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EVT-701: inhibition du complexe I de la chaine respiratoire comme stratégie thérapeutique en cancer

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A mi familia, mi sur y a mis cuatro puntos cardinales

Resumé en français

Cibler le métabolisme énergétique a récemment été identifié comme une approche thérapeutique prometteuse pour lutter contre le cancer. Les cellules cancéreuses ont des caractéristiques métaboliques particulières qui les rendent différentes des cellules « normales ». Le métabolisme oxydatif mitochondrial (OxPhos) a été proposé comme une vulnérabilité métabolique et un des mécanismes de résistance thérapeutique impliquée dans la rechute pour plusieurs types de tumeurs. Le fonctionnement de la chaine respiratoire mitochondriale a pour finalité la production de l'ATP mitochondrial, source d'énergie pour les cellules. Elle a également pour but de soutenir la prolifération cellulaire grâce au maintien des niveaux des intermédiaires métaboliques essentiels pour la biosynthèse nucléotidique. Différents types de cancer du sous-type OxPhos montrent une surexpression des gènes du métabolisme mitochondrial. Ces cancers OxPhos sont ainsi plus vulnérables au ciblage thérapeutique de la respiration mitochondriale. De plus, les cellules cancéreuses peuvent augmenter leur métabolisme oxydatif en réponse à la chimiothérapie, comme démontré pour les leucémies aigües myéloïdes (LAM). Ceci ouvre une opportunité unique pour lutter contre la chimiorésistance et les rechutes. Il existe ainsi un besoin évident de développer des agents thérapeutiques sûrs et sélectifs pour cibler la dépendance à l'OxPhos des cellules cancéreuses. Ces travaux doctoraux présentent l'EVT-701, un nouvel inhibiteur du complexe I de la chaine respiratoire (ETC I) et ses effets anti-cancéreux et notamment anti-leucémiques. Ce composé est sélectif, toléré et démontre une activité significative dans plusieurs modèles de tumeurs solides. Nous avons aussi évalué l'effet de l'EVT-701 dans un contexte de chimiorésistance et caractérisé son effet immuno-modulateur dans un modèle murin de LAM. Ainsi, nous avons mis en évidence un nouveau rôle de l'ETCI dans la régulation de l'expression des immune checkpoints molécules PD-L1 et CD39 dans des cellules de LAM. Enfin, ces travaux confirment le rôle de l'ETCI dans le cancer, renforcent l'intérêt thérapeutique de moduler son activité et montrent son rôle dans l'immuno-modulation, jusqu'alors méconnu pour les LAM.

English abstract

Targeting energetic metabolism has emerged in the recent years as an attractive and promising anti-cancer therapy. Cancer cells show metabolic features that distinguish them from normal cells, going beyond the initially described Warburg effect. Reliance on oxidative phosphorylation (OxPhos) has been identified as a vulnerability of many cancer types. Functional electron transport chain (ETC) is essential for mitochondrial ATP production, as well as for supporting cell proliferation by maintaining appropriate levels of metabolic intermediates essential for nucleotide biosynthesis. Cells from different cancer types overexpress OxPhos and mitochondria genes, displaying enhanced vulnerability to the inhibition of mitochondrial respiration. Furthermore, some molecular alterations such as LKB1 deficiency also render cancer cells susceptible to ETC inhibition. Interestingly, chemotherapy has also been reported to increase OxPhos metabolism in many cancer types including acute myeloid leukemia (AML), paving the way for a novel therapeutic opportunity to eradicate chemoresistance and prevent patient relapse. Overall, this enhanced OxPhos dependence strengths the need for new selective and safe therapeutic agents. This thesis introduces a novel ETCI inhibitor, EVT-701, that is selective and safer than other drug candidates in this target class. It shows anti-cancer activity in several solid cancer tumor models as single agent. Furthermore, we assessed the anti-leukemic effects of EVT-701 in an AML model of resistance to chemotherapy and we discovered a new regulatory role of ETCI on immune checkpoints PD-L1 and CD39 in leukemic cells. In summary, my PhD work highlights the role of ETCI in cancer, the interest to target it, as well as it identifies an immunomodulatory role for ETCI in AML.

List of publications

1. Luna-Yolba, R., Visenstin, V., Hervé, C., Chiche, J., Ricci, J-E, Méneyrol, J., Paillase, MR. and Alet, N. (2021). EVT-701 is a novel selective and safe mitochondrial complex I inhibitor with potent anti-tumor activity in models of solid cancer. *Pharmacology Research & Perspectives*.

2. Luna-Yolba, R., Marmoiton, J., Gigo, V., Boet, E., Sahal, A., Alet, N., Abramovich, I., Gottlieb, E., Visenstin, V., Paillase, MR. and Sarry J-E (2021). Disrupting mitochondrial electron transport chain complex I decreases immune checkpoints in murine and human acute myeloid leukemic cells. *Cancers*.

