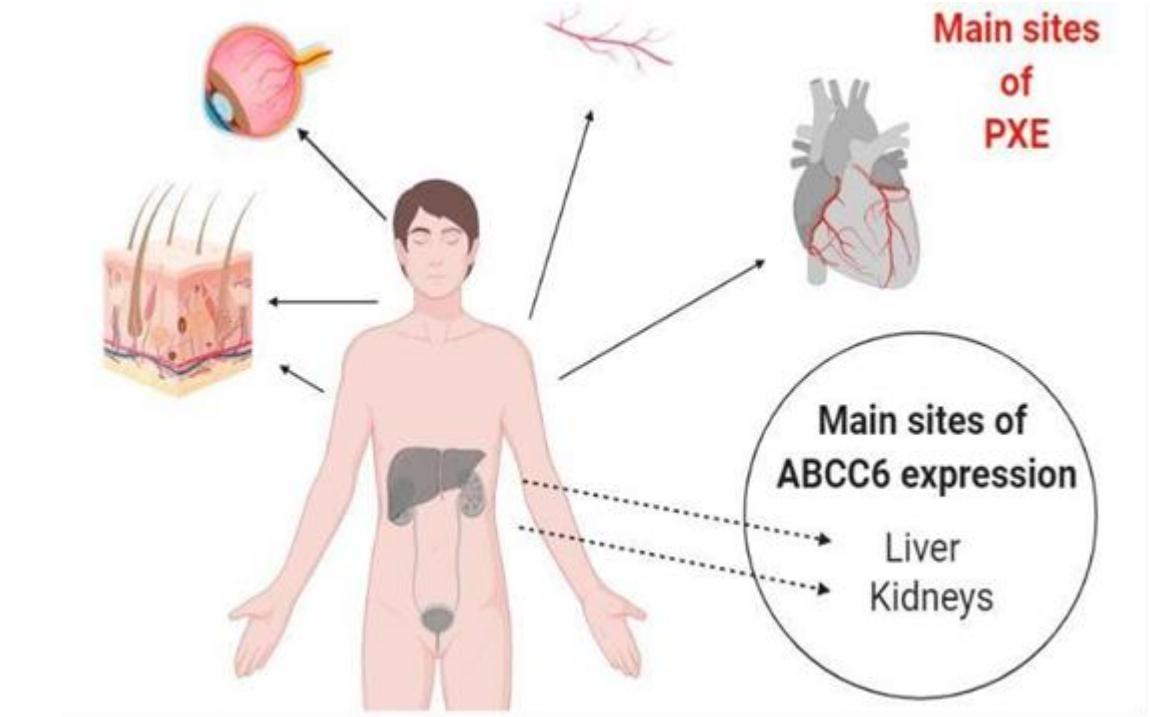


Figure 7: Pictorial presentation of ABCC6 expression and affected tissues in Pseudoxanthoma elasticum (PXE) (Bisaccia et al., 2021)

It shows an autosomal recessive inheritance and an estimated prevalence between 1:100000 and 1:25000, with slight female predominance (Germain, 2017). From the first description of the disease, by the French dermatologist Ferdinand-Jean Darier, who first coined the term “Pseudoxantoma elasticum” in 1896 (Darier, 1896), it took more than a century to identify the underlying genetic cause, when in year 2000 ABCC6 was independently recognized by different groups as the sole defective gene in PXE, among a set of five-six gene, which were previously mapped within a locus of approximately 500 kb (Ringpfeil et al., 2000; Le Saux et al., 2000; Bergen et al., 2000; Struk et al., 2000).

Nowadays, more than 400 mutations have been identified, mostly single nucleotide changes, leading to a panel of missense, nonsense, splice site and frameshift mutations, in addition to small and large insertions/deletions, which are potentially able to determine two possible scenarios: 1) failure to hydrolize ATP and to transport substrates and 2) abnormal protein folding leading to intracellular retention and/or reduced trafficking (Shimada et al., 2021). 71.5% of the total individual mutations identified consist of two recurrent mutations, that is R1141X and del23–29 (Pfendner et al., 2007).

At the time when the first pathological mutations in ABCC6 gene were described as causing PXE, its physiological substrate was yet to be known - and still remains to be

undoubtedly proven - but together with observations that small amount of mRNA were found in many peripheral tissues, it led to the so-called “PXE cell hypothesis”, which postulated that loss of function of this peripheral ABCC6 could explain the pathogenesis of the disease (Hendig et al, 2008).

However, a second kind of hypothesis was subsequently elaborated, which assumed that PXE would be caused by the absence of an unknown compound secreted by ABCC6 from the liver, where it is mostly expressed, into the circulation, where it counteracts peripheral calcification. Supporters of this “metabolic hypothesis” used *Abcc6* KO mice to prove their theory with some convincing experiments. This model proved particularly useful because mineralization of connective capsule surrounding vibrissae is an early biomarker of mineralization processes (Klement et al., 2005). Jiang et al. used tissue transplantation experiments to test whether local tissue factors contributed to the calcification in Abcc6 −/− mice. When they transplanted muzzle skin of Abcc6 −/− mice onto wild-type mice, the calcification was strongly diminished, while transplantation of the muzzle skin of wild-type mice onto Abcc6 −/− mice resulted in calcification (Jiang et al., 2009). The same authors had previously demonstrated that serum of Abcc6-/- mice had lesser ability to prevent the mineral deposition induced by inorganic phosphate in a cell culture system (Jiang et al., 2007). In another work, by using parabiotic animals, they showed that linking the circulation of a wild-type mouse with that of an Abcc6 −/− mouse, calcification in the PXE mouse was halted, whereas linking two Abcc6 −/− mice had no effect on disease symptoms (Jiang et al., 2010). All together these experiments corroborated the hypothesis that the absence of circulating serum factors due to the loss of ABCC6 activity was more important than local factors in the calcifying tissues. Since the liver contains the bulk of ABCC6 within the body, it should also be the source of that factor, as also suggested by incidental observations on human liver transplantations resulting in full-blown PXE, even though the ABCC6 gene in the recipients was normal (Bercovitch et al., 2011).

But it was not until 2013 that the most likely candidate substrate was identified. Before that date, it was proposed that putative substrate of ABCC6 was vitamin K, secreted as glutathione or glucuronide conjugate, based on the fact that vitamin K is needed for the gamma-carboxylation of glutamate residues in proteins known to be required for counteracting calcification of connective tissue and that PXE-like symptoms can occur in patients with mutations in the gamma-glutamyl carboxylase gene and in rats treated with vitamin K antagonists (Borst et al., 2008). However, such hypothesis proved to be wrong, as

every attempt to prevent calcification in animal models through vitamin K supplementation invariably failed (Gorgels et al., 2011; Jiang et al., 2011; Brampton et al., 2011; Fülöp et al., 2011). Instead, the work of Jansen and colleagues was enlightening in this field. They first overexpressed human and rat ABCC6 in HEK293 and clearly showed that ABCC6-conditioned medium was able to prevent calcification in *in vitro* experiments. Untargeted LC-MS metabolomics analysis showed that a range of nucleotides and nucleotides, such as AMP and GMP were more abundant in supernatant of ABCC6-transfected cells, compared to control. None of these compounds was deemed to be able to prevent mineralization, but AMP is produced from ATP by ectonucleotide pyrophosphatase-phosphodiesterase (ENPP) enzyme (Stefan et al., 2005), leading to inorganic pyrophosphate (iPP) as a byproduct, which is a powerful inhibitor of mineralization (Orriss et al., 2016). iPP was subsequently found in this supernatant using a specific enzymatic assay, in concentration sufficient to prevent mineralization, while it was significantly reduced in plasma of *Abcc6*$^{-/-}$ mice. Finally, in order to prove that nucleoside triphosphates were secreted by ABCC6-containing cells, they incubated cells in the presence of the ectonucleotidase inhibitors ARL67156 and β,γ-methyleneadenosine triphosphate (AMP-PCP), which diminished level of iPP while at the same time raising that of ATP. However, since further vesicular transport assays failed to prove that ATP was transported by ABCC6, the authors concluded that its actual substrate should have been an unidentified factor X that induces the release of nucleotides triphosphate (Jansen et al., 2013). In their following work they rejected their previous factor X hypothesis, ultimately demonstrating that ABCC6 was responsible for ATP efflux with the help of liver perfusion experiments and hepatocytes cultures. This loss of ATP efflux from the liver was the cause of reduced iPP in serum of PXE patients, which they found to be 2,5-fold lower than in healthy people (Jansen et al., 2014). This may seem not a great difference, especially considering that blood levels of iPP are in the range of 1.19-5.65 µM (Russell et al., 1971), several orders of magnitude lower than that of Ca2+ and phosphate, which, on the contrary, do not significantly vary as product (Ca x P) in patients (Hendig et al., 2006), but this is apparently sufficient to cause PXE-related symptoms. Little amount of iPP is usually required to prevent biomineralization, since it does not only antagonize the ability of calcium to crystallize with phosphate to form hydroxyapatite ($Ca_{10}(PO_4)(OH_2)$), but also binds strongly to the surface of hydroxyapatite crystals, thus preventing their nucleation and further growth (Fleisch et al., 1966).

Concomitant conditions and risk factors could integrate with iPP levels, thus partially explaining the extreme variability of PXE phenotype. Fetuin-A is a liver-derived plasma protein which binds amorphous calcium phosphate, forming colloids of "calciprotein particles" (CPP). These CPPs, besides ensuring solubilization of microcrystals of calcium phosphate, also facilitate their rapid clearance by macrophage system (Herrmann et al., 2012). In PXE patients, serum concentrations of Fetuin-A are significantly reduced, even though this could be caused by increased consumption, due to the augmented formation of calcium phosphate micro-precipitates in absence of iPP (Hendig et al., 2006). In fact, overexpression of fetuin-A has been used as a strategy to reduce ectopic mineralization of connective tissue in *Abcc6 −/−* mice (Jiang et al., 2010). Matrix gla protein (MGP) is produced by vascular smooth muscle cells (VSMCs) and effectively prevents arterial calcification by adsorbing to growing hydroxyapatite (HA) crystals, while mice lacking the gene encoding matrix gla protein (MGP) exhibit massive mineral deposition in blood vessels and die soon after birth. γ-carboxylation of specific residues of glutamic acid is essential for MGP activity (O'Young et al., 2011) and is catalyzed by gamma-glutamyl-carboxylase (GGCX) in a vitamin K dependent manner (Schurgers et al., 2013). Mutations in GGCX gene are known to cause PXE-like skin lesions, in addition to deficit of several coagulation factors (Vanakker et al., 2007), while anticoagulant drug Warfarin, inhibitor of vitamin K recycling, recapitulates some of PXE typical vascular features in mice (Li et al., 2013). Another interesting correlation has been found between PXE and β-thalassaemia. This is a genetic disease caused by a reduced or absent synthesis of the β-globin chains of hemoglobin, resulting in excess of α-globin chain precipitating and damaging red blood cells precursors (Origa, 2017). Presence of typical PXE features has been observed in patients with β-thalassaemia and other hemoglobinopathies, such as sickle cell disease, since long time (Aessopos et al., 2002). These are not limited to ocular and skin lesion in the form of angioid streaks and yellow papules, but also involve cardiovascular system (Baccarani-Contri et al., 2001; Cianciulli et al., 2002). The high prevalence of these findings, together with a recent observation of a significant downregulation of ABCC6 in a mouse model of β-thalassaemia (Martin et al., 2011), seems to suggest a tight link between the two conditions, coupled by a common pathomechanism. It has been proposed that the reduced ability to bind iron in the hemoglobinopathy could result in excess of oxidative stress, because of reactive hydroxyl radical ($OH^{\bullet}$) through the Fenton reaction, which can cause a direct damage to the elastic fibers of connective tissues (Voskou et al., 2015). But oxidative stress is also a hallmark of

PXE, as unbalanced reactive oxygen species (ROS) and product of peroxidation were found in both fibroblasts and serum of PXE patients (Pasquali-Ronchetti et al., 2006; Garcia-Fernandez et al., 2008) (Figure 8).

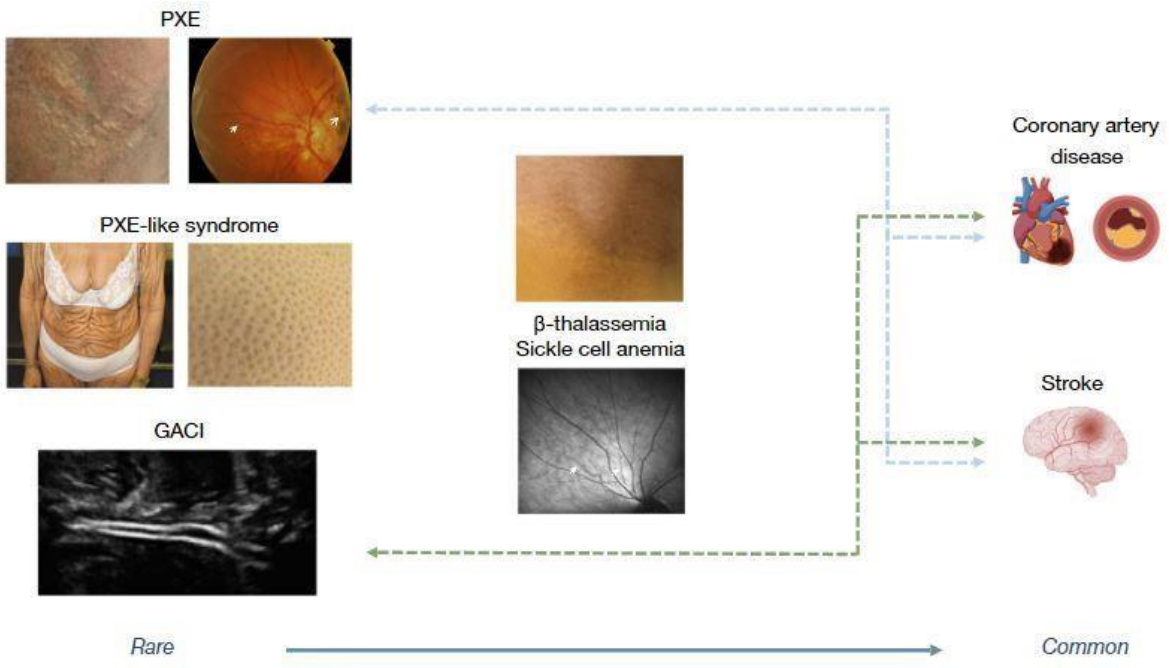


Figure 8: Mendelian and complex ABCC6-related disorders: from rare to common entities. Left panel: cutaneous, ophthalmological, and/orvascular presentation of the Mendelian disorders PXE (papular skin lesions; angioid streaks and hemorrhaging),PXE-like syndrome (excessive skin folds and peau d'orange aspect of the skin) and GACI (excessive hypermineralization of the vessel wallon ultrasound). Middle panel: skin (papular lesions) and eye (angioid streaks) manifestations of a PXE phenocopy in beta-thalassemia. The dotted lines indicate the high prevalence of common ABCC6-related manifestations such as stroke in rare MendelianABCC6 disorders (Verschuere et al., 2020)

It is nowadays widely accepted that the most prominent physiological activity of ABCC6 is to mediate the efflux of ATP from the liver. This fact has important repercussions, not only for iPP homeostasis, but also because this ABCC6-mediated efflux may contribute to the extracellular purinergic signaling, which will be discussed in next section. It is however undoubtful that this ATP is the primary source of serum iPP, but other players than ABCC6 also participate to the fine balance of iPP/iP ratio. The first one is ENPP1 enzyme, a membrane glycoprotein, expressed in several tissues, such as liver, bone, and cartilage (Huang et al., 1994), which hydrolyzes phosphodiester bond of nucleosides triphosphates producing iPP. Loss of this function due to mutations in ENPP1 gene result in generalized arterial calcification of infancy (GACI), a rare early fatal disease characterized by an extensive ectopic calcification and intimal proliferation of arteries, which often results in death in utero and infancy (Rutsch et al., 2001). A distinct but related condition is arterial calcification

due to deficiency of CD73 (ACDC), an autosomal recessive disorder due to loss-of-function of NT5E, the gene encoding CD73, an enzyme with ectonucleotidase activity which converts AMP to adenosine. This is an adult late-onset calcification disturb affecting mostly lower extremities blood vessels and articular joints (St Hilaire et al., 2011). Even though CD73 is not directly involved in production of iPP, *in vitro* studies suggest that reduction in adenosine due to the loss of CD73 function results in gain of tissue-nonspecific alkaline phosphatase (TNAP) activity, which metabolize iPP to inorganic phosphate (Jin et al., 2016). ABCC6 is therefore the upstream modulator of an extracellular purinergic pathway that inhibits mineralization by modulating the iP/iPP ratio in connective tissues through the sequential activity of ENPP1, CD73 and TNAP enzymes. Based on this paradigm, PXE is not an isolated disease but rather part of a spectrum of ectopic mineralization disorders, together with GACI and ACDC, which share some common features (Figure 9). These similarities are even more evident between PXE and GACI, since a minority of GACI patients carries mutations in ABCC6 gene, with no evidence of pathological ENPP1 alleles. These patients show a typical severe phenotype associated with myocardial infarction and death in early infancy, indistinguishable from classical cases of GACI due to mutations of ENPP1, which, in turn can develop clinical signs of PXE, including angioid streaks and calcifications of elastic skin fibers (Nitschke et al., 2012). However, PXE is usually a mild condition compared to GACI since while in absence of ABCC6 serum iPP is only reduced to 40%, probably because other transporters could mediate the efflux of ATP, ENPP1 is the sole responsible to produce iPP.
This is maybe the reason why levels of iPP are so low that they are undetectable in GACI patients and animal models (Nitschke et al., 2018; Lomashvili et al., 2014).

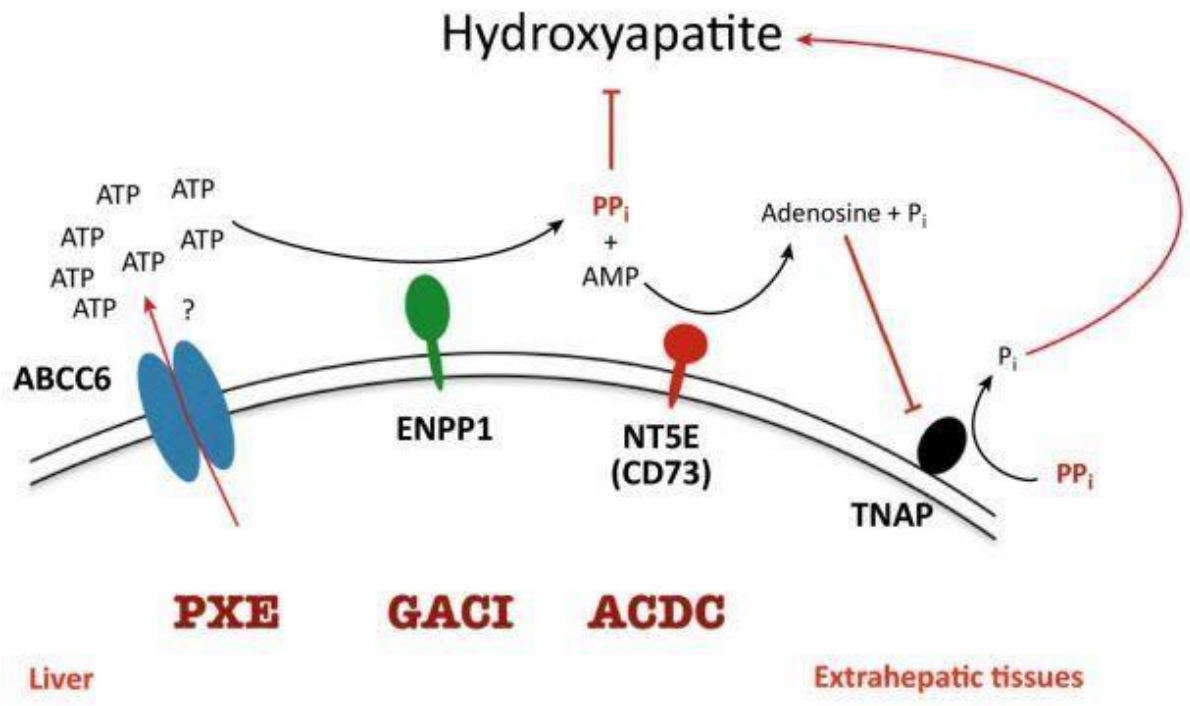


Figure 9: The known major mechanisms regulating plasma pyrophosphate level (Borst et al., 2019)