Oral communications

1. Characterization of AML resistance and evaluation of new therapeutic approaches. METACAN ITN. Leuven, September 2019

2. EVT-701 as therapeutic agent in AML. METACAN ITN. Zoom, September 2020

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Abbreviations

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1,3-BPG: 1,3-biphosphoglycerate
2PG: 2-phosphoglycerate
3PG: 3-phosphoglycerate
3PHP: 3-phosphohydroxypyruvate
4E-BPs: eukaryotic initiation factor 4E-binding proteins
5mC: methlycytosine
6PGL: 6-phosphogluconolactone
6PG: 6-phosphogluconic acid

A

ACC: acetyl-CoA carboxylases
ACSS2: cytoplasmic acetyl-CoA synthetase
ACLY: ATP-citrate lyase
ACO2: Aconitase 2
ADC: antibody drug conjugate
ADC: arginine decarboxylase
ADME: absorption, distribution, metabolism, and excretion
ADP: Adenosine diphosphate
α-KG: α-ketoglutarate
AIF: Apoptosis Inducing Factor
ALD: aldolase
ALL: acute lymphoblastic leukemia
ALT: alanine transaminase
AML: acute myeloid leukemia
AMPK – AMP-kinase
AMP – Adenosine monophosphate
AOM: azoxymethane
APL: acute promyelocytic leukemia
AR: allelic ratio
AraC: cytosine arabinoside, cytarabine
ASCT: Alanine, Serine, Cysteine Transporter
ASL: argininosuccinate lyase
ASNS: asparagine synthetase
ASS: argininosuccinate synthase
ATM: Ataxia telangiectasia mutated
ATO: arsenic trioxide
ATP – Adenosine triphosphate
ATRA: α-trans retinoic acid
AZA: azacytidine

B

BCAA: branched-chain amino acid
BCL-2: B-cell CLL–lymphoma 2
BCR: B-cell receptor
BCSCs: breast cancer stem cells
bHLH LZ: basic-helix-loop-helix-leucine zipper
BLI: bioluminescence

C

Carbamoyl-P: carbamoyl phosphate
CAC: colitis-associated colorectal cancer
CAD: Carbamoyl phosphate synthase II, Aspartate transcarbamoylase and Dihydroorotase
CAFs: cancer-associated fibroblasts
CAMKK2/ CAMKKβ: calcium/calmodulin-dependent protein kinase kinase 2
CASTOR1: cellular arginine sensor for mTORC1
CBF: core-binding factor
cDC: conventional dendritic cell
CDP: common DC progenitor
CDK: cyclin-dependent kinase
CDKN: cyclin-dependent kinase inhibitors
CEBPA: CCAAT/enhancer binding protein α
ChREBP: carbohydrate-responsive element-binding protein
CK: creatine kinase
CLP: common lymphoid progenitor
CMLP: common myelolymphoid progenitor
CMP: common myeloid progenitor
CMPK1: cytidine monophosphate kinase 1
CoA: coenzyme A
COX: cyclooxygenase
COX: cytochrome c oxidase
CPGIII : coproporphyrinogen III
CPOX: coproporphyrinogen oxidase
CPT: carnitine palmitoyl transferase
CR: complete remission
CReP: constitutive repressor of eIF2a phosphorylation
CT: carnitine transporter
CYP: cytochrome P450
Cys: cysteine

D

d: days
DAC: decitabine
DC: dendritic cell
DSS: dextran sodium sulfate
DCK: deoxycytidine kinase
dCTP: deoxycytidine triphosphate
DHAP: dihydroxyacetone phosphate
DHO: dihydroorotate
DLBCL: Diffuse large B cell lymphoma
DMPK: Drug Metabolism and PharmacoKinetics
DNA: deoxyribonucleic acid
DNMTs: DNA-methyltransferases
DNMT3A: DNA (cytosine-5)-methyltransferase 3A
DRP1: Dynamin-related protein 1

E

E4P: erythrose-4-phosphate
EC: endothelial cells

EF2K: elongation factor-2 kinase
EFS: event-free survival
EGFR: epidermal growth factor receptor
ELOVLs: elongation of very long fatty acids
ENO1: enolase 1
ENT1: equilibrative nucleoside transporter 1
EPO: erythropoietin
ER: endoplasmic reticulum
ETC: electron transport chain

F
F1P: fructose-1-phosphate
F1,6BP: fructose-1,6-biphosphate
F6P: fructose-6-phosphate
FA: fatty acid
FAB: French, American and British
FABPs: FA binding proteins
FAD/$FADH_2$: flavin adenine dinucleotide
FADSs: FA desaturases
FAO: fatty acid oxidation
FAT: FA translocase
FATPs: FA transport proteins
FAS: fatty acid synthesis
FASN: fatty acid synthase
FBS: fetal bovine serum
FDA: Food and Drug Administration
FDXR: Ferredoxin Reductase
FECH: ferrochelatase
FIHs: Factors inhibiting HIFs
FLT3:– FMS-like tyrosine kinase 3
FLCN-FNIP2: folliculin- folliculin-interacting protein 2
FLVCR: feline leukaemia virus subgroup C receptor
FTDH: formate dehydrogenase

G
G1P: glucose-1-phosphate
G6P: glucose-6-phosphate
GA3P: glyceraldehyde 3-phosphate
GADD: Growth arrest DNA damage-inducible protein
GAMT: guanidinoacetate methyltransferase
GC: glutamate carrier
GCN2: general control nonderepressible 2
G-CSF: Granulocyte colony stimulating factor
GFAT: glutamine fructose-6-phosphate amidotransferase
GlcN: glucosamine
GlcN6P: glucosamine-6-phosphate
GlcNAc-6P: N-acetylglucosamine-6-phosphate
GLS: glutaminase
GLUD: glutamate dehydrogenase
GLUT: glucose Transporter
Glu: glutamate
Gly: glycine
GM-CSF: granulocyte-monocyte colony-stimulating factor
GMP: granulocyte and macrophage progenitor
GNPNAT: glucosamine-phosphate N-acetyltransferase
GOT: glutamate-oxaloacetate transaminase
GPCRs: G protein-coupled receptors
GPT: glutamate-pyruvate transaminase
GS: glutamine synthetase
GSK3: Glycogen synthase kinase 3
GVHD: graft-versus host disease

H
2-HG: 2-hydroxyglutarate
HCT: hematopoietic cell transplantation
HER2: human epidermal growth factor receptor 2
HIF: hypoxia-inducible factor
HK: hexokinase
HLA: human leukocyte antigen
HMA: hypomethylating agents
HMGR: 3-hydroxy-3-methylglutaryl-CoA reductase
HRI: heme-regulated inhibitor
HSCs: hematopoietic stem cells
HSP: heat shock protein

I
IAP: inhibitor of apoptosis
IDAC: Intermediate dose AraC
IDH: isocitrate dehydrogenase
IL: interleukin
IMP2: insulin-like growth factor 2 mRNA-binding protein 2
IRF1: Interferon regulatory factor 1
IRG1: immune-responsive gene 1
IRS-1: insulin receptor substrate 1
ISR: Integrated stress response
ITD: internal tandem duplication
IV: intravenous

J
JNK1: c-Jun N-terminal protein kinase 1

K
KMT2A : Lysine Methyltransferase 2A
KRAS: Kirsten rat sarcoma viral oncogene homologue

L
LAT1: L-type amino acid transporter 1
LDAC: low dose cytarabine
LDHA : lactate dehydrogenase-A
LDLR: low-density lipoprotein receptor
LSC: leukemic stem cell

M
M-CSF: monocyte-macrophage colony-stimulating factor
Man-PMs: mannose-modified macrophages-derived microparticles
MCP: mast cell progenitor
MCT: monocarboxylate transporter

MDM2: mouse double minute 2 homologue
MDP: macrophage and DC progenitor
MHC: major histocompatibility complex
MIEAP: Mitochondria Eating Protein
MILIP: Myc-Inducible Long noncoding RNA Inactivating P53
MRD: minimal residual disease
MDS: myelodysplasia syndrome
ME: malic enzyme
MEFs: mice embryonic fibroblasts
MEP: megakaryocyte and erythroid progenitor
MFC: multiparameter flow cytometry
MFF: mitochondrial fission factor
MOMP: mitochondrial outer membrane permeabilization
MPN: myeloproliferative neoplasm
MPT: mitochondrial permeability transition

N
NAD/NADH – Nicotinamide adenine dinucleotide
NADP/NADPH – Nicotinamide adenine dinucleotide phosphate
NAM: nicotinamide
NAMPT: nicotinamide phosphoribosyltransferase
NDPKs: nucleoside diphosphate kinases
NHL: Non-Hodgkin lymphoma
NK: natural killer
NLN: neurolysin
NMN: nicotinamide mononucleotide
NO: nitric oxide
NOS: nitric oxide synthase
NPM1: Nucleophosmin 1

O
OAA: oxaloacetate
OMM: outer mitochondrial membrane
OS: overall survival

P
PARP: poly (ADP-ribose) polymerase
pDC, plasmacytoid DC
PDGFR: platelet-derived growth factor receptor
PDH: pyruvate dehydrogenase
PDK: pyruvate dehydrogenase (acetyl-transferring) kinase
PDPK1: phosphoinositide-dependent protein kinase 1
PEP: phosphoenolpyruvate
PERK: PKR-like endoplasmic reticulum kinase
PGAM: phosphoglycerate mutase
PGC-1α: PPARG coactivator 1α
PGDH: Phosphoglycerate dehydrogenase
PGE_2:Prostaglandin E_2
PGI: phosphoglucose isomerase
PGK: Phosphoglycerate kinase
PHDs: prolyl hydrolyxases
PHGDH: D-3- phosphoglycerate dehydrogenase
PI3K: phosphatidylinositol 3-kinase
PIP2: phosphatidylinositol 4,5-bisphosphate
PIP3: phosphatidylinositol 3,4,5-trisphosphate
PK: pyruvate kinase
PKC: protein kinase C
PKR: doublestranded RNA-dependent protein kinase
PML: promyelocytic leukemia
PMN: polymorphonuclear
PPAR: Peroxisome proliferator-activated receptors
PPAT: phosphoribosyl pyrophosphate amidotransferase
PPOX: protoporphyrinogen oxidase
PPP: pentose phosphate pathway
PRDX3: peroxiredoxin 3
PRPP: phosphoribosyl pyrophosphate
PRPS: phosphoribosyl pyrophosphate synthetase
PSAT: phosphoserine transaminase
P-Serine: phosphoserine
PSPH: phosphoserine phosphatase
PTEN: phosphatase and tensin homologue
PTP: permeability transition pore