Currently there are no valid treatment options for PXE and related calcification disorders. The most obvious approach is restriction of dietary minerals (primarily calcium, magnesium, and phosphate) that influence calcification process. Despite earlier suggestions that clinical PXE severity correlates with calcium ingestion (Renie et al, 1984), a high calcium diet did not increase calcification in *Abcc6* $^{-/-}$ mice (Gorgels et al, 2010). Conversely, a diet providing high amount of magnesium, a mineral with inhibitory properties towards calcification (Alfrey et al., 1974), had a positive effect on vascular calcification in animal and *in vitro* models (Gorgels et al., 2010; LaRusso et al., 2009) but clinical trial failed to prove any improvement in the disease from magnesium supplementation (Rose et al., 2019). Worsening of calcification parameters with high dietary phosphate set the rational basis for the use of phosphate binders in PXE (LaRusso et al., 2008). However, clinical trial with sevelamer hydrochloride showed only a rather modest improvement of clinical scores (Yoo et al., 2011). Bisphosphonates are analogs of pyrophosphate with a high affinity for hydroxyapatite, which are incorporated in the site of active bone remodeling, where they inhibit both calcification and reabsorption, and are used in disorder of low bone mass and

high bone turnover (Drake et al., 2008). Since etidronate has been previously used for the treatment of GACI (Huesa et al., 2015), a clinical trial was performed to test safety and effectiveness in PXE. This trial reported no adverse effect from etidronate administration, but efficacy data were somewhat mixed (Kranenburg et al., 2018). Using pyrophosphate to treat PXE was proposed soon after the discovery that mutation of ABCC6 affect iPP production (Jansen et al., 2013), but before that, iPP was already suggested as a therapeutic option for ectopic mineralization (O'Neill et al., 2011). A study on the effect of daily injections of iPP proved it was effective in preventing the development of pseudoxanthoma elasticum-like spontaneous calcification in *Abcc6* $^{-/-}$ mice but failed to reverse already established lesions (Pomozi et al., 2017). However daily injections are not practical for lifelong treatments and the main limitation of directly using iPP as a drug was that it was not considered orally bioavailable. Unexpectedly a recent study demonstrated that not only oral iPP is bioavailable, yet in low amount, but it also effective in attenuating ectopic calcification (Dedinszki et al., 2017). Alkaline phosphatases (ALPs) are a group of isoenzymes with tissue-restricted expression. The TNAP member of the group refers to the isoenzyme encoded by ALPL gene, which is expressed in bone, liver, kidney, and central nervous system. They can hydrolyze or transphosphorilate a wide range of compounds, but only few natural substrates are recognized, such as PPi, pyridoxal 5'-phosphate (PLP), and likely phosphoethanolamine (PEA) (Millán et al., 2016). By converting iPP into iP, TNAP activity is of primary importance in the control of calcification. In fact, loss of this activity is associated with a severe form of hypophosphatasia and rickets (Weiss et al., 1988; Savinov et al., 2015), while use of TNAP inhibitors has been proposed to counteract vascular smooth muscle cell calcification (Narisawa et al., 2007). Recently it has been shown that fibroblasts from PXE patients have an increased TNAP activity. This study is very meaningful, not only because it apparently rejects the hypothesis of liver-derived circulating factor, suggesting as local factors may be at least as important, but also offers a new strategy of treatment for PXE. However, even if TNAP inhibitors reduced calcification *in vitro*, they did not significantly increase iPP levels in *in vivo* experiments, so their role in PXE therapy requires further studies (Ziegler et al., 2017). Gene therapy has also been proposed: Fetuin-A is one of possible candidate genes, since, as previously mentioned, it has anti-mineralization properties and its overexpression in *Abcc6* $^{-/-}$ mice resulted in a significant yet not persistent reduction of ectopic calcification (Jiang et al., 2010). More recently, administration of an adenovirus vector carrying ABCC6 cDNA to PXE mice every 4 weeks was proved to induce high levels of ABCC6 expression in

the liver and normalization of plasma iPP levels. Signs of mineralization were minimal in younger mice, but, as per many of proposed therapies listed here, if ABCC6 gene therapy was administered to older mice, it exerted no effect on existing mineralization (Huang et al., 2019). Ataluren (PTC-124) is a drug that suppresses translation termination at in-frame premature termination codons (PTCs) to restore translation of a full-length, functional polypeptide, which has been previously used in diseases caused by non-sense mutations as Duchenne muscular dystrophy and cystic fibrosis (Keeling et al., 2011). In a zebrafish model of PXE, PTC-124 managed to rescue the expression of functional ABCC6, thus making a promising strategy yet limited to cases carrying non-sense mutations (Zhou et al., 2013). Sodium phenylbutyrate (4-PBA) is an aromatic fatty acid normally used to treat urea cycle disorders and thalassemia which shows several biological activities. One of these is acting as a pharmacological chaperone (Iannitti et al., 2011). For such reason, it has been used for the treatment of diseases caused by improper translocation of proteins to the plasma membrane, including other ABC transporters (Rubenstein & Zeitlin, 2000; Sorrenson et al., 2013). 4-PBA has also been repurposed in PXE, demonstrating its ability to rescue expression and functionality of ABCC6 in *in vitro* and *in vivo* models but, as per PTC-124, it is very likely that its activity is allele-specific (Le saux et al., 2011; Pomozi et al., 2017) (Figure 10).

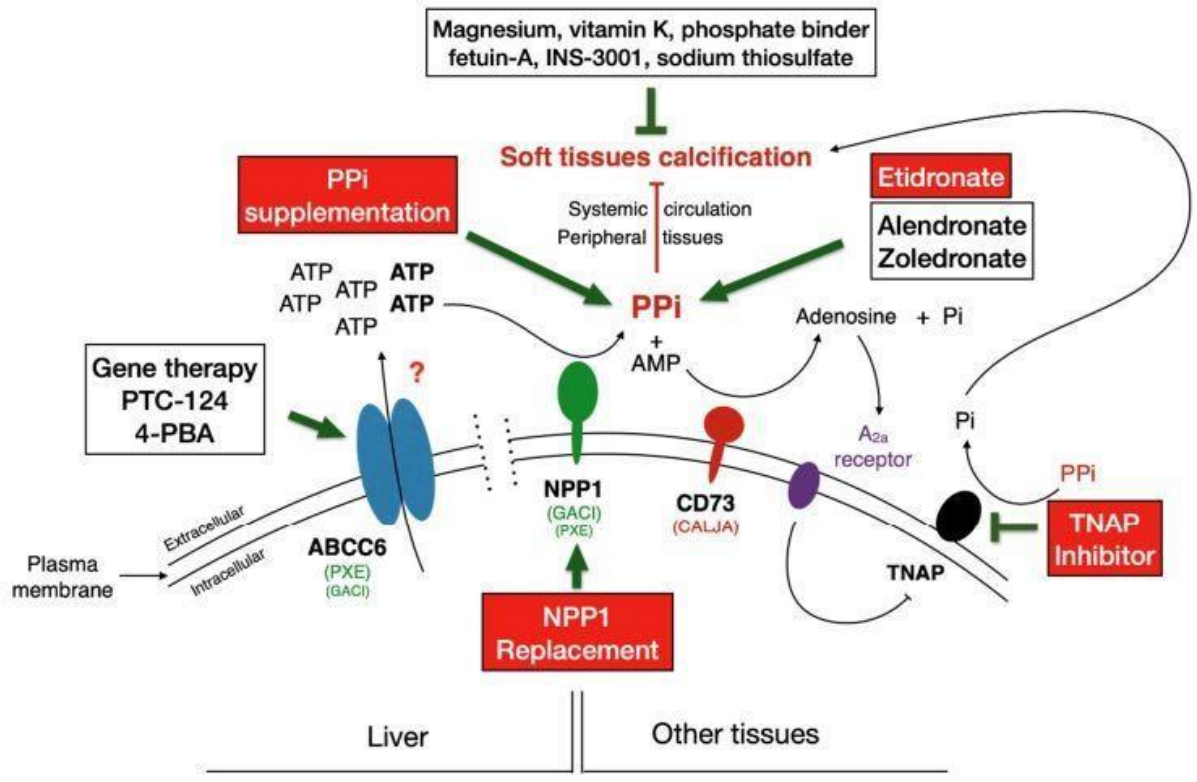


Figure 10: Tested therapeutic Interventions targeting different steps in the ABCC6 pathway to prevent calcification in PXE/GACI. (Shimada et al., 2021)

Here there were presented multiple therapeutic approaches which aim to correct abnormalities in mineralization processes caused by loss of ABCC6 function as a mediator of iPP efflux from the liver. However, as more and more evidences are emerging of the role of ABCC6 in several other functions, as lipid homeostasis (Brampton et al., 2021; Ibold et al., 2021), inflammation (Mention et al., 2018), senescence (Miglionico et al., 2017; Tiemann et al., 2020) and extracellulare purine metabolism (Kauffenstein et al., 2018), it is becoming evident that ABCC6 itself could be considered in some cases a pharmacological target and great interest is arousing from studying its contribution on mechanisms of autocrine and paracrine cell interactions.

Purinergic system

Purinergic system plays a major role in the extracellular signaling, in both pathological and physiological conditions. First evidence of adenine nucleotides functioning as signaling molecules date back to the work of Drury and Szent-Györgi in 1929 (Drury & Szent-Györgyi, 1929), but in fact it was not until the early 70's that the concept of ATP as a signaling molecule was proposed by G. Burnstock (Burnstock, 2006). Nowadays it has been widely accepted that not only ATP, but also other purine and pyrimidine nucleotides (ADP, adenosine, UTP, UDP), may act as cellular messengers and two classes of receptors have been cloned, namely P1 and P2. The first class is sensitive to adenosine and includes four G-protein coupled receptors (GPCRs): A1R and A3R are mainly coupled to the Gi/o subunit and thus inhibit adenylate cyclase and cAMP production; A2AR and A2BR are mainly coupled to the Gs subunit and stimulate cAMP synthesis through adenylate cyclase activation (Borea et al., 2018). The second class of receptors is in turn divided in two subfamilies: the first one (P2X) is made of ligand-gated cation channels, named P2X1-7, whose activation can promote rapid depolarization associated with Ca+2 and Na+ influx, and K+ efflux (Coddou et al., 2011); the second one (P2Y) is composed of eight GPCRs, which can bind different types of G-proteins and couple to ion channels. ATP is the natural ligand of all P2XRs and can also activate P2Y2 and P2Y11 receptors (Abbracchio et al., 2006). Purine receptors are widely expressed in a variety of cell types. In fact, purinergic signaling does not only participate to a multitude of physiological processes, like neurotransmission, sensory transmission, hormone secretion and specialized function of different organs and of the immune system, but also to

pathological conditions, like pain, nervous system diseases, infections, inflammation, and cancer.

Intracellular nucleotides can be released through a variety of mechanisms. In condition of cell stress nucleotides can simply leak out of damaged membrane and act as a danger signal, which attract inflammatory cells to the site of damage (Trautmann, 2009). In a more specific manner, nucleotides can be released from intact cells via exocytosis of cytosolic granules or through specific channels and transporters. Connexins and pannexins are families of protein which assembly to form hexameric membrane hemichannels that mediate extracellular release of small molecules. These channels are opened following multiple stimuli, including intracellular calcium increase, plasma membrane depolarization, activation of the P2X7R, redox potential changes, and mechanical stress. The P2X7 receptor itself can form a large membrane pore allowing passage of molecules up to 900 Da. Channels belonging to the calcium homeostasis modulator (CALHM) family have been recently claimed as important contributors to extracellular ATP release. In addition, Maxi-anion channels (MACs) are voltage-dependent, large-conductance, ATP-permeable anion channels, which are activated by different stimuli, including hypoxia, high glucose, and osmotic swelling (Giuliani et al., 2019). ABC transporters members like the P-glycoprotein have been claimed to actively transport ATP across plasmatic membrane (Abraham et al., 1993).

ATP and adenosine are key component in the context of the Tumor Microenvironment (TME) as signal molecules but, due to the low concentration and the rapid dynamic of their removal from the extracellular space, measuring their concentration has been for long time challenging. Moreover, concentration of ATP is not uniform throughout the interstitium, since cells are surrounded by a halo of higher concentration within the unstirred layer closer to the cell surface (Beigi et al., 1999). Recently, by using a chimeric luciferase probe linked to the plasmatic membrane, it has been demonstrated that ATP concentration is in the low nanomolar in the healthy tissues, while it increases to the micromolar range in the site of inflammation, tissue damage and in the TME (Falzoni et al., 2013; Pellegatti et al., 2005).

Concentration of adenosine is normally low as well, but it can rapidly increase up to 100-fold in hypoxic tissues, in response to inflammation and in the TME. The main source of adenosine in the TME is the hydrolysis of ATP by ecto-nucleoside triphosphate diphosphohydrolases (CD39) enzyme into AMP, which is in turn converted in adenosine by ecto-5-nucleotidase (CD73). CD39 and CD73 are thus the key regulators of adenosine

production in the TME. CD39 is mainly expressed in endothelial cells and subsets of leukocytes, while CD73, which is the rate-limiting enzyme in the production of adenosine, is present of lymphocytes, endothelial cells and epithelial cells and has also been found overexpressed in several types of cancers (Stagg & Smyth, 2010) (Figure 11).

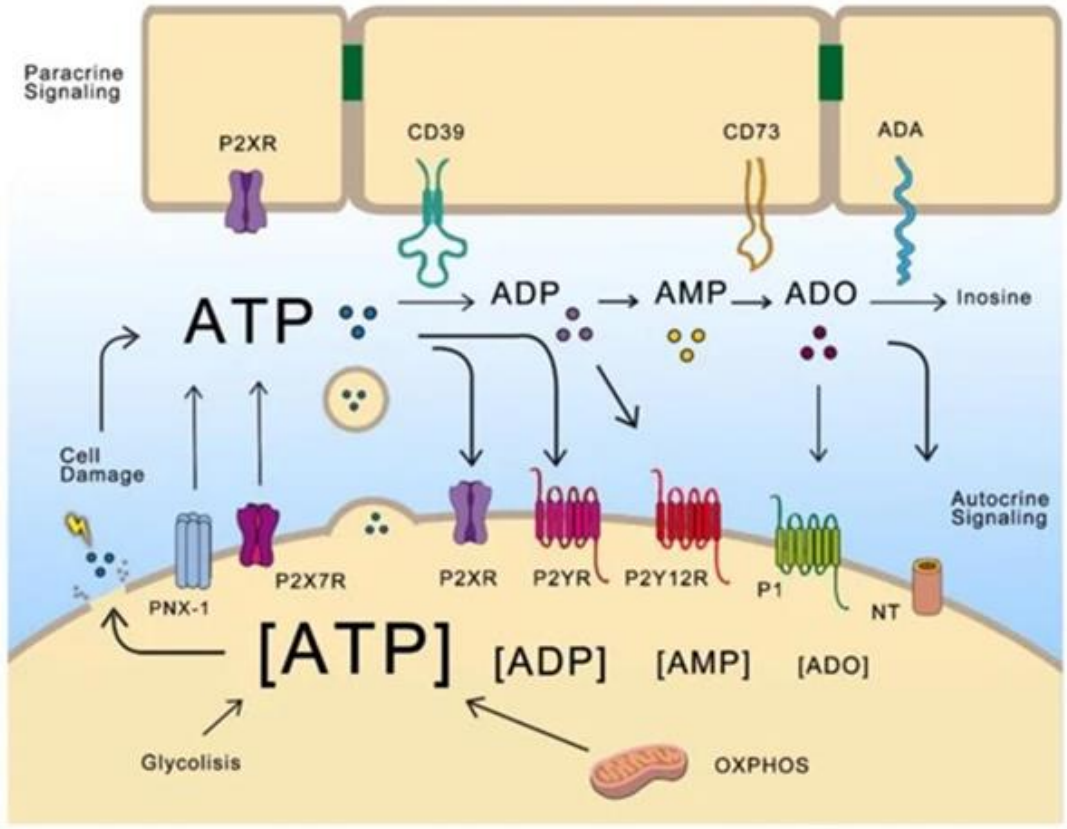


Figure 11: Nucleotides as autocrine and paracrine messengers. (Campos-Contreras et al., 2020)

It is nowadays accepted that both ATP and adenosine play a key role in the TME, by exerting a pleiotropic effect, on both tumor and host cells. An extensive literature has been produced on purinergic signaling in cancer, which is way too large to be reported here in details and it would be even beyond the scope of this introduction. However, trying to give just a few hints, it is worth to say that the activity of ATP and adenosine on tumor immunity is often depicted like yin and yang, with the first activating and the second suppressing the immune response to cancer (Feng et al., 2020).

In the highly hypoxic and acid environment of the tumor, a variety of cells with immunosuppressive activity are recruited, such as tumour-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), regulatory T cells (Treg), while activity of cells

with the potential to kill cancer cells is reduced. In such context, ATP acts as a damage-associated molecular pattern, recruiting neutrophils, macrophages, and dendritic cells (DCs) contributing to damage resolution. ATP drives the activation of the NLRP3 proinflammatory inflammasome pathway in M1s (Mariathasan et al., 2006) pro-inflammation and anti-tumor subtype, which secretes TNFα and has a low level of ecto-nucleotidase activity (Zanin et al., 2012), and improves maturation, migration and antigen presenting ability of DCs (Paustian et al., 2013; Ben Addi et al., 2010). It also regulates T cells activation, cytokine production and lymphocyte metabolism (Zhao et al., 2016; Borges da Silva et al., 2018). These effects are mainly mediated by P2X7 receptor. Indeed tumors growing in P2X7 null mice showed a decrease in CD8+ cells and an increased number of Tregs, overexpressing the fitness markers OX40, PD-1, and CD73, while systemic administration of selective P2X7 blocker increased CD4+ effector cells and decreased their expression of CD39 and CD73 (De Marchi et al., 2019).

On the contrary, adenosine acts as an important negative regulator of T cells functions, by reducing their mobility, migration, and adhesion. In fact, A2AR activation on T CD4+ cells promotes their differentiation to Treg cells and facilitates their suppressive function (Ohta et al., 2014), while its inhibition enhances T CD8+ function and reduces the population of Treg within the tumor (Ma et al., 2017). In DCs which express A1R, A2AR and A3R, adenosine has significant effects on activation, maturation, and cytokine production. Adenosine-differentiated DCs have impaired allostimulatory activity and express high levels of immune suppressor and tolerogenic factors (Bergamin et al., 2015; Cekic et al, 2012). On macrophages adenosine promotes the alternative polarization to M2, which, differently from M1, produce IL-10 (Csóka et al., 2012; Koscsó et al., 2013). In addition, adenosine can regulate the function of a subpopulation of regulatory B cells (Vijayamahantesh et al., 2016) and inhibit the lytic activity of natural killer (NK) towards tumor cells (Young et al., 2018).

The second main function of purine nucleotides in the context of TME is acting as an autocrine-paracrine signal, linking the release of extracellular ATP from cancerous cells (eATP) with the subsequent activation of cell proliferation and tumor growth. However, the overall activity of these signals depends on the presence and diversity of specific receptors in cells releasing the nucleotide and neighboring ones. The best characterized among purine receptors is P2X7R, which is known to be an apoptosis inducer (Fu et al., 2009; Coutinho-Silva et al., 1999). However, many reports are there supporting a role of P2X7R in proliferation and/or survival, so that DiVirgilio's group proposed that P2X7R can act as a

growth-promoting receptor, on the basis of multiple evidences: 1) P2X7R exogenous expression in several cell lines incremented proliferation; 2) the TME contains high amounts of ATP (hundreds of µM) to activate P2X7R; 3) various cancerous tissues from different organs show high expression levels of P2X7R; and 4) P2X7R is a positive regulator of aerobic glycolysis (Campos-Contreras et al., 2020; Di Virgilio et al., 2009). Among adenosine receptors, ADOA2bR is the best characterized. It has been found overexpressed in tumors deriving from many organs, where it seems to function as proliferation promoter (Ma et al., 2010; Kasama et al., 2015; Zhou et al., 2017). In addition, extensive evidence indicates that purinergic signaling participates to the modulation of EMT, which drives tumor invasiveness, by directy regulating EMT-related genes (Qiu et al., 2014), by regulating ERK (Li et al., 2015) or AKT pathway (Xia et al., 2015), in a mechanism involving Ca^{2+}-activated SK3 potassium channels (Jelassi et al., 2011) etc. P2X7R and P2Y2R are involved more often, but involvement of other receptors has been described too (Figure 12).

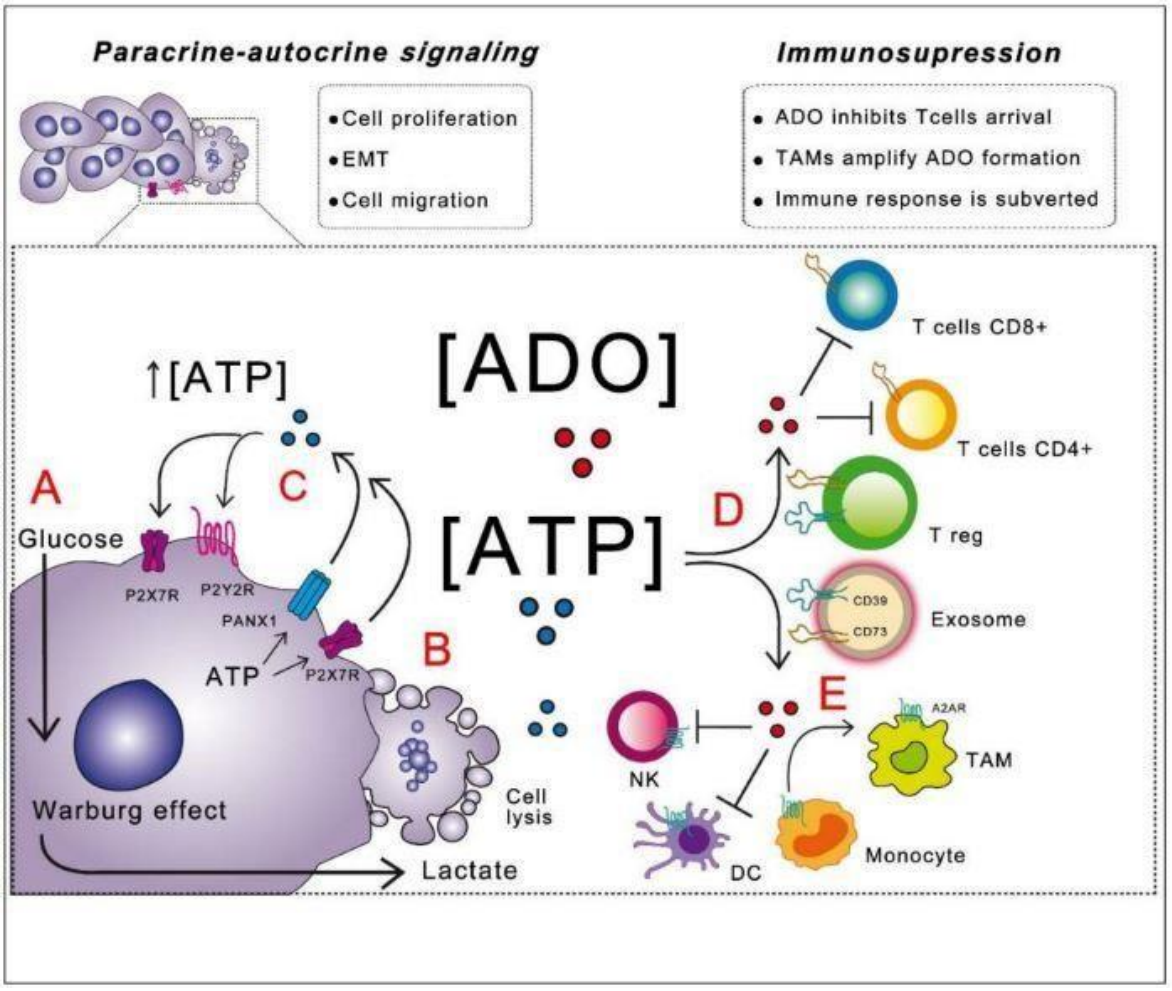


Figure 12: Purinergic signaling and tumor microenvironment (TME). (Campos-Contreras et al., 2020)

Regenerative Medicine and stem cells

According to a recent definition, Regenerative Medicine is an "*interdisciplinary field of research and clinical application focused on repair, replacement or regeneration of cells, tissues or organs to restore impaired function resulting from any cause*" (Mason & Dunnill, 2008). Restoration of health status is the main aim of Medicine and during the past hundread years, a plethora of different medical interventions have been described, reflecting some yet rudimental knowledge of our innate ability to regenerate. We can find a notable allegory of human regenerative capacity in the myth of Prometheus: the Titan God stole fire from Olympus and donated to humanity, and as consequence he was chained to a rock, with an eagle feeding on his own liver every night. Notably, Prometheus' liver regenerated the day after, just to endure the same fate day by day. Ancient Greeks must have been aware of the liver's capability to regenerate, as they used to call the liver *ἧπαρ* (hepar), after *ἠπάομαι*, which means "to repair oneself" (Steinhoff, 2011).

Few centuries later, we coined the term Regenerative Medicine to describe a set of methods and resources from various and heterogeneous fields, such as transplants, biomaterials, scaffolds, drug therapies and many others. However, there is no doubt that a large part of Regenerative Medicine research revolves around the use of stem cells (SCs). These cells are peculiar components endowed with two unique features: self-renewal and multi-potency. The latter is the ability to differenciate into different cell types, while the former is the property to divide symmetrically or asymmetrically to generate one or two identical cells. Self-renewal, a feature shared with progenitor cells, characterized by restricted potential, is essential for stem cell pool to be maintained within adult tissues, even in the event of injury. Multiple mechanisms contribute to the regulation of self-renewal at cellular and molecular level. A unique transcriptional program and cell cycle regulation allows stem cells to prevent differentiation and rapidly proliferate (He et al., 2009). DNA damage accumulates over time as a consequence of aging, exposure to chemicals and physical mutagens and oxidative stress, while stem cells maintain their genome integrity thanks to efficient DNA repair mechanisms and higher resistance to ROS molecules. Moreover, stem cells characteristically express telomerase, which enable them to unlimited replication and maintained telomere length (Sharpless & DePinho, 2007). External microenvironment is equally important in regulation of function and maintenance of stem cell properties. Signals originated by the interaction between extracellular matrix and cells,

or the activity of soluble factors secreted within specialized niches, preserve stem cell multi-potency and proliferative potential (Morrison & Spradling, 2008).

Stem cells can be classified according to their potency and origin. Totipotent cells have the capacity to mature into any cells or tissues, originating a complete organism. Totipotency is restricted to fertilized oocyte, the zygote, which gives rise to both the placenta and the embryo (Condic, 2014). Few days after fertilization, the zygote give rise to the morula, which further develop into the blastocystis. The cells of the inner cell mass (ICM) are known as embryonic stem cells (ESCs) and have been isolated and implemented in several ex vivo studies. ESCs are pluripotent, they can originate all three germ layers and retain their characteristics when cultured *in vitro* (Thomson et al., 1998; Evans et al., 1981). Extraembryonic tissues, like outer layers of the placenta, originate from the trophoblast, which establish in a former stage of morula and is required to support embryo/fetus growth for the nine months of human pregnancy (De Miguel et al., 2010). In later stage, during gastrulation, an involution forms within the epiblast and this event initiates the differentiation process, with the formation of the three germ layers: the ectoderm, which gives rise to epidermis, the nervous system, and to the neural crest; the endoderm, which gives rise to the epithelium of the digestive system and respiratory system, and organs associated with the digestive system, such as the liver and pancreas; the mesoderm, which gives rise to many cell types such as muscle, bone, and connective tissue. At this stage it is believed that the embryo possesses three types of SCs specific for each germ layer. These cells are multipotent, as they have a narrower potency, compared to pluripotent stem cells (PSCs), restricted to specific tissues deriving from the same germ layer (Ratajczak et al., 2007). A typical example of multipotent stem cells which can be found in adults is mesenchymal stem cells (MSCs), which can differentiate into mesoderm-derived tissues (Augello et al, 2010) (Figure 13). At the lowest levels of this hierarchy there are oligopotent and monopotent stem cells, which can originate one or few lineages within the same tissue, such as myeloid and lymphoid lineages deriving from bone marrow-resident hematopoietic stem cells (HSCs).

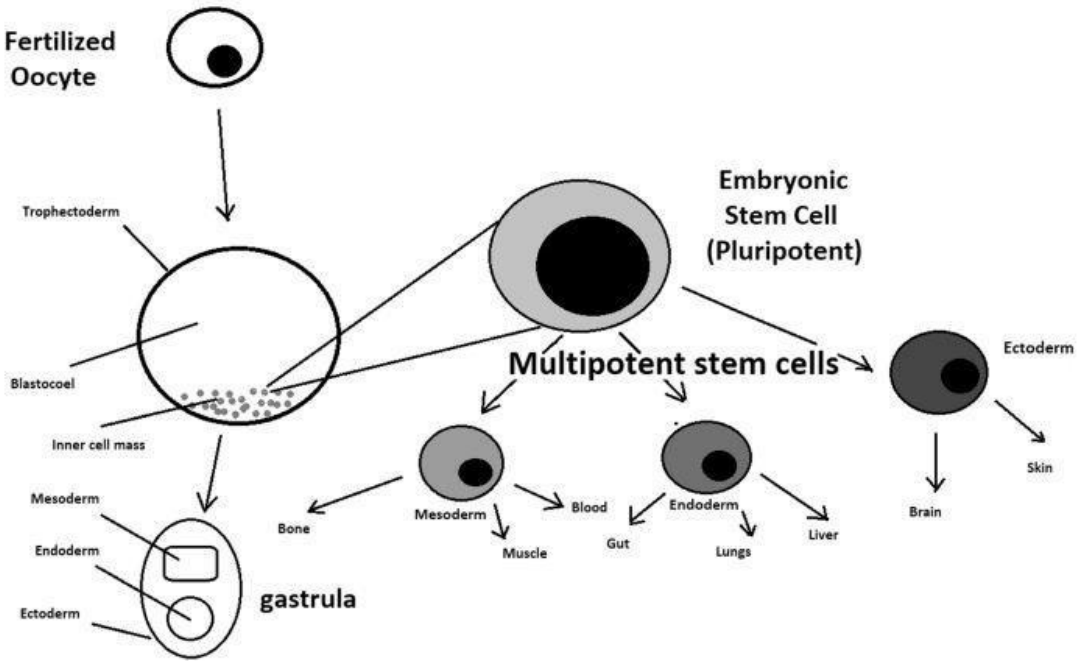


Figure 13: Embryonic origin of stem cells. (Zakrzewski et al., 2019)

From the historical point of view, HSCs have been the most characterized and well-established among various types of stem cells in cellular therapy. Transplantations of bone marrow containing HSCs have been used for more than 60 years to treat hematological malignancies (Thomas et al., 1959). Although first attempts failed due to the poor knowledge of human leukocyte antigen (HLA) genes, it is nowadays a very common procedure, with more than 50.000 bone marrow tranplants performed annually worldwide.

MSCs are multipotent cells and can be isolated from various tissues, including bone marrow, adipose tissue, umbilical cord and dental pulp. Their most prominent propert is immunomodulatory activity (Hass et al., 2011). Their clinical use has been studied for almost 30 years (Lazarus et al., 1995), and the number of clinical applications involving MSCs has grown exponentially in the last few years. During these studies, therapeutic administration of MSCs has proven to be well tolerated and initially used in prevention of graft-versus-host disease (GVHD). Since then, multiple applications have been proposed, reporting beneficial effects in various conditions, including: regeneration of damaged myocardium following ischemic events; neurological disorders caused by inflammatory demyelinating disease of the central nervous system (CNS) or spinal cord injury (SCI); bone and cartilage diseases, like osteogenesis imperfecta or osteoarthritis; diseases cause by injuries to the liver, kidneys or

lung; chronic inflammatory and autoimmune diseases, such as lupus erythematosus or Crohn's disease. The therapeutic potential of MSCs has been ascribed to 1) differentiation into various cell lineages, 2) secretion of soluble factors crucial for cell survival and proliferation, 3) modulation of immune response, and 4) migration to the site of injury. However, despite the promise and expectancies regarding the clinical implementation of MSC-based therapies, several pitfalls limit their application, such as donor heterogeneity and the lack of standardized procedures for *ex vivo* expansion, both limiting greatly MSC properties (Wang et al., 2020; Squillaro et al., 2016).

One of the first clinical trials involving therapeutic use of fetal tissues reported a dramatic improvement of symptoms after transplanting fetal substantia nigra into the caudate nucleus of two Parkinson disease (PD) patients (Madrazo et al., 1988). Positive outcomes from this study, together with similar results from different groups and the discover of human ESCs (Thomson et al., 1998), encouraged researchers worldwide to apply these approaches to the treatment of PD. At the same time, transplantation of fetal tissues and ESCs raised ethical controversies worldwide, which led goverments to introduce some restrictions in the use of fetal material and ESCs for therapeutic and research purposes (Ishii & Eto, 2014; McHugh, 2004).

The afore described issues served as a boost for alternative sources of pluripotent stem cells, which led to the successful reprogramming of somatic cells into pluripotent cells using somatic cell nuclear transfer (SCNT) (Tachibana et al., 2013). SCNT technique became notorious to the public many years earlier, instrumental for the clonation of Dolly the sheep (Campbell et al., 1996). Later on, it allowed Yamanaka and colleagues' pioneeristic work to induce pluripotency into mouse fetal and adult fibroblasts (Takahashi & Yamanaka, 2006), and human cells (Takahashi et al., 2007). By forcing expression of four factors, later called Yamanaka factors (Oct3/4, Sox2, Klf4, and cMyc), somatic fibroblasts can be reprogrammed in induced pluripotent stem cells (iPSCs). Similar results were achieved by another group, by using a different combination of factors (OCT4, SOX2, NANOG, and LIN28) (Yu et al., 2007).

However, many challenges still need to be solved to grant safety for the treatment of human diseases by pluripotent stem cells (Yamanaka, 2020). The first risk is tumorigenicity: PSCs have the potential of indefinite number of replications, a double-edge sword, eventually leading to teratoma. The expression of the four factors is sufficient by itself to induce tumors as some of them are powerful oncogene (Okita et al., 2007). Indeed, tumors may arise from proliferation of differentiated progeny (Nakamura & Okano, 2013) or

incorrect or incomplete patterning (Malchenko et al., 2014). Several strategies have been proposed to minimize such risk, including differentiating cells at high level of purification before transplantation (Souied et al., 2017; Kikuchi et al., 2017). This may be achieaved by positive selection (cell sorting) or removing undifferentiated cells (negative selection) (Sougawa et al., 2018). I*n vitro* expansion inevitably causes genetic alterations. The most relevant alteration is chromosomal abnormality and can be easily monitored by kariotiping (Amps et al., 2011). More difficult to evaluate and controversial in their significance are single nucleotide variation (SNV) and copy number variation (CNV), which require next-generation sequencing to be detected. These alterations appear in PSCs at higher rate compared to somatic cells (Rouhani et al., 2016) and often accumulate in cancer-related gene (Merkle et al., 2017).

The second challenge is represented by immunogenicity. Although some researchers reported iPSCs failing to induce teratoma when subcutaneously transplanted into mice, with signs of rejection (Zhao et al., 2011), this was likely an isolated case as other groups did not measure any evidence of immunogenicity after transplantation of autologous iPSCs (Araki et al., 2013; Guha et al., 2013; Souied et al., 2017). Autologous transplantation of iPSCs could offer a remarkable advantage in term of immune acceptance. However, due to the monetary and time cost, production of autologous iPSCs is unpractical and unreliable yet. Two kinds of strategies have been proposed to overcome the need of immunosuppression associated with allogeneic PSC transplants. First, matching HLA haplotipes, like it is currently and widely occurring in bone marrow transplantation. Indeed, collaboration with biobanks may generate HLA-matched iPSCs (Umekage et al., 2019). The second strategy is called “HLA cloaking” and consists in the inactivation of all class I MHC using CRISPR technology to delete their common component beta 2-microglobluin (B2M) gene and in the introduction of a chimeric molecule consisting of HLA-E and B2M. Such strategy prevents lytic activity of NK cells, which would normally target any cell lacking HLA-I antigens (Lanza et al., 2019). Finally, the last challenge with both ESC and iPSC, is cell heterogeneity, in terms of gene expression and differentiation potential (Osafune et al., 2008; Kajiwara et al., 2012). Such limit could be circumvented when background differences are erased by overexpressing specific transcription factors, turning “primed” PSCs into a naïve state (Takashima et al., 2014). Alternatively, a combination of chemical inhibitors for growth factors have been proposed (Theunissen et al., 2014).

Besides ESCs, adult stem cells (ASC) or iPSCs, another source of stem cells is represented by perinatal cells. Perinatal SC can be isolated from placenta and fetal annexes. Placenta is a complex and temporary organ, composed of fetal and maternal components. Placenta is required for the development of the fetus and ceases to function around 40th week of pregnancy. Perinatal tissues perform several tasks, as supplying fetus with nutrients, removing metabolic products, and acting as a selective barrier to protect the fetus from pathogens, xenobiotics and maternal hormones and immune system. As a source of stem cells, placenta has some unique feature: perinatal cells have been less exposed to infection and DNA damage, but most importantly, do not raise ethical issues, because can be obtained from placentae at full term pregnancies, commonly discarded. Placenta can be divided into three sections: the fetal-derived amnion and chorion, and the maternal-origin decidua. The amnion is the innermost layer, whose primordial cells, the amnioblasts, differentiate from the epiblasts prior to grastrulation. The inner sheet of amniotic membrane is constituted of a single-cell epithelium layer, whose cells, called amniotic epithelial cells (AECs), derive from the division of amnioblast, which do not belong to any germ layer, and for this reason are supposed to maintain memory of pluripotency (Torre & Flores, 2020; Qiu et al., 2020) (Figure 14). AECs are now being considered a valuable source of stem cells thanks to a unique combination of features.

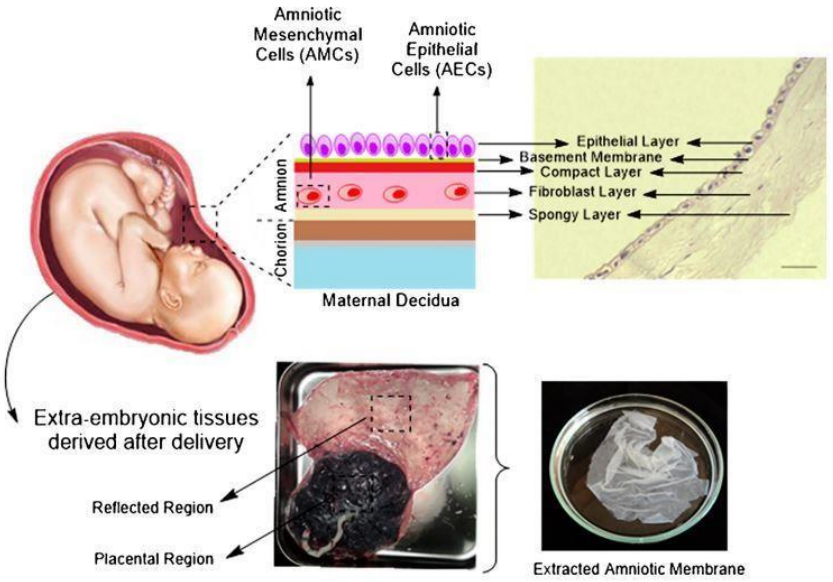


Figure 14: Amniotic membrane is the nearest layer of the fetal membranes to the fetus. It consists in five layers: epithelium (containing amniotic epithelial cells, AECs), basement membrane, compact layer, fibroblast layer (containing amniotic mesenchymal cells, AMCs) and spongy layer (adjacent to the chorion). Extra-embryonic tissues include the placental disk and fetal membranes. After peeling off the amniotic membrane from the chorionic membrane, the amniotic epithelial and mesenchymal cells are extracted by using enzymatic digestion from two placental and reflected regions of amniotic membrane (Niknejad et al., 2016)

Of note, more than 100 million AECs can be easily harvested from one term human amnion, which can be further expanded, allowing sufficient cell supply for clinical use (Miki et al., 2005). A standardized protocol to generate clinical grade AECs, according to current good manufacturing practices (cGMP) has been described (Gramignoli et al., 2016). According to their origin, AECs express ESC-associated pluripotency markers, such as OCT-4, NANOG, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-80, selectively lost *in vitro* (Miki et al., 2005). However, the most convincing evidence supporting the hypothesys of AEC pluripotency features were described upon injection in mouse blastocyst, forming chimeric mouse embryoid bodies in which AEC were distributed among all three germ layers (Tamagawa et al., 2004). Furthermore, AEC are considered quite safe compared to stem cells from other sources, as they proved to be non-toxic and, most important, non tumorigenic (Yang et al., 2018). This absence of tumorigenic activity is at least in part ascribed to the lack of telomerase expression, which limits AEC proliferative capacity (Miki et al., 2005). AECs have also a very low immunogenicity. This is quite intuitive, given amnion role in protecting the fetus from maternal immunity, confirmed by direct implantation into volunteers and animals without any sign of rejection (Kubo et al., 2001; Akle et al. 1981). Low immunogenicity was initially attributed to the fact that AECs do not express HLA-I (HLA-A, -B, and -C and β2 microglobulin) or HLA-II molecules (Ackle et al., 1981). Nowadays it is accepted that AECs do express classical HLA molecules at low levels and that their peculiar immunoprivileged properties should be attributed, at least in part, to the expression of non-polymorphic antigens, such as HLA-G (Strom & Gramignoli, 2016; Hammer et al., 1997). AECs show outstanding anti-inflammant properties. They suppress lymphocytes responsiveness (Liu et al, 2012; Bailo et al., 2004), inhibit cytotoxic activity of NK cells and monocytes, promote maturation of regulatory T cells (Treg) (Tan et al., 2015; Li et al., 2015), and improve phagocytic activity of neutrophils. At the same time, AEC attenuate oxidative burst capacity, protecting fetus from both microbial treats and oxidative stress (Alipour et al., 2020). These properties result from AEC-derived cytokines, including: 1) apoptosis inducers, as tumor necrosis factor-α (TNF-α), Fas ligand (FasL), and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (Li et al., 2005); 2) macrophage migration inhibitors, such as transforming growth factor-β (TGF-β) and macrophage migration-inhibitory factor (MIF) (Liu et al., 2012; Li et al., 2005); 3) antiangiogenic and anti-inflammatory proteins, including interleukin-1 receptor antagonist (IL-1ra), interleukin-10 (IL-10), tissue inhibitors of metalloproteinase (TIMPs) (Li et al., 2015; Hao et al., 2000). Finally, AEC-derived exosome

promotes migration and proliferation of fibroblasts, with opposite effects on wound healing and fibrosis (Zhao et al., 2017). Such unique combination of properties makes AEC promising in a boad variety of conditions, including neurodegenerative diseases, autoimmune disorders, fibrosis, and wound healing.