Q
q: every

R
R5P: ribose-5-phosphate
Ru5P: ribulose-5-phosphate
RHEB: Ras-related small G protein Ras homologue enriched in brain
RPE: ribulose 5-phosphate epimerase
RPI: ribose-5-phosphate isomerase
ROS: reactive oxygen species
RSC: respiratory supercomplexes
RTKs: receptors tyrosine kinases

S
S6K1: p70 S6 kinase 1
S7P: sedoheptulose-7-phosphate
SAT1: spermidine/spermine N1-acetyltransferase 1
SCAFI: supercomplex assembly factor 1
SCO2: Synthesis of Cytochrome c Oxidase 2
SC: subcutaneous
SCD: stearoyl-CoA desaturases
S-CoA: free coenzyme A
SHMT: serine hydroxymethyltransferase
SIRT1: sirtuin 1
SLC1A4: Solute Carrier Family 1 member 4
SLC1A5: solute carrier family 1 member 5
SLC29A1: carrier family 29 member 1
SMARCC1: SWI/SNF Related, Matrix Associated, Actin Dependent Regulator Of Chromatin Subfamily C Member 1
SOD2: enzyme Superoxide Dismutase 2
SRC-2: steroid receptor coactivator 2
SREBPs: sterol regulatory element binding proteins

T

TA: transaminase
TAMs: tumor associated macrophages
TALDO: Transaldolase
TCA: tricarboxylic acid
TCR: T cell receptor
TDG: thymine DNA glycosylase
TET: ten eleven translocation
THF: tetrahydrofolate
TIF-1A: transcription initiation factor 1A
TIGAR: TP53-inducible glycolysis and apoptosis regulator
TIL: tumor infiltrating cells
TKT: Transketolase
TME: tumor microenvironment
TNBC: triple negative breast cancer
TNF α: tumor necrosis factor α
TPI: triose phosphate isomerase
TSC: heterotrimeric tuberous sclerosis complex

U

UBF: factors upstream binding factor
UDP-GlcNAc: uridine diphosphate N-acetylglucosamine
UPR: unfold protein response

V

VDAC: voltage-dependent anion channel
VEGFR: vascular endothelial growth factor receptor

W

WBC: White blood cell counts
WHO: World Health Organisation

X

X5P: xylulose-5- phosphate

Y

Y: years

Z

1. Introduction

1.1. Metabolic adaptations in cancer

Cancer cells must adapt their metabolism to meet their energetic demand, maintain viability and generate new biomass for the biosynthesis of new macromolecules needed for cell proliferation (Pavlova and Thompson, 2016; Vazquez et al., 2016). Indeed, one of the emerging hallmarks of cancer is the reprogramming of energy metabolism (Hanahan et al., 2000). It is also known that energy metabolism is modulated by proteins that are involved in one way or another in programming the core hallmarks of cancer; such as hypoxia inducible factor 1 (HIF-1) transcription factor, or oncogenes (Vazquez et al., 2016).

Since Otto Warburg described in 1925 the "aerobic glycolysis", known today as Warburg Effect, as a feature of cancer cell metabolism that supports large biosynthetic processes required for proliferation, other metabolic alterations have been identified in cancer context. Nowadays, it is accepted that cancer cells adapt their metabolism to support different biological needs along disease progression as well as during the response to therapy. Hereafter are described the three main cabon sources used by cancer cells to support tumor progression and resistance.

1.1.1. Glucose metabolism

Glycolysis

Glycolysis is the metabolic pathway that converts glucose to pyruvate and yields energy in the form of two molecules of ATP (**figure 1**). Glucose enters the cells through glucose transporters (GLUTs). Once in the cytoplasm, glucose is phosphorylated to glucose-6-phosphate (G6P) by hexokinase (HK). G6P can either enter into the pentose phosphate pathway (PPP) to generate NADPH and carbons for nucleotide biosynthesis or continue the glycolytic flux, where it will become fructose-6-phosphate (F6P) by the action the phosphoglucose isomerase (PGI), for being later on phosphorylated by the phosphofructokinase-1 (PFK-I), resulting in fructose-1,6-biphosphate (F-1,6-BP). Then, aldolase (ALD) will excise F-1,6-BP into two molecules containing three carbons each: dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (GA3P). DHAP and GA3P can be interconverted one in another by the triose phosphate isomerase (TPI), and while DHAP can enter the synthesis of glycerol-3-phosphate for lipid biosynthesis, GA3P continues the glycolytic pathway, where glyceraldehyde-3-phosphate dehydrogenase (GAPDH) renders 1 NADH per molecule and 1,3-biphosphoglycerate (1,3-BPG). Phosphoglycerate kinase (PGK) will render 1 ATP per molecule in the transformation of 1,3-PG into 3-phosphoglycerate by (3-PG). 3-PG can either enter the serine/glycine biosynthesis or the one carbon cycle

(that provides methyl groups for several biological processes such as synthesis of DNA, polyamines, amino acids..), or be converted to 2-phosphoglyceric acid (2-PGA) by phosphoglycerate mutase (PGAM), the substrate of the enolase 1 (ENO1) that will transform it into phosphoenol pyruvate (PEP). Subsequently, PEP is converted into pyruvate by the pyruvate kinase (PKM2), producing 1 ATP per molecule of pyruvate. The NADH generated during the process, will then be oxidized to regenerate NAD^+, either in the mitochondria during oxidative phosphorylation or by the lactate dehydrogenase-A (LDHA), which converts pyruvate to lactate (Stryer et al., 2010; Pavlova et al., 2016).