Safety of amnion cells in humans has been proved once implanted in patients with lysosomal storage disease (Sakuragawa et al., 1992) or Niemann-Pick disease type B (Bembi et al., 1992). More recently, AECs proved to be well tolerated and to slightly improve respiratory function in a phase I clinical trial premature infants with bronchopulmonary dysplasia (Lim et al., 2018), with no evidence of tumor formation during a 2-year follow-up (Malhotra et al., 2020).

So far, I have tried to roughly depict the role of stem cells in regenerative medicine, which is not an easy task, given the complexity of this topic and the speed of progress in this field. However, the spectrum of possible applications of stem cells is not limited to a cell-based approach of medical interventions, as they proved to be also a valuable tool for disease modeling and drug discovery screening. Thanks to the advances in reprogramming of primary cells into iPSC and differentiation processes into a panel of different cell types, together with the feasibility of genetic manipulation using any of the multiple technologies currently available, from gene knockdown with RNA interference to genomic editing tools, such as zinc-finger nucleases, TALENs, and the CRISPR/Cas9 systems, stem cells have provided a variety of cell models for human biology and disease studies (Musunuru, 2013). At the same time, iPSCs are renewable and scalable sysyems, which is particularly convenient for high throughput drug screening and toxicological studies. For these purposes, iPSCs offer many advantages over traditional models as they can recapitulate diseases which do not have any clear mouse counterpart. Moreover, they can be used a substitute of primary cells which are difficult to culture and to obtain from human donors, and for which standard culture cell lines do not exist. This concept has been widely adopted in several fields, including research into diabetes (Kharat et al., 2021), cancer (Kim, 2015), neurodegenerative diseases (Bonaventura et al., 2021) and more, but where the potential of stem cells appeared to be more outstanding is perhaps cardiotoxicity assessment (Stella Stoter et al., 2020). This is a potentially letal event, one of the first causes of drugs withdrawal from the market. Several preclinical assays are approved by EMA and FDA for testing cardiotoxicity. However, ethical concerns have been raised with the use of animal models, which accounts for their restricted use. The fact that adult human cardiomyocytes

can only be obtained through biopsy or explanted hearts, and are extremely difficult to culture, in addition to the fact heart lacks stem/progenitor cells (Li et al., 2018), pose significative limitations to such tests. In this context, PSC could be considered as the only source of human cardiomyocytes. Studies on iPSC–derived cardiomyocytes (hiPSC-CM) allowed to investigate, for instance, the mechanisms of cardiac toxicity for tyrosine kinase inhibitors (Sharma et al., 2017) or anthracyclines (Burridge et al., 2016). Cardiotoxicity often arises due to drug-induced electrical perturbation of the cell interfering with its contractile function. Even though stem cells-derived cardiomyocytes are not fully mature, they still recapitulate some aspects of phenotype required to produce specific forms of toxicity (Denninget al., 2016). Different is the case of primary hepatocytes, which are difficult to source, costly, functionally variable, undergo severe stress during the isolation process and lose key functions when cultured, yet they are still considered the gold-standard single cell model of drug-induced liver injury (Hewitt et al., 2007). Hepatocytes exhibit hundreds of specific functions, far more than any other cell type within the human body, and stem cell-derived hepatocyte-like cells are not mature enough to emulate all of hepatic functions, resembling fetal phenotype rather than adult (Baxter et al., 2015). Nevertheless, some studied clearly showed that generating fully mature hepatocytes-like cells (HLCs) is not always mandatory, as even the limited grade of differentiation which can be obtained with current protocols may be sufficient. PSC-derived HLCs can still express hepato-specific cytochrome P450 (CYP) metabolizing enzymes and tranporter proteins, leading to HLCs useful in drug screening and toxicological studies (Takayama et al., 2014; Ulvestad et al., 2013).

Aim of the thesis:

The overall aim of this thesis was to investigate potential roles of ABCC6 transporter, as a biomarker and a potential target in cancer treatment, as well as to evaluate the feasibility of adopting some innovative tools of regenerative medicine as a substitute of primary cells culture to study the effect ABCC6 inhibition in normal cells.

The specific aims dealt in each section were:

- To evaluate the effect of ABCC6 gene silencing and pharmacological inhibition with organic anions transport inhibitor Probenecid on ATP efflux, cytoskeleton rearrangement and migration rate in liver cancer-derived HepG2 cell line and breast cancer-derived MDA-MB-231 (Paper I)
- To evaluate the effect of ABC transporters inhibitor natural flavonoid quercetin on ABCC6 expression and cell pathways involved in control of cell motility in HepG2 cells (Paper II)
- To evaluate the role of ABCC6 in the modulation of purinergic system and cell pathways involved in cell migration of colon cancer-derived Caco2 and HT29 cell lines (Paper III)
- To study the effect of Quercetin and Probenecid on hepatocytes-like cells generated from AECs (experience at Karolinska Institutet, Sweden)

Materials and Methods

Mammalian cell lines cultures

Human hepatocarcinoma cells HepG2, human triple-negative breast cancer MDA-MB-231, human colorectal adenocarcinoma cell lines Caco2 and HT29 were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Thermo Fisher Scientific; Waltham, MA) containing 4.5 g/L glucose, supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, penicillin (100 U/mL), and streptomycin (100 µg/mL) at 37°C, in a humidified atmosphere with 5% CO^2. Cells were passaged 2 or 3 times a week by trypsinization and maintained in exponential growth throughout the experiments. Cells were detached with Trypsin-EDTA solution 0,025% and centrifuged to discard Trypsin (optional for HepG2 and MDA-MB-231) at each passage.

All chemicals were bought from Sigma (St. Louis; MO). Unless otherwise specified, chemicals were dissolved in Dimethyl sulfoxide (DMSO) as a stock solution. Final concentration of DMSO never exceeded 0,5% in culture medium. ATP was dissolved in water.

RNA extraction and cDNA synthesys

In Paper I, II and III RNA extraction was performed using Quick-RNA™ MiniPrep kit (Zymo Research; Irvine, CA), while purification was obtained through various steps of centrifugation on filter columns, according to manifacturer's protocol. RNA concentration and purity was measured spectrophotometrically by reading absorbance at 260, nm using a Multiskan™ GO Microplate Spectrophotometer (Thermo Fisher Scientific). Absorbance ratio at 260/280 and 260/230 nm were used as indicators of contamination by proteins of phenols respectively. Throughout the work in Dr. Gramignoli's lab at Karolinska Institutet RNA isolation was performed through traditional phenol/chloroform method using TRIzol™ reagent (Thermo Fisher Scientific) accordind to manifacturer's instruction, followed by RNA precipitation and purification with ethanol and sodium acetate.

cDNA synthesis was carried out on 1 µg of template RNA with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystem; Waltham, MA), on a peqSTAR 96 Universal Thermo Cycler (EuroClone; Siziano, PV, Italy), using the program suggested by the producer of the kit.

Real time PCR

In Paper I, II and III amplification and relative quantification of cDNA templates was performed through Real Time PCR with a 7500 Fast Real-Time PCR System (Applied Biosystems) using iTaq— Universal-SYBR® Green Supermix (Bio-Rad; Hercules, CA). Primers were designed to span exon-exon regions to avoid unwanted genomic DNA amplification and PCR products were subjected to a melting-curve analysis to confirm amplification specificity. The comparative threshold cycle method (ΔΔCt) was used to quantify relative amounts of product transcripts with β-actin as the endogenous gene reference. Here is reported a list of primers sequenced used in this thesis (Table 2).

Gene	Accession Number	Forward Primer	Reverse Primer
β-ACTIN	NM_001101.3	5'-CCTGGCACCCAGCACAAT-3'	5'-GCCGATCCACACGGAGTACT-3'
ABCC1	NM_004996.4	5'-GCTGATGGAGGCTGACAAGG-3'	5'-GATGCTGAGGAAGGAGATGAAGAG-3'
ABCC2	NM_000392.5	5'-CCCTTGTCCTGGAAGATGTT-3'	5'-AGAGCCTTCATCAACCAGG-3'
ABCC3	NM_001144070.2	5'-CCACACCACAACCACCTTCAC-3'	5'-CTCGGCGTCCAGCACATTG-3'
ABCC4	NM_005845.5	5'-GCACACCAGGATTTACATTCAGAG-3'	5'-CCAGACGGACGGCAAACC-3'
ABCC5	NM_005688	5'-CCACCATCCACGCCTACAATAAAG-3'	5'-ACAGCCAGCCACCGCATC-3'
ABCC6	NM_001171.5	5'-AAGGAACCACCATCAGGAGGAG-3'	5'-ACCAGCGACACAGAGAAGAGG-3'
ABCG2	NM_001348985	5'-ATCACTGATCCTTCCATCTTG-3'	5'-GCTTAGACATCCTTTTCAGG-3'
NT5E	NM_002526	5'-GGGCGGAAGGTTCCTGTAG-3'	5'-GAGGAGCCATCCAGATAGACA-3'
ENPP1	NM_006208.3	5'-CCGTGGACAGAAATGACAGTTTC-3'	5'-ATGGACAGGACTAAGAGGAATTCTAAA-3'
TNAP	NM_000478.6	5'-TACAAGCACTCCCACTTCATCTG-3'	5'-GCTCGAAGAGACCCAATAGGTAGT-3'
P2RY1	NM_002563.5	5'-TGTGGTGGTGGCGATCTCC-3'	5'-TCGCAGGTACTCGTCTGAGG-3'
P2RY2	NM_176072.3	5'-TCAGCATTGTGTTCTTTGGG-3'	5'-CTGGGAAATCTCAAGGACTG-3'
P2RY14	NM_001081455.2	5'-TCAGCATTGTGTTCTTTGGG-3'	5'-TGCTGTAACTCACTGACTGG-3'
ADORA2A	NM_001278497.2	5'-AACCTGCAGAACGTCACCAA-3'	5'-GTCACCAAGCCATTGTACCG-3'
ADORA3	NM_000677.4	5'-GTCAGATACAAGAGGGTCAC-3'	5'-GTCAGTTTCATGTTCCAGCC-3'
ADORA2B	NM_000676.4	5'-GAGACACAGGACGCGCTGTACG-3'	5'-CGGGTCCCCGTGACCAAACT-3'

Table 2 Primers used in reverse transcription-quantitative polymerase chain reaction assay

Throughout the work in Dr. Gramignoli's lab at Karolinska Institutet RT-PCR experiments were conducted using TaqMan™ Universal PCR Master Mix (Applied Biosystem). Each assay was performed using specific predesihned TaqMan probes bought from Thermofisher Scientific.

Western Blot

Identification and relative quantification of cellular proteins was performed through western blot analysis. For cytosol proteins analysis cell pellets were suspended in Radioimmunoprecipitation assay (RIPA) buffer (0.1% sodium dodecyl sulfate, 1% NP-40, and

0.5% sodium deoxycholate in PBS at pH 7.4) supplemented with a protease and phosphatase inhibitor cocktail (Roche, Penzberg, Germany) and lysed by sonication. Cell lysate was then centrifuged at 13000 rpm at 4°C and supernatant was collected. Total protein content in samples was determined spectrophotometrically by staining with Coomassie Brilliant Blue G-250 according to Bradford method and measuring absorbance at 595 nm. For membrane proteins (ABCC6) analysis, cell pellets were directly suspended in Laemmli sample buffer (60 mM Tris–HCl pH 6.8, 10% glycerol, 2% SDS, 1% β-mercaptoethanol, and 0.002% bromophenol blue) and sonicated. Proteins were resolved by SDS-PAGE (sodium dodecyl sulfate - polyacrylamide gel electrophoresis) and transferred on nitrocellulose membranes Amersham™ Protran® (GE Healthcare; Chicago, IL). After 1 hour incubation with proper blocking solution, bovine serum albumin (BSA) 5% or nonfat dried milk (NFDM) 5% in PBS or TBS with 0,1% of Tween-20, membranes were incubated overnidght at 4°C with primary antibodies: 1:400 anti-β-actin A5441 (Sigma-Aldrich); 1:50 MRP6 Monoclonal Antibody M6II-31 (Invitrogen; Waltham, MA); 1:500 anti-CD73 monoclonal 1D7 (Invitrogen); 1:1000 anti-α-tubulin T9026 (Sigma-Aldrich); 1:1000 anti-AKT (pan) C67E7 (Cell Signaling; Danvers, MA); 1:2000 anti-phospho-AKT (Ser 473) D9E (Cell Signaling); 1 µg/mL anti-ERK1/2 3HCLC (Thermofisher Scientific); 1 µg/mL anti-phospho-ERK 1/2 (Thr185, Tyr187); (Thermofisher Scientific). The membranes were washed three times with PBST or TBST and incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h, and signal were visualized by the ECL™ Western Blotting Detection Reagents (GE Healthcare) or the SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Scientific), using the Chemidoc TM XRS detection system equipped with Image Lab Software for image acquisition (BioRad). Densitometric analysis of immunoreactive bands was performed by using GelAnalizer 2010 software (Debrecen, Hungary).

Generation of stable ABCC6 knockdown cells

To silence ABCC6 expression in HepG2 cells we used shRNA (short hairpin) technology. Plasmid vectors carrying shRNA sequences for ABCC6 were packaged into lentiviral particle, which allow permanent silencing through the integration of specific short hairpins sequences into the host cell genome. Lentiviral particles were bought from Vectorbuilder Inc (Chicago, IL) and custom designed through their host software on their website. Given the length of ABCC6 transcript, three shRNA sequences binding different regions were required to reach adequate knockdown score. Sequences used were:

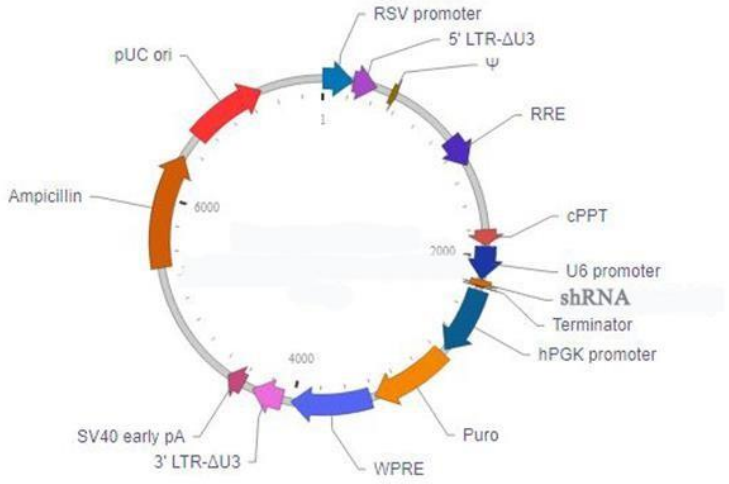
- shRNA 1: AGATCGAGTTCCGGGACTTTG (exon 26)
- shRNA 2: CAACAAGGCAATAGCATTTAA (exon 6)
- shRNA 3: TCCCTGCCTCCACAGAATAAA (exon 14)

As a negative control of gene silencing, scrambled non-inhibiting shRNA was used with following sequence:

- Scramble shRNA: CCTAAGGTTAAGTCGCCCTCG

The enhanced green fluorescent protein (EGFP) was used as a reporter gene to control infection efficiency, while a gene of resistance to the antibiotic Puromycin was included into the vector to allow clonal selection (Figure 15).

a



b

Name	Description
RSV promoter	Rous sarcoma virus enhancer/promoter
Δ5' LTR	Truncated HIV-1 5' long terminal repeat
Ψ	HIV-1 packaging signal
RRE	HIV-1 Rev response element
cPPT	Central Polypurine tract
U6 Promoter	Human U6 small nuclear 1 promoter
hABCC6[shRNA]	shRNA
Terminator	Pol III transcription terminator

Name	Description
hPGK promoter	Human phosphoglycerate kinase 1 promoter
EGFP:2TA:puro	EGFP and Puro linked by T2A
WPRE	Woodchuck hepatitis virus posttranscriptional regulatory element
ΔU3/3' LTR	Truncated HIV-1 3' long terminal repeat
SV40 early pA	Simian virus 40 early polyadenylation signal
Ampicillin	Ampicillin resistance gene
pUC ori	pUC origin of replication

Figure 15: (**a**) Map and (**b**) list of components of lentiviral vectors used. Custom components listed in red

Briefly, $1{,}5x10^5$ HepG2 cells were seeded on each well of a 12 well plate. After 24 h a suspension of lentiviral particles at a suitable multiplicity of infection (MOI) of 10 containing ABCC6-shRNA or scrambled-shRNA plasmid vectors was added to the cells. The day after medium was changed with fresh one and cells let grow for further 24 h. Then cells were

treated for 12 days with puromycin 2 µg/mL to remove non-infected cells and single clones were collected using cloning cylinders in 200 mm plates and expanded in multi-well plates. Level of silencing was evaluated by RT-PCR and confirmed by western blot and clones with at least 75% silencing were used for further analysis.

Extracellular ATP bioluminescence assay

Measurement of extracellular ATP in cell culture medium was performed using the ATP Bioluminescence Assay Kit CLS II (Roche), according to the manufacturer's protocol. $1.5x10^5$ HepG2 cells or $1x10^5$ MDA-MB-231 cells were seeded into each well of a 12 well plate and after 24 h, the culture medium was removed, cells were washed with PBS and complete DMEM medium without Phenol Red (Sigma-Aldrich), in presence or absence of probenecid 250 µM, was added. After 48 h, 100 µL of the culture medium was gently collected in ice-cold sterile tubes, centrifugated at 1200 rpm for 5 min at 4°C to precipitate cell debris, and ATP in the supernatant was measured after dilution 1:10 in sterile distilled ATP-free water and the addition of the luciferin/luciferase reagent. Luminescence was immediately determined through a Glomax luminometer (Promega, Madison, WI) with an integration time of 0.5 s. ATP concentrations were calculated by interpolating luminescence values into a calibration curve obtained with serial dilution of a known standard. ATP levels were then normalized per micrograms of proteins. Resulted were presented as a percentage of normalized ATP content of the control cells medium.

Confocal fluorescence microscopy

Possible variation in organization of the actin filaments of the cytoskeleton were analyzed through confocal microscopy, by staining cells with a fluorescent conjugate of phalloidin, a natural toxin found in *Amanita Phalloides* mushrooms which selectively binds filamentous actin with high affinity, preventing its depolimerization. $1,5x10^5$ HepG2 or $1x10^5$ MDA-MB-231 cells were seeded on coverslips and treated with 250 µM probenecid (in some of the experiments a 500 µM of ATP or 100 µM adenosine was added to the culture medium). Cells were fixed in 4% PFA for 10 min at room temperature, washed three times with PBS, permeabilized with 0.5% v/v Triton X-100 in PBS for 5 min, washed three times with PBS, and blocked by saturation buffer (1% bovine serum albumin in PBS) for 20 min. Then, cells were incubated with Phalloidin Alexa Fluor 488 (ex./em.: 495/518 nm, Invitrogen), which gives a green fluorescence, diluted 1:1000 in saturation buffer for 1h in

the dark and washed three times with PBS. In experiments with silenced cells, Phalloidin Alexa Fluor 568 (ex./em.: 578/600 nm, Invitrogen) was used, because emission spectrum is not overlapping with that of EGFP, at a dilution of 1:500. Where indicated, the nuclei were stained with 1.5 µM of propidium iodide (ex./em.: 535/617 nm, Invitrogen) in PBS for 10 min. Pictures were taken with a confocal fluorescence microscope (Leica TSC-SP2 HCX PL APO, x63/1.32–0.60 oil objective) and acquired using the Leica Confocal Software W (Leica Microsystem, Wetzlar, Germany).

Migration assay

Collective migration rate was assessed through the *in vitro* scratch test. 1* 10^6 cells were seeded in a 6-well plate and cultured in DMEM containing 10% FBS and let grow until they reached a nearly confluent cell monolayer. Cells were there treated with either 250 µM Probenecid or 165 µM presence or absence of 500 µM ATP or 400 µM adenosine for 12 h in DMEM containing 10% FBS. As control, cells treated with vehicle alone (DMSO) were used. Then, a linear wound was generated in the cellular monolayer using a sterile 10 µL plastic pipette tip. Any cellular debris was removed by washing with PBS, and the medium replaced with low FBS (1%), still keeping treatments with test molecules. Pictures of the scratch were taken every 12 h by using a Nikon Eclipse TS 100 inverted microscope equipped with a Nikon Coolpix P6000 digital camera (objective magnification 10x) (Minato, Tokyo, Japan). In Paper I, comparison between ABCC6-shRNA and scramble-shRNA cells was made in the same way, without the addition of Probenecid and need of DMSO in control cells. The scratch area was measured by the free processing software Icy image. The relative difference in the scratch area filled during observation time, expressed in arbitrary surface area units, between treatments groups, was used to assess their effect on cells migration.

Intracellular Reactive Oxygen Species (ROS) assay

In Paper II, the effect of Quercetin on intracellular oxidative stress of Hepg2 cells was assessed through the 2',7'-dichlorofluorescein diacetate (DCFH-DA) assay. This is a non-fluorescent cell-permeable compound which is rapidly deacetylated by cytosolic esterases into the dichlorodihydrofluorescein (DCFH) carboxylate anion, which is retained inside the cells and in turn oxidized into the fluorescent dichlorofluorescein (DCF) in the presence of ROS (ex./em.: 495/520 nm). $1x10^4$ cells were seeded in each well of a 96-well black polystyrene plate with clear bottom. The day after, cells were treated with progressive

dilutions of Quercetin for 24 h and then incubated with dichlorofluoresceindiacetate (DCFH-DA) at a final concentration of 10 µM in PBS for 30' at 37°C. Fuorescence was measured by using a GloMax MultiDetection System (Promega) equipped with a blue filter (ex.:490 nm; em.: 510–570 nm). As a positive control of ROS presence, a 2 h treatment with 500 µM H_2O_2 prior to DCFH-DA addition was used. Results are presented as a percentage of the fluorescence of negative control (cells treated with the vehicle DMSO only).

Viability assay

In Paper II and III, the effect of Quercetin or Probenecid was assessed through the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolim bromide (MTT) assay. The assay is based on enzymatic conversion of MTT into a purple-colored formazan compound which absorbs light at 570 nm. This is a NAD(P)H-dependent reaction which is catalyzed by oxidoreductase member succinate dehydrogenase, whose activity is dependent on mitochondrial respiration. 2 * 10^4 cells were seeded in each well of a 96-well polystyrene clear plate. The day after, cells were treated with progressive dilutions of Quercetin or Probenecid for 24 or 48 h, and then incubated with fresh medium containing 0.75 mg/mL MTT for 4 h at 37°C. The formazan crystals were finally dissolved for 1 h at room temperature on agitation in a mixture 1:1 of DMSO and isopropanol with 1% of Triton X-100. Absorbance was measured using a microplate reader Multiskan ^{TM} GO (Thermofisher Scientific) The viability of cells was assessed by comparing the light absorbance at 570 nm, after subtraction of the background at 630 nm of treated and control cells (treated only with vehicle DMSO), defined as 100% cell viability.

Viability of freshly isolated AEC cells was assessed by Trypan Blues exclusion test. The assay is based on the ability of Trypan Blue to enter cells through damaged membranes, allowing selective staining of dead cells with compromised membrane, upon binding to intracellular proteins. Immediately after isolation, an aliquot of cells suspension was mixed with an equal volume of 0,4% (w/v) trypan blue in PBS. Viable (unstained) and dead (blue) cells were counted with the aid of a hemocytometer and viability was obtained by dividing the number of viable cells per that of total count.

Amniotic epithelial cells isolation

Isolation of amniotic epithelial cells was performed according to current Good Manufacturing Procedures (Gramignoli at al., 2016). Discarded placentae from

uncomplicated cesarean resections (preferred to natural birth due to sterility concerns) at 37–42 week of gestational age were obtained from Karolinska University Hospital (Stockholm, Sweden) upon the approval by the Institutional Review Board. Briefly, amnion membrane was surgically removed from the inner surface of placenta and washed with saline solution to remove blood clots. The amnion was then digested for 30' at 37°C with TrypLE™ (Thermofisher Scientific) or 0,5% Trypsin (Thermofisher Scientific) to release epithelial cells. Cells suspension was washed two times in saline solution to get rid of remaining enzyme and digested tissue and filtered through a 100 µm sterile filter to separate aggregates. A second digestion (optional) may be performed if the yield is very low (usually less than 10^7 /g of wet tissue). Viability was assessed on an aliquot of cells suspension prior to further experiments by Trypan Blue exclusion assay. Standard culture medium for AECs consisted of EpiLife™ (Thermofisher Scientific), supplemented with eparin-free pooled platelet lysate Stemulate® (Sexton Biotechnologies, Indianapolis, IN), 1 mM nonessential amino acids (NEAA), 2 mM GlutaMAX , 1x Antibiotic/Antimycotic (all from Thermofisher Scientific) and 55 µM 2-mercaptoethanol (2-ME) (Sigma-Aldrich).

In vitro hepatocellular differentiation of AECs

Cell culture was maintained at 37°C in a humidified atmosphere with 5% CO2. 1x10^6 of viable freshly isolated or cryopreserved amniotic epithelial cells (AECs) were seeded on collagen-treated 12 well plates. 3x10^6 AECs were suspended into 750 µl of matrigel (MG) or decellularized extracellular matrix (dECM) and seeded on 12 well plates. AECs were grown for few days till 100% confluence in proliferative standard medium supplemented with 10 ng/ml epidermal growth factor (EGF). Then cells were cultured for 14 days with hepatocytes differentiative medium based on DMEM/F12 (Thermofisher Scientific) supplemented with HGF 20 ng/ml hepatocytes growth factor (HGF), 1% NEAA, 1% Glutamax, 0,5% antibiotic/antimycotic, 1% DMSO, 5% Stemulate®, renewed every 48 or 72 hours. After that, cells were cultured for further 5 days with addition of 30 µg/ml Gentamicin sulfate, 15 ng/ml Amphotericin-B , 0,1% Dexamethasone, 0,1% Hydrocortisone, 0,1% Insulin Transferrin Selenium (ITS) Supplement), 0,1% Ascorbic acid, 1% lipid mixture (all from Lonza; Basilea, Sweitzerland) for hepatic maturation. The last 3 days 10 µM Rifampicin and 25 µM β-Naphthoflavone (Sigma) were added to culture medium to induce hepatic enzymes. Control undifferentiated cells were kept in standard maintenance medium throughout the protocol.

Phase I metabolism: ethoxyresorufin-O-deethylase (EROD) assay

Phase I metabolic activity was assessed through the ethoxyresorufin-O-deethylase (EROD) assay. The assay is based on enzymatic conversion of non-fluorescent 7-ethoxyresorufin into fluorescent resorufin (ex./em.: 572/584 nm). Hepatocyte-like cells were incubated for 60' at 37°C with 20 µM 7-ethoxyresorufin (Sigma-Aldrich) in William's E Medium without phenol red (Thermofisher Scientific) in presence of 1,5 mM salicylamide (Sigma-Aldrich) to prevent conjugation of the product, and fluorescence in 100 µl aliquots of culture medium was meausured in black non-treated polystyrene microplate, by using a CLARIOstar Plus microplate reader (BMG LABTECH; Ortenberg, Germany), at ex/em wavelength bands of 545-20/600-40. Results were expressed in Relative Fluorescence Units (RFU) and presented as the fold change of fluorescence compared to non-metabolized 7-ethoxyresorufin working solution.

Phase II metabolism: resorufin conjugation assay

Phase II metabolic activity was assessed by resorufin conjugation assay. The assay is based on conjugation of resorufin by Uridine 5'-diphospho-glucuronosyltransferase (UDP-glucuronosyltransferase, UGT) microsomal enzymes and consequent loss of fluorescence. Hepatocyte-like cells were incubated with 50 ng/ml resorufin in Hank's Balanced Salt Solution (HBSS) for 30' at 37°C. Resorufin fluorescence was measure immediately on aliquots of working solution as time 0 and after incubation time, as above described. Results were expressed in Relative Fluorescence Units (RFU) and presented as a percentage of fluorescence of resorufin working solution.

Statistical analysis

Each experiment was repeated three times at least. Otherwise specified, results were expressed as the mean ± the standard error of the mean (sem). Statistical significance was assessed by Student's T Test or ANOVA with appropriate correction for multiple comparisons. $p<0.05$ was chosen as the minimum level of significance. Data were analyzed using GraphPad Prism software version 8.4.2. (GraphPad; San Diego, CA).

Results and Discussion

ABCC6 gene silencing and pharmacological inhibition affects cytoskeleton and motility of Hepg2 cells via Purinergic Pathway (Paper I)

The role of ABCC6 in the pathogenesis of PXE has been extensively studied. From the first evidences that mutations in the ABCC6 gene are the genetic cause leading to PXE (Le Saux et al., 2000), and the demonstration that ABCC6-mediated release of ATP from the liver is the main source of circulating mineralization inhibitor PPi (Jansen et al., 2014), the exact mechanism linking the transport activity to the ectopic mineralization processes has been undoubtedly recognized. However, a growing number of data seem to suggest that ABCC6 could be involved in pathogenesis of cancer, besides of its canonical role of drug resistance transporter. In fact, by supplying ATP in the Tumor Microenvironment (TME), its expression in tumor cells could directly influence those processes which are controlled by extracellular purinergic system, which ultimately have repercussions on tumor progression. Moreover, previous experiments conducted in our lab showed that ABCC6 knockdown in hepatocellular carcinoma-derived Hepg2 cells leads to a senescent phenotype and a dysregulation in genes involved in mineralization (Miglionico et al., 2017; Miglionico et al., 2014). One of such gene, whose expression was significantly downregulated, was CD73, which is also considered a potential target in cancer therapy. CD73 was in fact found to be overexpressed in many kinds of tumors, thus contributing to the promotion of tumor growth, metastasis, angiogenesis, immune suppression and drug resistance (Gao et al., 2014). Furthermore, same results on CD73 expression were obtained in HepG2 cells after treatment with Probenecid (Martinelli et al., 2018), a well-known uricosuric drug and unspecific ABCC6 inhibitor (Iliás et al., 2002).

In this first work we aimed to study the contribution of ABCC6-mediated ATP efflux to cytoskeleton rearrangement in cancer cells. As a part of a strategy to prevent the dissemination solid tumors, targeting cell migration is a promising approach to reduce cancer invasiveness. Cell movement is a very complex phenomenon, driven by a finely coordinated rearrangement of actin filaments in the cytoskeleton. During this process some tight cell-cell junctions are lost, while new interactions with extracellular matrix are formed and contractile force is generated by polymerization of actomyosin filaments. At the same time cells undergo a dramatic change in shape, with the formation of protrusive structures at the leading hedges of motile cells, while at the opposite extremity, the body is retracting.

These structures, called lamellipodia and filopodia, are generated by the polymerization of filamentous actin and further organization in tight bundles (filopodia) or cross-woven webs (lamellipodia), in which filaments are oriented at a certain angle with respect to the direction of movement (Olson et al., 2009; Yilmaz et al., 2009).

In this study, we focused on two different models of cancer: the aforementioned HepG2 cell line and the triple negative breast cancer MDA-MB-231 cell line, as a model of extra hepatic tumor. As shown in figure 16, ABCC6 expression in these cells was negligible compared to the formers, while NT5E was considerably higher. We also silenced ABCC6 expression in HepG2 cells by using shRNA technology. After infection with custom lentiviral particles carrying ABCC6-specific shRNA sequences and a 12-day clonal selection with puromycin, we isolated those clones with at least 80% reduction of ABCC6 mRNA and we used them for further experiments, after silencing of ABCC6 protein was confirmed by western blot (Figure 16).

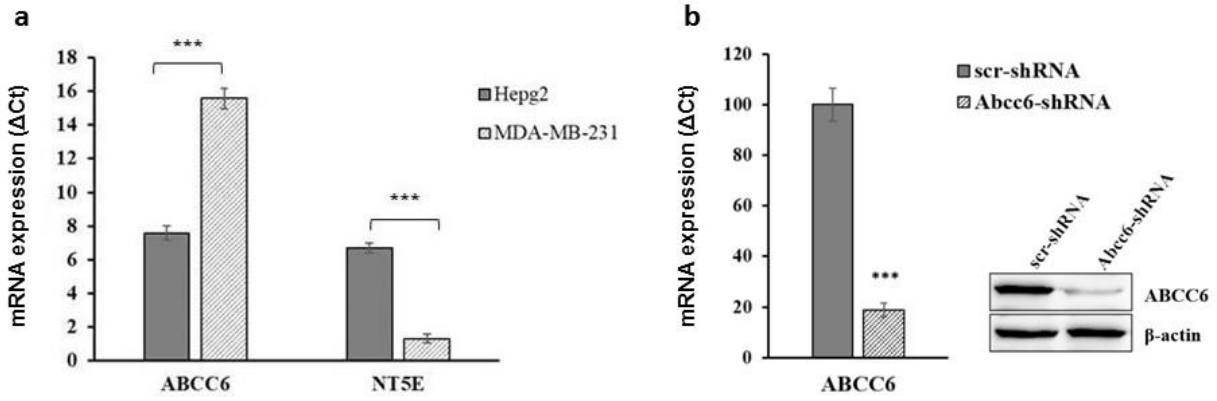


Figure 16: (**a**) ABCC6 and NT5E gene expression in HepG2 and MDA-MB-231 cells. mRNA levels were normalizes to β-actin as a reference gene. (**b**) Densitometric analysis and representative western blot of ABCC6 protein in ABCC6-shRNA and control scrambled-shRNA HepG2 cells. Protein levels were normalized β-actin content. Results are expressed as the mean standard error (ES) of at least three different experiments. *** $p < 0.001$

In order to assess the effect of ABCC6 knockdown or pharmacological inhibition on cytokeleton we performed a series of experiments, in confocal microscopy, by staining actin filaments with a fluorescent phalloidin conjugate. In the first subset of experiments we evaluated the effect of ABCC6 silencing on HepG2 cells. As shown in Figure 17a, control Scrambled shRNA HepG2 cells exhibit many filopodia, while these structures are almost completely absent in ABCC6 knockdown cells. However, when ATP 500 µM or Adenosine 100

µM is added to the culture medium, normal architecture of filopodia is restored. In the next subset of experiments, we showed how treatment with Probenecid roughly mimicked the effect of ABCC6 silencing in HepG2 cells, since it almost completely abolished filopodia, compared to control cells treated with vehicle alone (Figure 17b). On the contrary, no effect of Probenecid treatment was found on MDA-MB-231 cytoskeleton (Figure 17c).

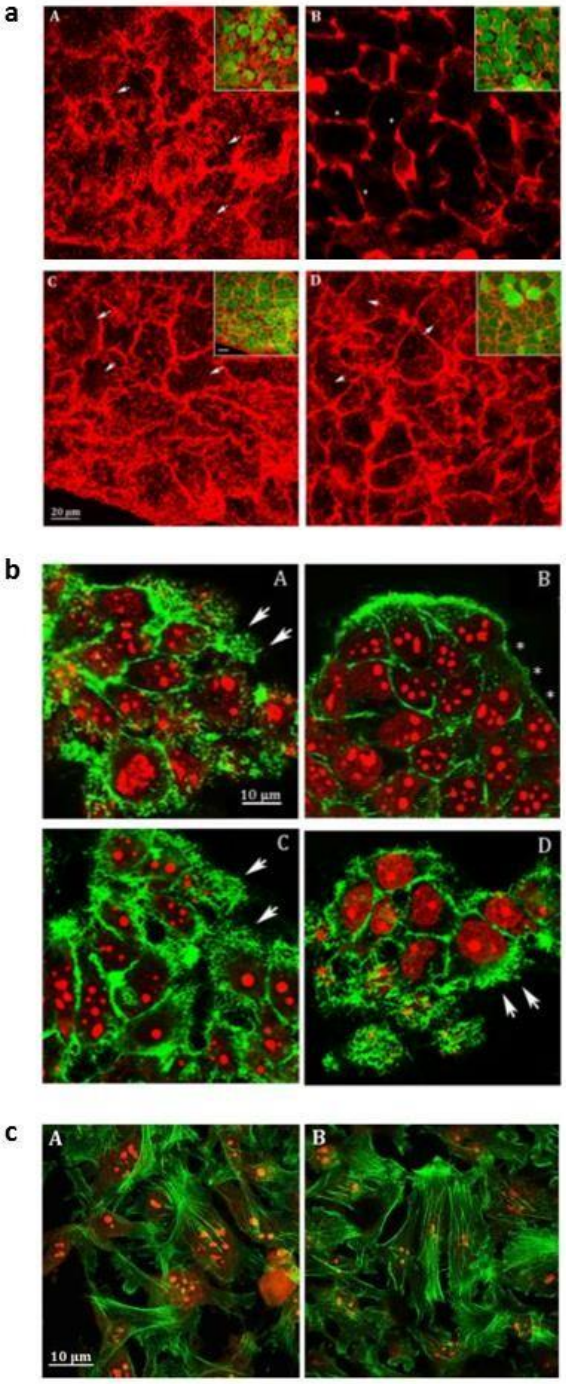


Figure 17: (**a**) Representative confocal image of (A) scrambled HepG2 cells; (B) Abcc6-shRNA HepG2 cells; (C) Abcc6-shRNA HepG2 + 500 µM ATP; (D) Abcc6-shRNA HepG2 + 100 µM adenosine. F-actin was stained with Phalloidin Alexa Fluor 568. In the insets, superposition of cytoskeleton (red) and EGFP (green) to monitor the infection efficiency. (**b**) Representative confocal image of HepG2 cells treated with (A) 0.25% DMSO (vehicle); (B) 250 µM probenecid; (C) 250 µM probenecid and 500 µM ATP; (D) 250 µM probenecid and 100 µM adenosine. (**c**) Representative confocal image of MDA-MB-231 cells treated with (A) 0.25% DMSO (vehicle) and (B) 250 µM probenecid. F-actin and nuclei were stained with Phalloidin Alexa Fluor 488 and propidium iodide, respectively.

Since our main goal was to demonstrate that a reduction of tumor cell invasiveness could be obtained through a modulation of ABCC6 transport activity, a series of scratch tests were performed by the group of Prof. Carmosino, from the University of Bari, which confirmed our original hypothesis, since both ABCC6 knockdown and Probenecid treatment caused a significant reduction in HepG2 cells migration rate. Once again addition of ATP to the culture medium restored migration rate to the level of control cells (Figure 18).

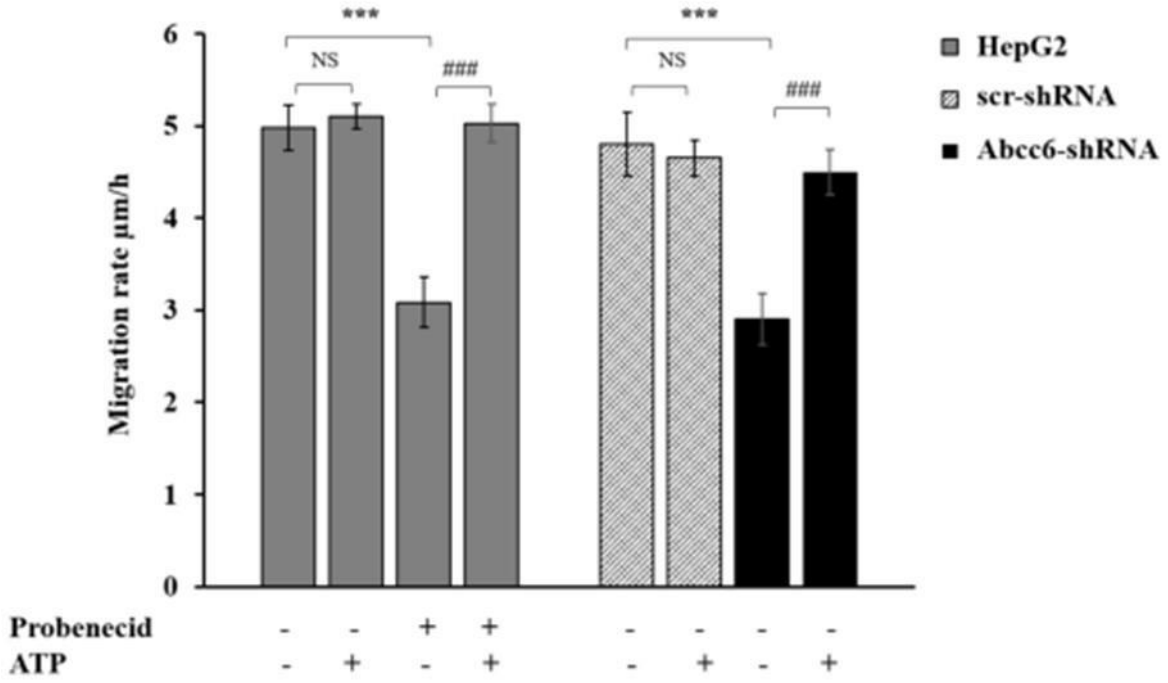


Figure 18: Effect of ABCC6 silencing (right an Probenecid treatment (left) on HepG2 cells' migration rate. DMSO-treated cells were used as a control. ATP 500 µM was added to both control and Probenecid-treated or ABCC6-silenced cells. Data are expressed as the mean and standard error of at least three different experiments. Statistical significance was assessed by one-way ANOVA followed by Dunnett's post hoc test *** $p < 0.001$

Finally, to demonstrate that the effect we had just seen on cytoskeleton and cell migration was dependent on ABCC6 mediated ATP efflux, we measured the amount of ATP released from ABCC6-silenced and Probenecid-treated cells and we observed a decrease of about 60% and 40% respectively in conditioned medium. In comparison, in MDA-MB-231 cells, which have a very low expression of ABCC6, Probenecid treatment at most slightly yet not significantly affected ATP efflux (Figure 19).