The process is regulated at several points. PFK is the rate-limiting enzyme of the glycolysis, being allosterically inhibited by high concentrations of ATP, low pH, PEP and citrate, while it is activated by high concentrations of AMP, ADP and F-2,6-BP (Hasawi et al., 2014; Lee et al., 2017). Another point of regulation is the HK, the first enzyme of glycolysis, which is inhibited by its product, glucose 6-phosphate. Also the pyruvate kinase (PKM2), the last enzyme of the process that is allosterically inhibited by ATP, which indicates that energy charge is high, as well as by alanine, which is synthesized from pyruvate by the alanine transaminase (ALT) indicating that building blocks are abundant (Li et al., 2015).

Several glycolytic enzymes have been reported to be upregulated and/or essential in cancer context. HK2 is upregulated in pancreatic cancer, lung cancer, hepatocyte cell cancer, breast cancer, and prostate cancer (Jiang et al., 2012; Patra et al., 2013; DeWaal et al., 2018), as well as in leukemia with activating mutations in the tyrosine kinase receptor FLT3, where HK2 upregulation is mediated by Akt, promoting glycolysis and rendering these cells sensitive to glycolysis inhibition (Ju et al., 2017). In human glioblastoma, AKT sustained activation derived from phosphatase and tensin homologue (PTEN) loss and epidermal growth factor receptor (EGFR)-dependent phosphatidylinositol 3- kinase (PI3K) activation, phosphorylates and increases stability of PFK1, by impairing its degradation through binding to TRIM21 E3 ligase, what leads to increased PKF1 activity and cell proliferation (Lee et al., 2017). PFK1 as well as ALDA, LDHA, PGK and enolase 1 are induced by HIF-1α (Semenza et al., 1994), and are often found stabilized in hypoxic regions of solid tumors. PKM2 seems essential in lung cancer, where its deletion in xenografts of human lung cancer results in reduced tumor growth (Christofk et al., 2008). The deletion of the glycolytic enzymes pyruvate kinase isoform M2 (PKM2) or lactate dehydrogenase A (LDHA) impairs leukemia initiation (Wang et al., 2014), highlighting the involvement of the glycolytic pathway at initial phases of the disease. Interestingly, it's been also reported that leukemic cells induce a diabetic state in the host by impairing insulin sensitivity and secretion through the production of insulin-like growth factor binding protein 1 (IGFBP1). This leads to a decrease in glucose uptake by host tissues, leaving it available for the leukemic cells (Ye et al., 2018), and denotes the importance of glucose for leukemic cells. These are only a few examples of how cancer cells and glucose metabolism are closely connected.

To conclude, glycolysis is increased in many cancers due to the fact that many glycolytic intermediates are precursors for biosynthetic pathways. For example, G6P is precursor for NADPH producing PPP, which is

used to sustain redox balance protecting from oxidative stress by reducing glutathione via glutathione reductase, and ribose-5-P for nucleotide biosynthesis, both essentials to maintain cell viability and proliferation.