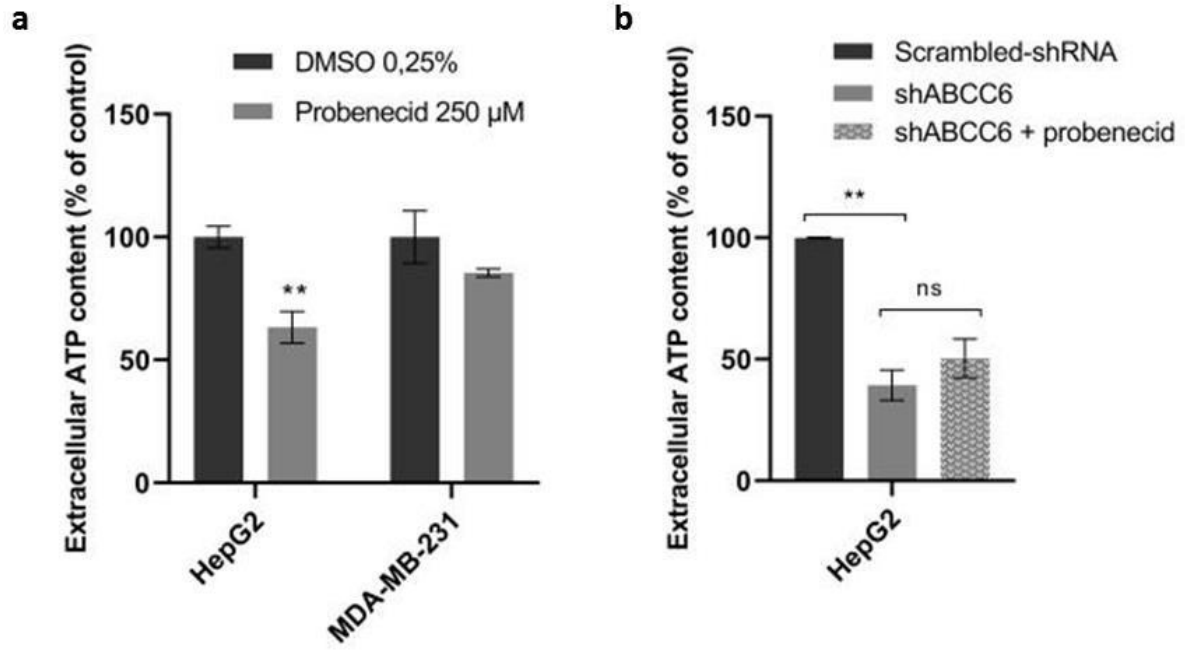


Figure 19: Extracellular ATP content of: (**a**) HepG2 and MDA-MB-231 treated with Probenecid 250 µM for 48h; (**b**) ABCC6-shRNA and ABCC6-shRNA HepG2 + Probenecid 250µM. **p<0,01; ***p<0,001

Quercetin affects ABCC6 expression through the modulation of AKT pathway (PaperII)

In the previous work we showed that a change in cytoskeleton and a reduction in migration rate could be obtained in hepatocarcinoma-derived HepG2 cells by the means of gene silencing or pharmacological inhibition of ABCC6 transporter. In order to block ABCC6 transport activity we used the inhibitor of organic anions transporters Probenecid.

In Paper II we aimed to primarily study the effect of Quercetin on ABCC6 expression in HepG2 cells. Quercetin is one of the most aboundant and extensively studied flavonoids, which owns many interesting biological properties. As a strong antioxidant compound, due to the ability to bind transition metal ions and scavenge free radicals, attributed to the catechol group in the B ring and the OH group at position 3 of the A ring (de Souza et al., 2004), Quercetin is very effective against O_2^- and $ONOO^-$ and can terminate lipid peroxidation, which has deleterious effects especially on the cardiovascular and nervous systems. Moreover, Quercetin has been proven to be useful in prevention and very effective an as anticancer drug both in *in vitro* and *in vivo* studies (Tang et al., 2020). Flavonoids are also well-known inhibitors of ABC transporters, so that their use has been proposed as a strategy to overcome drug resistance (Gonçalves et al., 2020). However, given their major

role in drug resistance, most of studies have been focused on P-gp, BCRP and MRP1, with very limited information on other members and ABCC6 in particular (Luo et al., 2010).

We first performed an MTT and a DCF assay to assess the effect of various concentrations of Quercetin on viability and ROS accumulation in HepG2 cells after 24 hours of treatment. Since our final goal was to deepen insight the mechanism Quercetin anticancer activity and to demonstrate that it could affect cells migration by modulating the purinergic system in an ABCC6 dependent way, we chosed for further experiments a concentration of 165 µM, which did not significantly harm the cells, but showed a strong antioxidant activity (Figure 20)

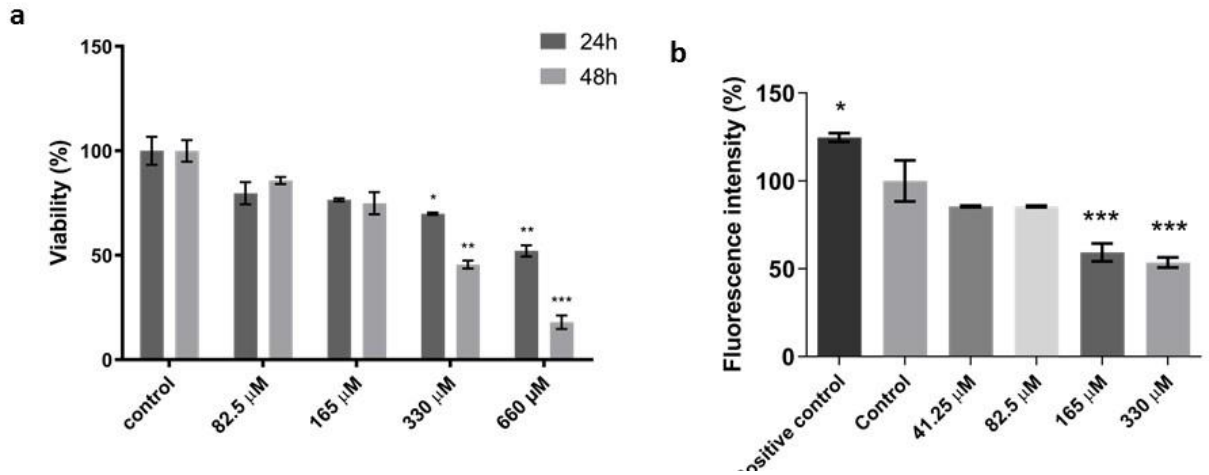


Figure 20: (**a**) Effect of Quercetin on HepG2 cells viability. Data are expressed as a percentage of the control and presented as the mean ± sem of at least three independent experiments. Statistical significance assessed by multiple t test followed by Holm–Sidak correction for multiple comparisons; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; (**b**) Effect of Quercetin on the intracellular level of Reactive Oxygen Species (ROS) in HepG2 cells. Data are expressed as a percentage of the control and presented as the mean ± sem of at least three independent experiments. Statistical significance assessed by one-way ANOVA followed by Dunnett's correction for multiple comparisons; * $p < 0.05$; *** $p < 0.001$

When we treated HepG2 cells with 165 µM of Quercetin for 24h we found the same pattern of variation in gene expression that our group previously previously reported for Probenecid treatment, that is a reduction in ABCC6 and NT5E expression. Moreover, among all main ABC transporters we considered, ABCC6 was the only one which was downregulated. However, while ABCC6 protein downregulation was confirmed by western blot, the same did not happen for NT5E (Figure 21).

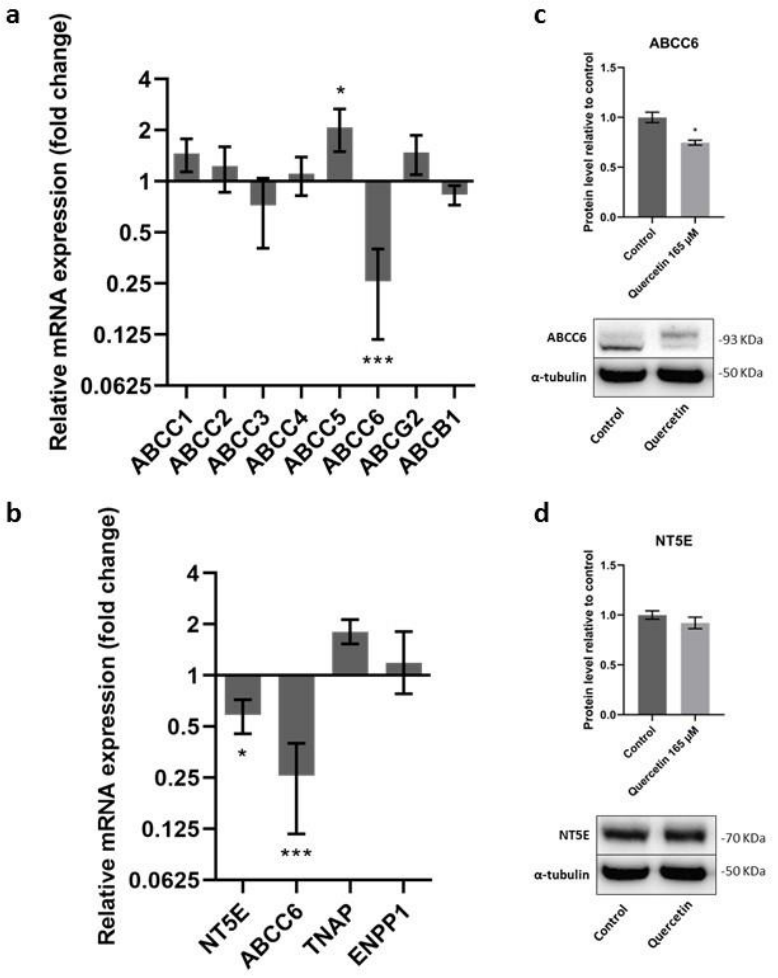


Figure 21: Effect of Quercetin on mRNA expression of some relevant ABC transporters (**a**) and on genes involved in the purinergic pathway (**b**). Results are expressed as the mean and 95% confidence interval of three different experiments. Statistical analysis was performed on dCt values by using multiple T-test followed by Holm–Sidak correction for multiple comparisons; * $p < 0.05$; *** $p < 0.001$. Effect of Quercetin on ABCC6 (**c**) and NT5E (**d**) protein expression. Results are expressed as the mean the standard error of three independent experiments. Statistical analysis was performed using Student's t test; * $p < 0.05$.

When it came to cytoskeleton and migration rate, as we hypothesized, Quercetin treatment affected both groups, causing loss of filopodia and a significant reduction in collective motility. Notably, when we added Adenosine 400 µM or ATP 500 µM to the culture medium, migration rate was not restored. Instead, we noticed a slight yet significant further decrease in motility when ATP was used together with Quercetin, thus suggesting that due to inhibition of NT5E activity, accumulation of AMP may hamper motility through the engagement of different receptors (Figure 22).

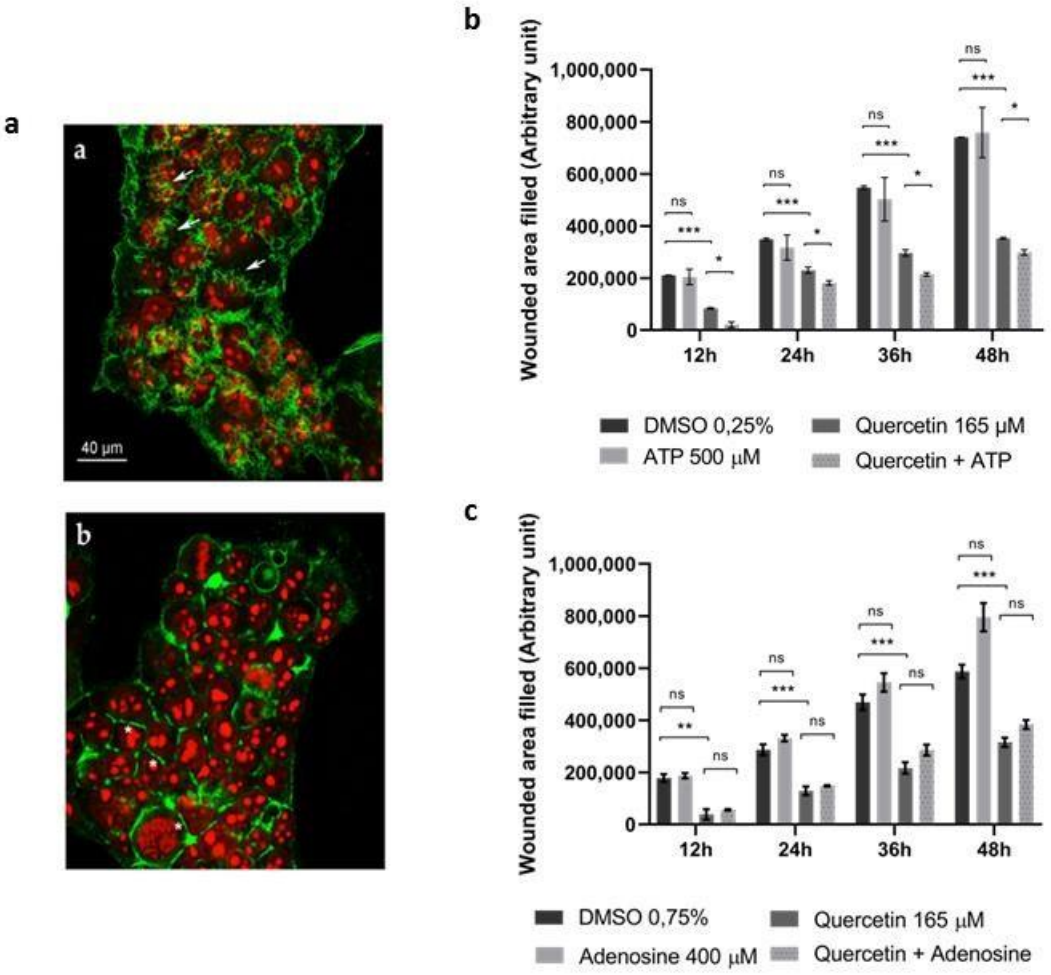


Figure 22: (**a**) Representative confocal image of HepG2 cells treated with 0,25% DMSO (a) or 165 µM Quercetin (b) for 24 h. F-actin and nuclei were stained with Phalloidin Alexa Fluor 488 and propidium iodide, respectively. Effect of Quercetin on HepG2 cells' migration rate. DMSO-treated cells were used as a control. ATP 500 µM (**b**), Adenosine 400 µM (**c**) was added to both control and Quercetin-treated cells. Data are expressed as the mean and standard error of at least three different experiments. Statistical significance was assessed by multiple t test followed by Holm–Sidak correction for multiple comparisons; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Finally, we tried to dig deeper into the cell pathway that could be involved in Quercetin mechanism of action in our model. As mentioned before, cells migration requires that different parts of cells machinery work together coordinately. In physiological conditions this is guaranteed by a redundant control of genes involved in the so-called Epithelial-to-Mesenchymal Transition (EMT), through specific transcription factors. In addition, several chemochines, growth factors and soluble mediators participate to this control, so that this process can be stopped once a specific function, such as tissue regeneration has been assolved. In tumors, unregulated activation of this machinery could

contribute to malignant cells dissemination (Giannelli et al., 2016). The phosphoinositide 3 kinase (PI3K)/Akt pathway and the extracellular signal-regulating kinase (ERK) member of mitogen-activated protein kinase (MAPK) are prototypical cell pathway linking extracellular signals to the activation of cellular proliferation and migration, whose dysregulation has been reported in cancer (Xue et al., 2013; Tanimura et al., 2017). Moreover, Quercetin has been shown to be effective in suppression of these pathways in several models of liver cancers, as a part of its mechanism of action (Ding et al., 2018; Wu et al., 2019).

Interestingly, in ABCC6 silenced cells both pathways appeared to be downregulated, as we found a reduction of phosphorylated active form of respective protein kinase in western blot experiments. In comparison, after 24h of treatment with 165 μM of Quercetin, we found a reduction only in p-AKT levels, not p-ERK (figure 22). This let us speculate that ABCC6 downregulation in Quercetin-treated HepG2 cells was the result of an AKT-dependent mechanism of adaptation to oxidative stress. In fact, it is well-known that the activation of AKT plays a key role in the cell response to oxidative stress, by inducing the expression of target genes, which support tumor cells' survival, as well as resistance to chemotherapy (Schöning et al., 2017; Martindale et al., 2002). It is noteworthy that in this study we used a concentration of Quercetin with strong antioxidant activity, which could have mitigated that response, ultimate leading to a decrease in ABCC6 expression (Gulhati et al., 2011).

Apart from that, this study showed once more a tight correlation among ABCC6 expression, cytoskeleton, and migration in liver cancer cells (Figure 23).

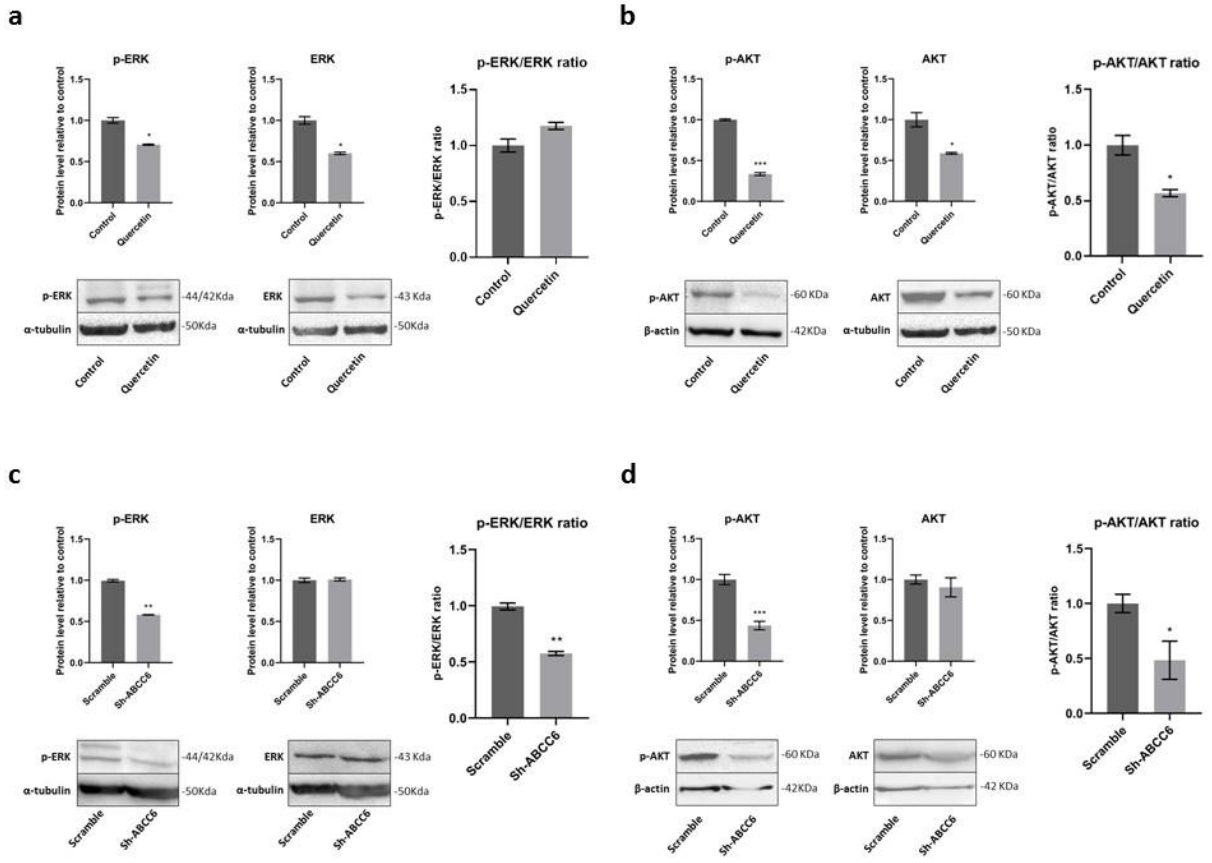


Figure 23: Effect of Quercetin treatment (**a**,**b**) or ABCC6 silencing (**c**,**d**) on phosphorylated AKT and ERK. α-tubulin or β-actin were used as a loading control. Data are presented as the mean and the standard error of the mean of three independent experiments. Results were analyzed by Student's t test; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

The expression level of ABCC6 transporter in colon cancer cells correlates with the activation of different intracellular signaling pathways (Paper III)

In paper I and II we studied the effect of ABCC6 gene silencing and pharmacological inhibition with Probenecid or Quercetin in liver cancer HepG2 cells, with a special emphasis on cytoskeleton rearrangement and migration rate as result of a modulation of extracellular purinergic signaling.