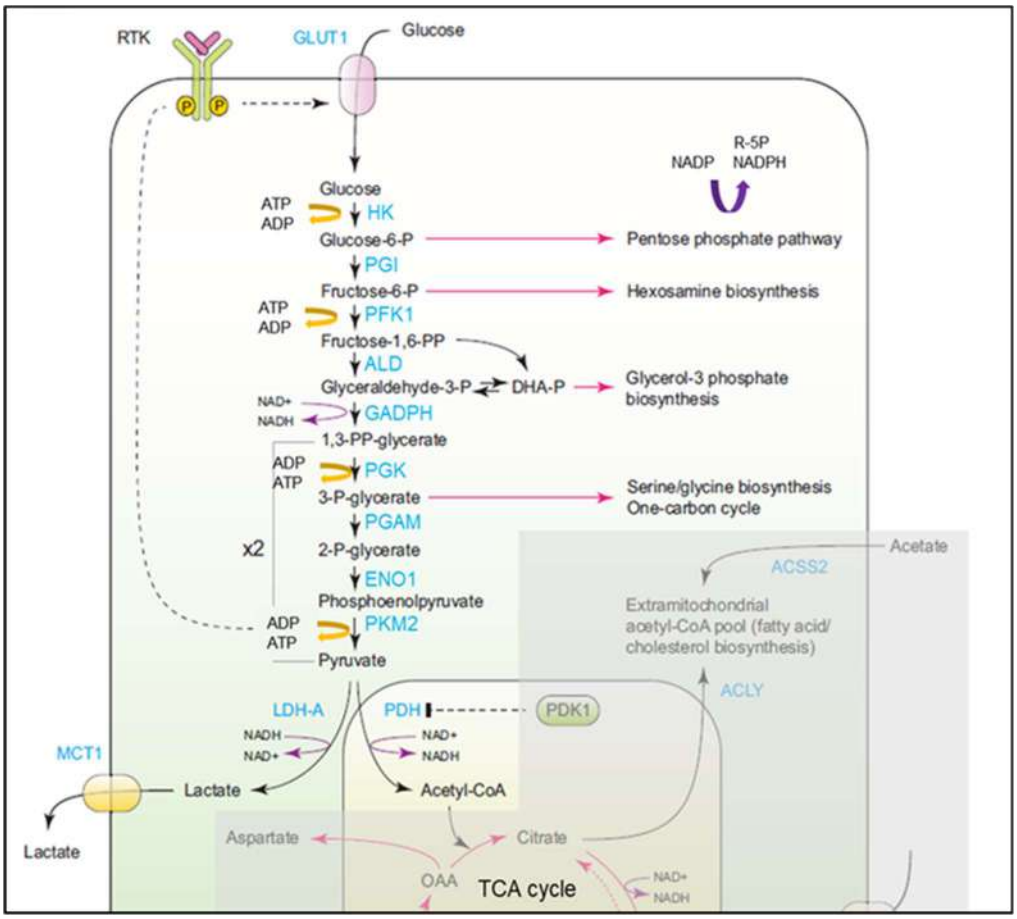

Figure 1. Glycolysis at a glance.
Adapted from Pavlova and Thompson, 2016.

Pentose phosphate pathway

The pentose phosphate pathway (PPP), represented in **figure 2**, is a key cellular pathway for cell survival since it is the major contributor to NADPH generation within the cell. NADPH is required for fatty acid synthesis as well as for cellular protection from oxidative stress, caused by reactive oxygen species (ROS). PPP participates also in nucleotide and amino acid biosynthesis. Therefore, it is an essential pathway for proliferating cells.

PPP is divided in two parts. The first one is known as the oxidative branch of the PPP, it is unidirectional and it is critical to maintain redox balance. PPP branches from glycolysis at its first step, where hexokinase generates glucose-6-phosphate (G6P). Glucose-6-phosphate dehydrogenase (G6PDH) converts G6P into 6-phosphogluconolactone and yields one NADPH. 6-Phosphogluconolactone will be

hydrolyzed by phosphogluconolactonase (6PGL) into 6-phosphogluconate. Then, 6-phosphogluconate dehydrogenase (6PGDH) will generate another molecule of NADPH and ribulose-5-phosphate (Ru5P) by decarboxylation. Subsequently, Ru5P enters the non-oxidative branch, where it can be converted into ribose-5-phosphate (R5P) by the ribose-5-phosphate isomerase (RPI), essential for nucleotide biosynthesis; or into xylulose 5-phosphate by ribulose 5-phosphate epimerase (RPE). Transketolase (TKT) generates G3P and sedoheptulose 7-phosphate (S7P) or fructose 6-phosphate (F6P) from xylulose 5-phosphate and ribose 5-phosphate or erythrose 4-phosphate. Transaldolase (TALDO) generates glyceraldehyde 3-phosphate and erythrose 4-phosphate from fructose 6-phosphate and sedoheptulose 7-phosphate. TALDO and TKT are therefore the connection between glycolysis and the PPP. The non-oxidative branch is reversible and the allosteric regulation of the enzymes in the pathway allow the branch to supply glycolytic intermediates or ribose-5-P accordingly to the metabolic demands of the cell (Patra et al., 2014; Stincone et al., 2014).

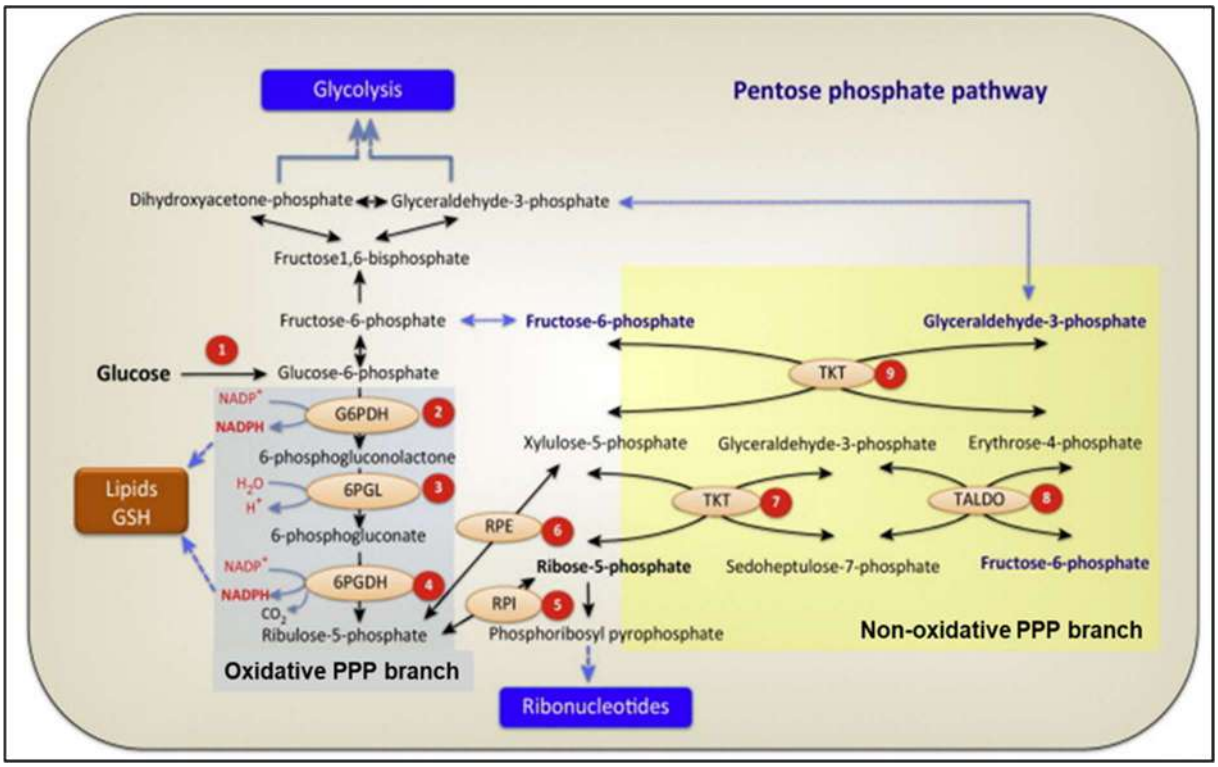

Figure 2. The pentose phosphate pathway (PPP).
Adapted from Patra et al., 2014.

Due to the involvement of PPP in the generation of NADPH, necessary for oxidative stress protection as well as in lipid synthesis, it is not strange that cancer cells make extense use of this pathway, being essential for tumor cells proliferation. G6PDH activity is regulated by oncogenes such as PI3K/AKT, Ras, Src, mTOR and by oncosuppressors such as p53 and TAp734 (Patra and Hay, 2014; Stincone et al., 2014; Mele et al., 2018). In acute myeloid leukemia (AML), it has been described that increased glycolytic flux and glucose addiction is mediated by mTOR activation, and it is used to sustain glucose for the PPP,

identifiying G6PDH as therapeutic target in AML (Poulain et al., 2017). In addition, increased expression of PPP enzymes, such as G6PDH or TKT correlate with poor prognosis in breast cancer and other cancer types (Lucarelli et al., 2015; Benito et al., 2017; Mele et al., 2018), and the inhibition of these enzymes results in decreased tumor cell growth (Benito et al., 2017). 6PGDH, the third enzyme of the PPP oxidative arm is as upregulated in colorectal cancer, thyroid tumors and lung cancer, and its suppression resulted in decreased tumor cell proliferation and tumor growth, mediated by decreased lipid and RNA biosynthesis and elevated ROS levels in cancer cells (Lin et al., 2015). In addition, the knock-down of 6-6PGDH within the oxidative branch of the PPP reduces NADPH production and has been reported to reduce AML cell growth as well (Bhanot et al., 2017), putting in evidence the importance of this pathway for maintaining redox balance to allow cancer cell proliferation. Upregulation of the fructose bisphosphatase 1 (FBP1) is important for AML induced by deregulated expression of ecotropic virus integration site 1 (EVI1). FBP1 decreases glycolysis while it enhances PPP by increasing F6P levels, and the individual knocking down of the PPP enzymes (G6PDH, 6PGDH, RPI) decreases the colony forming capacity of EVI1 transduced cells, indicating that PPP stimulation by FBP1 is essential for progression of EV1I leukemia (Mizuno et al., 2019).

In brief

Glucose contributes to energy generation as well as it sustains the biosynthesis of numerous macromolecules in the form of glycolytic intermediates. In addition, glucose supports NADPH generation through the oxidative branch of the PPP, as well as nucleotide biosynthesis through the non-oxidative PPP branch. Glucose metabolism is thus essential to support cancer cell viability and proliferation.

1.1.2. Amino acids

Amino acids play an essential role in cancer by contributing to different biosynthetic processes of macromolecules necessaries for proliferation, serving as energy source and being involved in maintaining redox balance. In addition, they can contribute to modulation of the immune response. In the next pages, the metabolism of the main amino acids with described involvement in cancer will be described.

Glutamine

Glutamine is the most abundant amino acid circulating in blood, and it is involved in many fundamental cell functions (**figure 3**), such as generation of metabolites to maintain mitochondrial metabolism, antioxidants, non-essential amino acids, pyrimidines, purines and fatty acids, being all of these processes important for proliferating cells (Yang et al., 2017).

Glutamine enters the cell by the solute-linked carrier family A1 member 5 (SLC1A5, also known as Alanine, Serine, Cysteine Transporter 2, ASCT2). Glutamine can also be generated from the condensation of ammonia and glutamate by the glutamine synthetase (GS), consuming ATP and contributing to amonia detoxification as well as to the neurotransmitter glutamate recycling (Matthews et al., 2010; Yang et al., 2016). Once inside the cell, it can either be used for biosynthesis of different molecules or it can be exported and exchanged by other amino acids such as leucine, whose presence activates mTOR via Rheb, by the antiporter L-type amino acid transporter 1 (LAT1). Another transporter involved in glutamine export is the cystine glutamate xCT antiporter (heterodimer of SLC7A11 and SLC3A2). More specifically, it exchanges glutamate derived from glutamine by cystine and when inside the cell, cystine, is reduced to cysteine. Cysteine, glutamate and glycine constitute the glutathione, a tripeptide (Glu-Cys-Gly) that neutralize peroxide free radicals, controlling therefore reactive oxygen species (ROS) and preventing cell damage. Cells can also get glutamine by breaking-down extracellular (micropinocytosis) or intracellular (autophagy) proteins normally under nutrient deprivation (Altman et al., 2016; Yang et al., 2017).