In Paper III we were mainly interested in confirming those results we got on HepG2 cells in a model of extra hepatic tumor. Accordingly, we moved on two cell lines derived from colorectal cancer, namely Caco2 and HT29. Very little evidence is there about the role of ABCC6 in colon cancer, with few reports of a downregulation or upregulation in colon adenocarcinoma, compared to normal tissue, depending on the studies (Hlavata et al., 2012; Maubon et al., 2007).

On the other hand, an extensive literature has been produced about the extracellular purinergic system in colon cancer. The evidence for presence of functional ATP receptors has been provided in HT29 cells (Nylund et al., 2003; Nylund et al., 2004), as well as for their overexpression in tumor specimens, compared to control healthy tissues (Nylund et al., 2007). Activation of P2X7 has been reported to promote invasion and migration in colon cancer cell lines, and expression of EMT-related genes and tumor growth *in vivo* (Zhang et al., 2020), even if higher concentrations of ATP may inhibit proliferation and induce apoptosis, reflecting the activation of different receptors with lower affinity (Coutinho-Silva et al., 2005), as also suggested by different authors (Souza et al., 2012; Yaguchi et al., 2010). Finally, the presence of active nucleotides metabolizing enzymes has been demonstrated in Caco2 and HT29 cells (Schachter et al., 2021; Bahrami et al., 2014).

First, we assessed the levels of expression of ABCC6 and NT5E and we found some relevant differences. In fact, ABCC6 mRNA expression was high in Caco2 cells, and was almost absent in HT29, while, on the contrary, the expression of nRNA of NT5E, which is the rate-limiting enzyme in extracellular ATP metabolism, was very high in HT29 cells and very low in Caco2 cells (Figure 24a). NT5E protein expression was not even detected in Caco2, as no bands appeared in Western Blot (Figure 24b). After 24h treatment, both mRNA and protein expression of ABCC6 was significantly downregulated by Quercetin in Caco2 cells (Figure 24c, d). In HT29 cells, we found a relevant, yet not statistically significant reduction of both ABCC6 and NT5E transcripts induced by Quercetin (Figure 2e), while NT5E protein was significantly downregulated by Quercetin (Figure 2f).

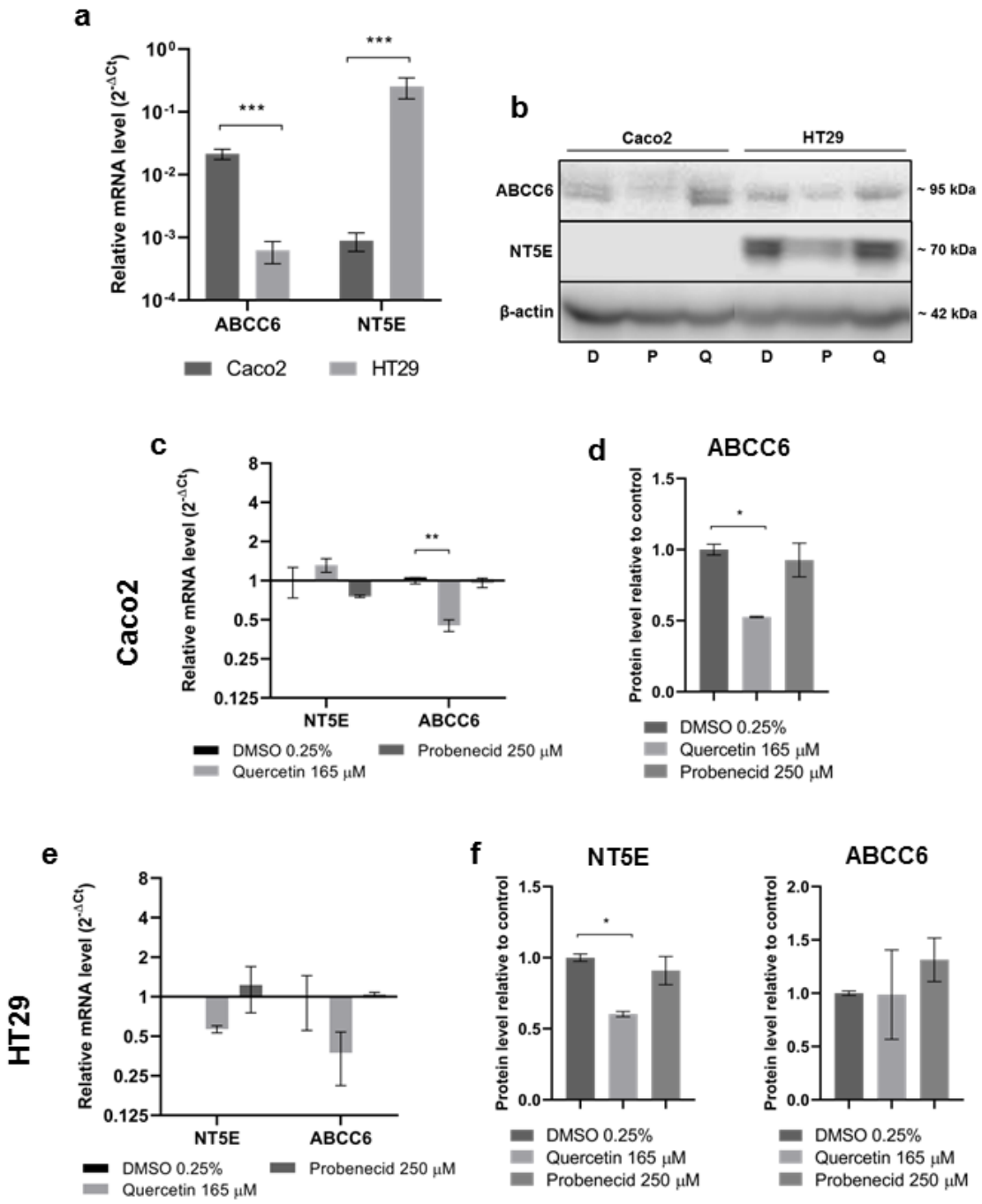


Figure 24: . (**a**) Relative mRNA expression levels of ABCC6 and NT5E gene in Caco2 and HT29 cells. Results are expressed as the 2-ΔCt and presented as the mean ± sem of at least three independent experiments. β-actin was used as a reference gene. Statistical analysis was performed by 2-way ANOVA followed by Holm Sidak correction for multiple comparisons. ***p<0.001; (**b**) Representative Western Blot of the effect of Quercetin and Probenecid on protein expression (D, DMSO 0.25%; Q, Quercetin 165 µM; P, Probenecid 250 µM). mRNA and protein expression levels of NT5E and ABCC6 in Caco2 (**c**,**d**) and HT29 (**e**,**f**) cells. Result are expressed as the 2-ΔCt and presented as the mean ± sem of at least three independent experiments. β-actin was used as a reference gene. Statistical analysis was performed by 2-way ANOVA followed by Dunnett's correction for multiple comparisons. *p<0.05. Cells treated with vehicle only (DMSO 0.25%) were used as a control β-actin was used as a loading control. Data are presented as the mean ± sem of three independent experiments. Statistical analysis was performed by one-way ANOVA followed by Dunnett's correction for multiple comparisons. *p<0.05.

Furthermore, when we measured mRNA levels of some relevant purinergic receptors, we found higher expression of three ATP receptors, P2Y1, P2Y2 and P2Y14, in Caco2 cells, while adenosine receptor A2A was slightly yet not significantly more expressed in HT29 cells (Figure 25).

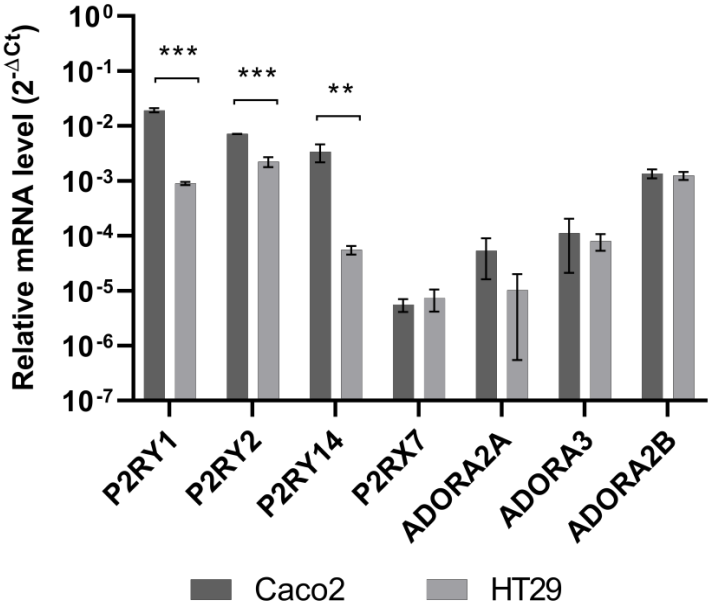


Figure 25: Relative mRNA expression levels of some relevant purinergic receptors in Caco2 and HT29 cells. Results were expressed as the 2-ΔCt and presented as the mean and the standard error of at least three replicates. β-actin was used as a reference gene. Statistical analysis was performed by two-way ANOVA followed by Holm-Sidak correction for multiplele comparisons. **p<0.01; ***p<0.001

Based on this premise we expected that Caco2 and HT29 cells responded differently to our treatments. In fact, when we treated them with 165 µM of Quercetin or 250 µM of Probenecid, the same concentration we used in previous works, which we proved not to be toxic in colon cells either, we observed a significant decrease in migration rate in both cases. However, when ATP was added to the culture medium, migration was restored in Caco2 only, with no additional effect on HT29 cells (Figure 26), thus suggesting that in Caco2 both treatments might modulate the purinergic signal through the involvement of P2 receptors.

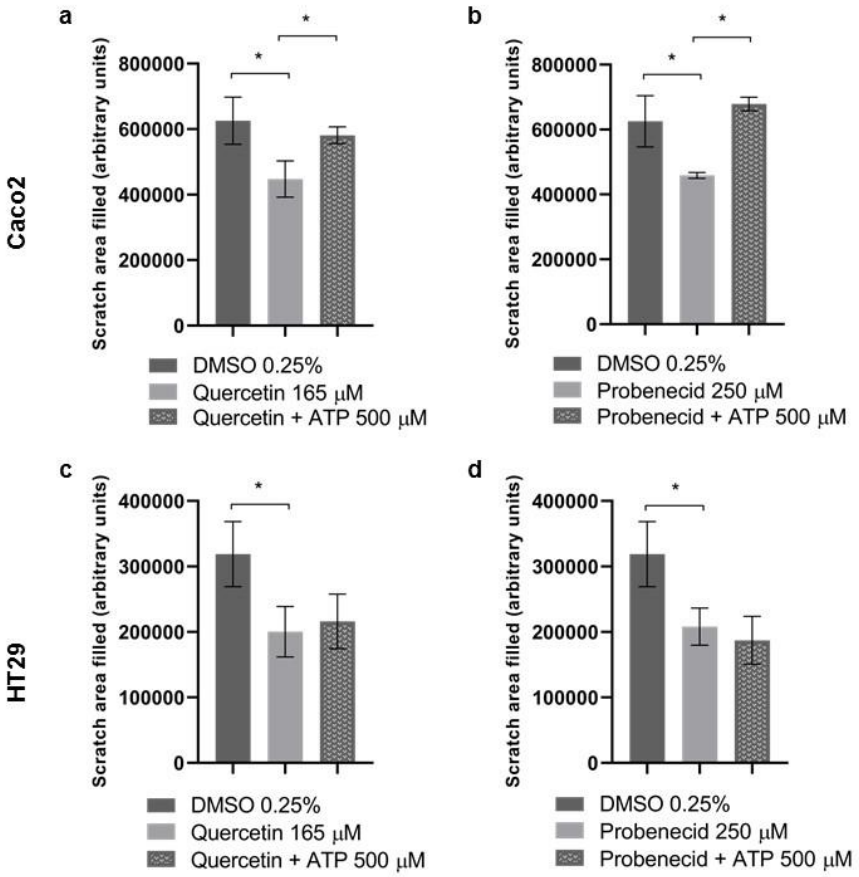


Figure 26: Effect of Quercetin and Probenecid on Caco2 and HT29 cells migration rate. Cells were treated with test molecules for 12 h. Then, a scratch was made in the cell monolayer, and pictures were taken after 24 hours, while the cells were still kept in contact with Quercetin or Probenecid. DMSO-treated cells were used as a control. ATP 500 μM was added to both Quercetin and Probenecid-treated cells. Scratch area filled after observation time was expressed as the mean and the standard error of at least three replicates. Statistical significance was assessed by one-way ANOVA 205 followed by Holm-Sidak correction for multiple comparisons. *p<0.01

To support this hypothesis is the evidence that, in Caco2, activation of P2Y receptors triggers Mitogen-activated protein kinases cascade, leading to the phosphorylation of Extracellular signal-regulated kinases 1 and 2 (ERK) and recruitment of downstream effectors (Buzzi et al., 2009; Buzzi et al., 2010), which can ultimately affect cell migration by controlling EMT-related genes expression (Avizienyte et al., 2005).

In HT29 cells, where ABCC6 expression is negligible, the activation of purinergic system may be controlled by some auxiliary players. It is not excluded that the greater production of adenosine due to the overexpression of NT5E may increase the engagement of P1 receptors. Moreover, in these cells, activation of AKT pathway may have a predominant role in controlling migration. It has been reported that its downstream effector, the Mammalian Target of Rapamycin complexes 1 and 2 (mTOR1/2), are often overexpressed in

advanced stage of colon cancer (Rychahou et al., 2006) and their inhibition has been proposed as a strategy to mitigate invasion and migration of colon cancer (Gulhati et al., 2011). Many authors reported that downregulation of PI3K/AKT/mTOR pathway effectively inhibits migration in HT29 cells (Han et al., 2015; Wu et al., 2019). In HT29 cells both Quercetin and Probenecid decreased the phosphorylation of AKT, which could explain an effect on migration independently from the involvement of P2 receptors (Figure 27).

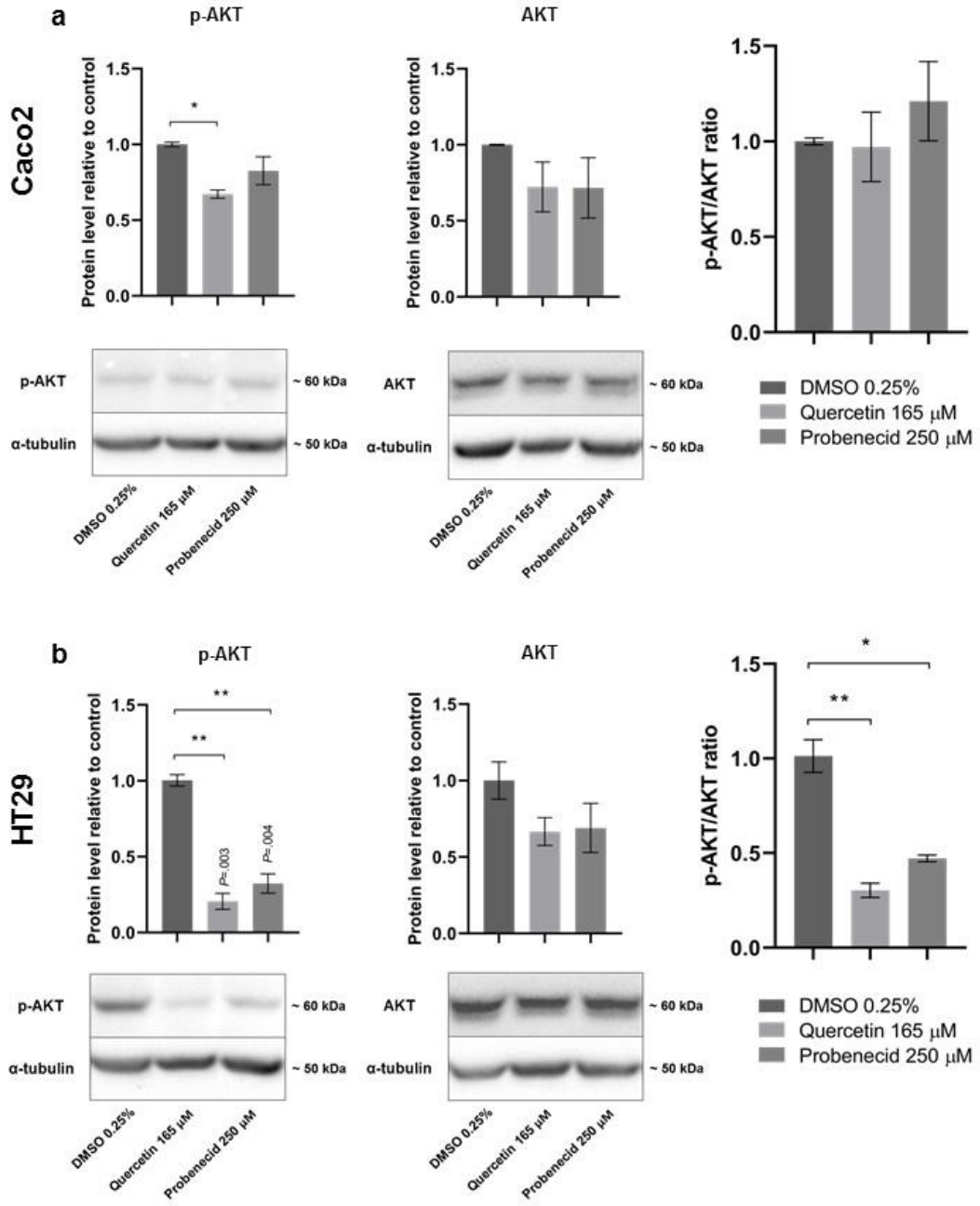


Figure 27: Effect of Quercetin and Probenecid treatment on AKT phosphorylation in (**a**) Caco2 and (**b**) HT29 cells. Cells treated with vehicle only (DMSO 0.25%) were used as a control. α-tubulin was used as a loading control. Data are presented as the mean ± sem of three independent experiments. Statistical analysis was performed by one-way ANOVA followed by Dunnett correction for multiple comparisons. *p<0.05**;** **p<0.01

This work confirmed that even in extra hepatic tumors in which ABCC6 is overexpressed, it may represent a target for a therapy aimed to reduce invasiveness through the modulation of purinergic signaling. In fact, in Caco2 cells, in which ABCC6 is highly expressed, it may have a major role in controlling cells migration by feeding the extracellular purine pool with ATP. On the contrary, in HT29 cells, lacking ABCC6 expression, the remarkable levels of NT5E expression, and probably higher ectonucleotidase activity, may increase the triggering of A2AR receptor by adenosine, which could have a predominant effect on PI3K/AKT activation (Ma et al., 2019).

AECs are a promising source of hepatocytes-like cells for cellular therapy of liver diseases and drug development studies, but *in vitro* differentiation is currently limited to an early stage (experience at Karolinska Institutet)

In previous works we studied the effect of ACC6 gene silencing and pharmacological inhibition in different cancer cell lines, demonstrating that targeting ABCC6 transport activity may be a promising strategy to reduce the invasiveness of tumors bearing its overexpression. However, blocking a specific function might carry a risk of toxicity, especially if that function is essential for the organism. Moreover, a potential drug has not only to be effective against malignant cells, but also must be proven to be not as toxic for healthy tissues. Major part of this work has been conducted on hepatocarcinoma cell lines. Hepatocytes are also the main site of expression of ABCC6. It has been long time recognized that the lack of ABCC6-mediated ATP efflux from the liver is the cause of ectopic mineralization in autosomal disease Pseudoxantoma elasticum. Yet except from a minority of cases of GACI due to a mutation of ABCC6, in which the extent of ectopic mineralization is dramatic and has a very early onset, in PXE patients this process often takes decades to be evident. Furthermore, while the lesions in soft tissues which are most affected by the disease have been characterized in detail, little is known about the consequences of impaired functionality of purinergic signaling in the liver, which surprisingly seems to be not harmed in PXE patients. For all these reasons it was extremely helpful to test our treatments on healthy cells, as a control.

As a part of my PhD program, I spent three months at Karolinska Institutet, in Stockholm, Sweden, under the supervision of Dr. Roberto Gramignoli. His Research Group, founded by Dr. Stephen Strom, which was the first to perform allogenic hepatocytes transplantation, is renowned for the advancements in hepatocytes isolation for cell-based therapy, and for the isolation and characterization of amniotic epithelial cells (AECs) from placentas. From early 90's hepatocytes transplantation (HTx) has emerged as a bridge or even an alternative to orthotopic liver transplantion (OLT) (Strom et al., 1997; Strom et al., 1997) for the treatment of many acute and chronic diseases including acute liver failure, cirrhosis, and metabolic liver disease. In comparison to OLT, HTx has a number of advantages: it is less invasive, being possible to be performed by multiple infusions, and does not require removal of native liver, which in fact supports liver regeneration and also serves as a back-up, allowing the return to the pre-transplantation state in case of cellular graft failure. However, the limited source and quality of hepatocytes remains a problem (Gramignoli et al., 2015).

Unfortunately, during the three-month stage at Karolinska Institutet, no fresh livers were available to isolate hepatocytes, and cryopreserved ones are generally affected by poor viability (Stéphenne et al., 2007). So we first looked at ABCC6 expression in different cell sources and, as we were expecting, we observed relevant levels only in cryopreserved human primary hepatocytes, whether adult or neonatal or even fetal, and in liver-derived cancer cell lines, while no expression was found in induced Pluripotent Stem Cells (iPSCs) and AECs (figure 28).

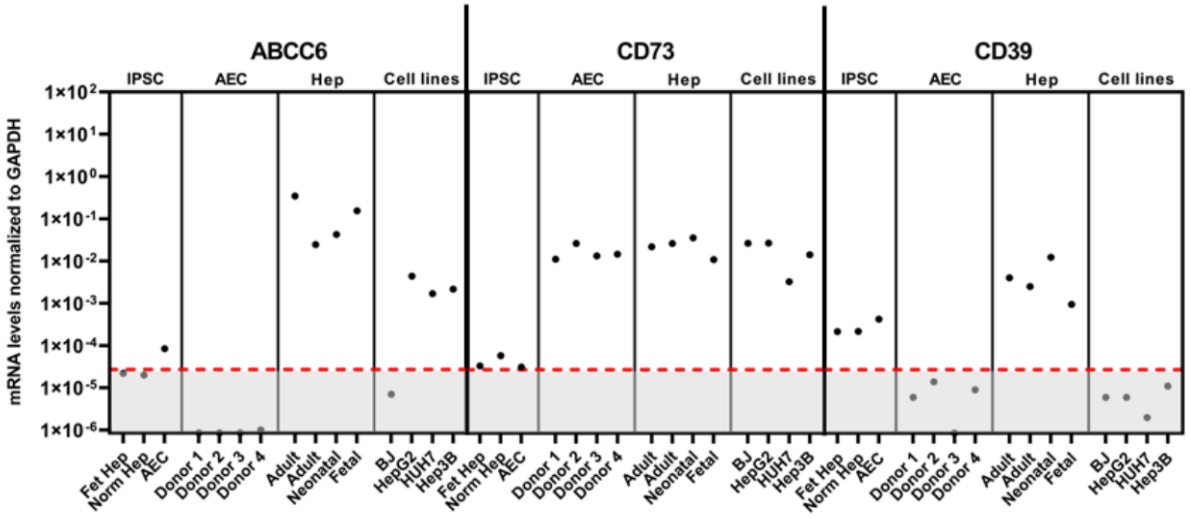


Figure 28: Gene expression level of ABCC6 and extracellular ectonuclease enzymes. Results were normalized to GADPH as a reference gene. Red dotted line separates reliable datas (above) from unreliables (below). Cutoff was set at a value of $3.25*10^{-5}$. This value was obtained from the formula: $2^{-(35-Ct)}$, where Ct is the mean Ct of the refence gene from all samples (19,84)

Dr. Gramignoli's group has reported that AECs have the ability to acquire hepatocyte features *in vitro* when co-cultured with mouse hepatocytes or using a 3D configuration (sandwich) in a culture system based on liver-derived extracellular matrix (L-ECM). Differentiated hAEC express mature hepatocyte marker genes and activities, including some of the major metabolizing enzymes, such as CYP 3A4, 3A7, 1A1, 1A2, 2B6, 2D6, and UDP-glucuronosyltransferase (UGT) 1A1. They also display metabolic functions typical of mature hepatocytes, such as the ability to metabolize ammonia and 17-hydroxyprogesterone coproate (17- OHPC), together with inducible CYP and phase II enzymes (Marongiu e al., 2011). In preclinical models of mouse maple syrup urine disease and Mucopolysaccharidosis type 1 (MPS1) show that AECs can be successfully used as a surrogate of hepatocytes for allogenic transplantation, being able to differentiate into hepatocytes-like cells and correct metabolic dysfunction (Skvorak et al., 2013; Skvorak et al., 2013; Rodriguez et al., 2017).

Then we decided to study the effect of Quercetin and Probenecid on hepatocytes-like cells derived from AECs. We did not know in advance whether these cells expressed ABCC6 or not, but the fact that even fetal hepatocytes express high levels of ABCC6 let us hope that differentiated AECs did that too, despite the grade of differentiated that can be obtained *in vitro* is limited to an early stage of maturation (Zabulica et al., 2019). The protocol we used to induce hepatocellular differentiation of AECs is based on a recent work which describes the differentiation of Human iPSCs into Hepatocytes (Takeishi et al., 2020). This protocol requires a first step of endoderm induction with Activin A, followed by a 14-day hepatic specification by culturing cells in the presence of DMSO and Hepatocyte growth factor (HGF) and a final four-day phase of hepatic maturation in presence of dexamethasone, hydrocortisone, free fatty acids, bile acids, cholesterol, and rifampicin. Our protocol, unlike the original, skipped pre-treatment with Activin A, which is not necessary for AECs, as it has been proved that it does not increase endoderm markers, but rather upregulates genes of stemness, ultimately not improving hepatic differentiation of AECs (Marongiu e al., 2011) (Figure 29).

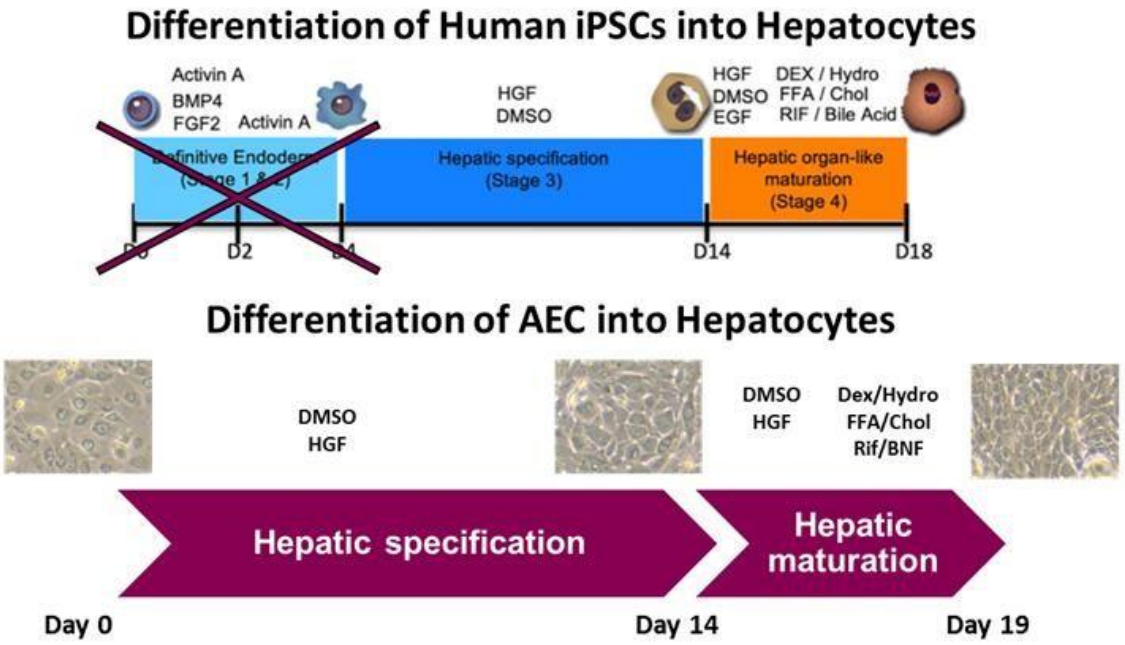


Figure 29: Comparison between differentiation protocols used (bottom) and protocol from Takeishi et al., 2020 (top)

So, we isolated fresh amniocytes from two donors according to the recently published procedures (Gramignoli et al., 2016), and followed our protocol of differentiation on cells cultured in different settings, including 2D culture on collagen-treated plates and three-dimensional cultures, with cells embedded in commercial matrigel (MG) or custom produced decellularized Extracellular Matrix (dECM). After final stage of maturation with various differentiative stimuli and treatment with inductors of cytochrome P (CYP) enzymes Rifampicin and β-Naphthoflavone, we assessed the activity of some liver-specific metabolic functions. Phase I metabolism was tested through the 7-Ethoxyresorufin-O-Deethylase (EROD) assay, which measures the rate of conversion of 7-ethoxyresorufin to resorufin, and is specific for CYP1A1 and 1A2, while for Phase II metabolism activity the resorufin conjugation assay, specific for UDP-glucuronosyltransferases (UGTs), was used (Gramignoli et al., 2012; Gramignoli et al., 2014). Results showed that at least in cells cultured in 3D, both in MG or dECM, some degree of metabolic activity was observed, although the activity was the same between treated and undifferentiated control cells, which were kept in maintenance medium. However, when we assessed the levels of expression of some liver-specific genes, ABCC6 included, they were very low to undetectable (Figure 30).

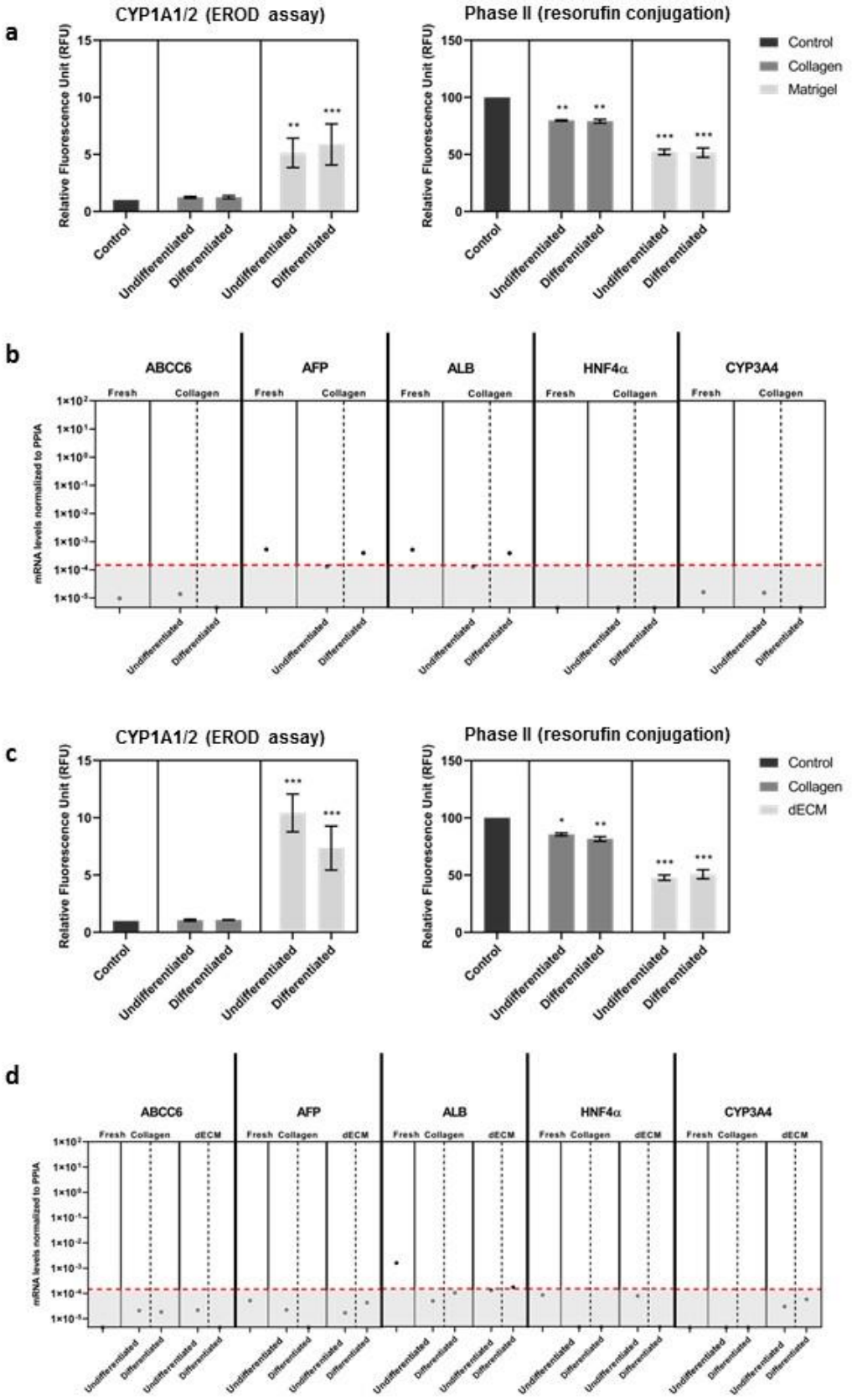


Figure 30: Phase I and Phase II metabolic activity in panel **a** (donor 1) and **b** (donor 2); Gene expression level of ABCC6 and relevant liver-specific genes in **c** (donor 1) and **d** (donor 2). Results were normalized to GADPH as a reference gene. Red dotted line separates reliable datas (above) from unreliables (below). Cutoff was set at a value of $1{,}48*10^{-4}$. This value was obtained from the formula: $2^{-(35-Ct)}$, where Ct is the mean Ct of the refence gene from all samples (22,27)

Up to now, *in vitro* differentiation of AECs into hepatocytes-like has been described in systems involving co-cultures with mouse primary hepatocytes or 3D configuration using Matrigel or Porcine liver-derived extracellular matrices, yet maturation which can be obtained is limited to a very early stage. In this study we used a different preparation of ECM and adopted a differentiation protocol which was first optimized on iPSCs. It is likely that higher concentration of HGF for longer time would be required in order to improve the results.

Conclusions and future perspectives

ABCC6 is a member of ATP-binding cassette membrane transporters. In past years the role of ABCC6 as a player in ectopic mineralization processes has emerged with the discovery that mutations in ABCC6 gene were associated with pathogenesis of autosomal genetic disorder *Pseudoxantoma Elasticum* (PXE) and with a minority of cases of the more severe and early onset *Generalized Calcification of Infancy* (GACI). These two diseases seem to be part of a broader spectrum of disorders which share a common pathway, where ABCC6 presumably acts upstream. In fact, it is now currently accepted that ABCC6-mediated efflux of ATP from the liver is the main source of circulating inorganic pyrophosphate, which is a powerful inhibitor of mineralization (Jansen et al., 2013). At the same time, multiple studies elucidated details of its structure, which shares some homology with other members of the same family and of its expression, the bulk of which is localized to the basolateral membrane of hepatocytes.

As more and more functions of ABCC6 are disclosed, it appears evident that it could be involved in different processes. Unlike other multidrug resistance proteins, which actively extrude drugs from the the cells, ABCC6 contribution to drug resistance is considered secondary. However, as ATP is an important extracellular signaling molecule and a key component of tumor microenvironment, controlling many aspects of cancer cells, from proliferation, to apotosis, migration and interation with host immunity, it is questionable wether this ABCC6-mediated ATP efflux can somewhat facilitate tumor progression and, if so, targeting this activity could be beneficial for cancer treatment.

In the work presented here we tried to address these questions, which is relatively a new concept because, despite a direct correlation between cancer progression and gene expression has been described for many ABC transporters, strangely no studies focused on this aspect of ABCC6 activity. In previous studies conducted in our lab it has been shown that ABCC6 sene silencing induced a gene dysregulation (Miglionico et al., 2014) and a senescent phenotype (Miglionico et al., 2017) in hepatocarcinoma cell lines HepG2, and that some of the effects on gene expression could be recapitulated by treating HepG2 cells with organic anions transporters inhibitors Probenecid (Martinelli et al., 2018), suggesting that a modulation of extracellular purinergic signaling could be involved.

The overall results of this work clearly show that inhibition of ABCC6-mediated ATP efflux from cancer cells reduces their motility and induces a rearrangement of the

cytoskeleton. As a model of ABCC6 expressing tumors we used HepG2 cell line (Paper I, II). To study the effect of ABCC6 knockdown we used shRNA technology (Paper I, II), which we compared with pharmacological inhibition with inhibitors of ABC transporters Probenecid (Paper I) and Quercetin (Paper II). We found that the both the effects on cytoskeleton and migration were likely due to a modulation of purinergic system. The main evidence supporting this hypothesis were the fact that: 1) ATP level in conditioned medium of Probenecid treated and ABCC6-silenced cells was reduced compared to control cells; 2) addition of ATP or adenosine restored architecture of cytoskeleton and migration rate (Paper I). However, we found the effect of Quercetin on cells motility somewhat different from Probenecid, reflecting a more pleiotropic activity of Quercetin, involving maybe ectonucleotidase enzymes. In paper III we studied the role of ABCC6 in the modulation of purinergic system in two cell models of colon cancers, Caco2 and HT29, the first one expressing high levels of ABCC6 mRNA compared to the second. As we expected, the two cell lines responded differently to treaments with our inhibitors, confirming that ABCC6 could have a role in controlling extracellular purinergic signaling even in extrahepatic cancers. Moreover, we studied the effect of ABCC6 gene silencing and pharmacological inhibitions on two cell pathways known to be activated by some of the purinergic receptors which are expressed on this cell lines (Paper II, III).

It is worth stressing once more that these are the first studies that try correlate ABCC6 transport activity with the modulation of migration in cancer cells. However, these findings, which are still quite preliminary, necessitate to be corroborated by *in vivo* studies, which only could reinforce their importance, assessing the effects of our treatments on cancers invasiveness and metastatc potential. Furthermore, it would be very interesting to study their impact on the activation of immune cells and ultimately on the restoration of host immunity against tumors, which is suppressed during tumor progression through a variety of mechanisms, involving, among others, purinergic signaling.

Finally, since most of this work was conducted on a liver cancer cell line, we tried to assess the feasibility of using hepatocytes -like cells (HLCs) derived from amniotic epithelial cells (AECs), as a substitute of primary hepatocytes for testing our treatments. AECs can be induced to differentiate *in vitro* into hepatocytes in certain conditions (Marongiu et al., 2011). However, our attempt was not successful as we did not find any variation in liver-specific gene expression after completion of our differentiation protocol. Different groups described protocols for *in vitro* differentiation of hepatocytes, but results are often limited

to a very early stage of maturation. The major challenges are: the difficulty to mimic in the short time of few weeks a development process which normally last for years; the fact that, as for primary hepatocytes, stem cell-derived hepatocyte phenotype may be unstable in culture and that it is difficult to emulate *in vitro* the complexity of the liver, which is normally exposed to relevant concentrations of intestinal products (Berger et al., 2015). It is likely that increasing the duration of exposure to HGF, as well as using different kinds of 3D configurations and incorporating other cell types, such as endothelial cells and Kupffer cells, would have an impact on maturation of hepatocytes. Nevertheless, it could be speculated that AECs could represent a promising opportunity for a cell-based therapy of PXE, as they do not form tumors and are completely safe compared other types of pluripotent stem cells and are able to differentiate into fully mature hepatocytes *in vivo.*

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Acknowledgements

"*It's just a ride in an amusement park...*"

In the time of completing my PhD, I could not find a more appropriate quote to describe my feelings about it. When I chose to go on it, it seemed so harsh to even figure the end of this ride. Then, after all ups and downs, and thrills and chills, this ride got to reach to the point, and I am asking which point is it.

When the brilliant comedian Bill Hicks. came out with this reflection, in early 90's, consumer society was at the apex of its burst. TV advertisements built up the illusion of welfare and shaped everyone's life. This powerful tool replaced our mind in its role as Creator of one's reality, by filling it with unnecessary needs and forcing us to look through a given filter, which entrapped our lives in the "work, eat, buy, consume, die" scheme.

Nowadays the potential of mass media escalated to another kind of level, as social media have occupied a great part of our lives in such a pervasive way. Information has been replaced by communication. This communication is often designed to target emotional centers in order to generate in the receiver a sense of anxiety and fear which makes people prone to acceptance, in the view of a given truth. It is noteworthy that in this era of communication, social homologation is something to be proudly shown, at every level of society. In this state of hypnotic fascination there is no more room for debate, as any carrier of critical thought is the real danger.

But what does it have to do with my PhD? I think that apart of doing lab experiments and writing papers, the main point one could get from such a challenge is to learn to always long for truth, no matter how complex it is, and to be courageous enough to support one's thesis, no matter how big is the price. This both in Science and Life. After all…

"*…it doesn't matter, because it's just a ride. And we can change it any time we want. It's only a choice…*"

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