Once inside the cell, glutaminases (the kidney-type glutaminase is encoded by the gene GLS whereas liver-type glutaminase by the GLS2) convert glutamine into glutamate and ammonium ion (NH_4^+). Then, glutamate can be converted into α-ketoglutarate (α-KG) by two different mechanisms: glutamate dehydrogenase (GLUD) transform glutamate into α-KG releasing ammonium or aminotransferases can transfer the amine group from glutamate to produce other amino acids and α-KG. There are different transaminases, such as the glutamate-oxaloacetate transaminase (GOT) that transfer the nitrogen from glutamate to oxaloacetate to produce aspartate, the glutamate-pyruvate transaminase (GPT) that transfer the nitrogen from glutamate to pyruvate to generate alanine, and phosphoserine transaminase (PSAT), involved in serine biosynthesis, that transfers nitrogen from glutamate to 3-phosphohydroxypyruvate to result in phosphoserine and α-KG. Then, α-KG generated by either of the before mentioned processes, can

enter the TCA cycle to produce ATP through the generation of the reducing equivalents NADH and $FADH_2$. This process of glutamine conversion into TCA metabolites is called glutaminolysis. Another way of generating reducing equivalents is by malic enzyme 1 (ME1): the aspartate generated by the GOT from glutamate can be transported to the cytoplasm by the mitochondrial aspartate malate shuttle, where ME1 converts it in malate generating NADPH. Alternatively, aspartate can be converted into asparagine by the asparagine synthetase (ASNS), incorporated into nucleotide synthesis or into the urea cycle. Mullen and colleagues highlighted the role of glutamine in maintaining TCA intermediate levels upon mitochondrial defects by transformation of glutamine-derived α-KG to isocitrate through a reductive carboxylation reaction catalyzed by isocitrate dehydrogenase (IDH), using $NADP^+$/NADPH (Mullen et al., 2011, Mullen et al., 2014). Subsequently, oxaloacetate derived from glutamine can condense with acetyl-CoA, generating citrate that can be used for lipid biosynthesis via ATP-citrate lyase (ACLY) and fatty acid synthetase (FASN) (Altman et al., 2016; Yang et al., 2017). Glutamine is also involved in protein synthesis by supporting the overall amino acid pools. It also takes part in trafficking and stress responses suppression, by contributing to the synthesis of uridine diphosphate N-acetylglucosamine (UDP-GlcNAc), part of the hexosamine biosynthetic pathway, required for protein glycosylation, proper ER-Golgi trafficking and suppression of the endoplasmic reticulum (ER) stress pathway (Altman et al., 2016).

Silencing of the glutamine importer SLC1A5 resulted in inhibition of mTOR signaling and abrogated cell growth and survival in an osteosarcoma cell line (Durán et al., 2012). Similar effects were described in non-small cell lung cancer (NSCLC) cells (Hassanein et al., 2012). In the human B cell model of Burkitt lymphoma P493, the inhibition of glutamine metabolism by the GLS inhibitor BPTES (Robinson et al., 2007) resulted in diminished tumor growth *in vivo*, while *in vitro* it efficiently killed hypoxic cancer cells by increasing ROS levels and reducing ATP charge (Le et al., 2012). In the immunocompetent MYC-driven mouse model of hepatocellular carcinoma model, the loss of one GLS allelle or GLS pharmacological inhibition by BPTES resulted in blunted tumor progression (Xiang et al,. 2015). Another glutaminase inhibitor, CB-839, displayed significant antitumor activity in triple negative and basal like $HER2^+$ breast cancer xenograft models, both as single agent or in combination with paclitaxel (Gross et al., 2014). Glutamine is also essential for ovarian cancer, where it was shown that cancer-associated fibroblasts (CAFs) presented upregulated glutamine anabolic pathway to and support tumor cells by the secretion of glutamine. The same study also showed that targeting stromal GS resulted in tumor regression (Yang et al., 2016). Similar phenomena has been reported in prostate cancer (Mishra et al., 2018). In prostate cancer, the oncogenic steroid receptor coactivator 2 (SRC-2) transcriptional regulator was found upregulated, which stimulated reductive carboxylation of glutamine-derived α-KG to generate citrate and promote lipogenesis for cancer cell survival and metastasis (Dasgupta et al., 2015). Interestingly, epithelial-to mesenchymal transition in breast cancer was prevented by GLS inhibition, resulting in less invasive cancer and potentally impairing metastasis (Lee et al., 2016).

Targeting glutamine uptake is a therapeutic approach also in AML. Tumor formation in AML xenografts is inhibited by preventing the entry of glutamine into the cell by knocking down the glutamine transporter SLC1A5, which denotes AML dependency on glutamine for tumor progression. The same work also showed that targeting glutamine uptake or glutamine levels with L-asparaginase (that also harbors glutaminase activity) inhibited mTORC1, and induced apoptosis in primary AML cells (Willems et al., 2013). In addition, other work proposed that glutamine levels regulate oxidative phosphorylation in AML, and they showed that pharmacologic inhibition or genetic knock down of glutaminolysis via GLS1 decreased respiration and led cells to apoptosis (Jacque et al., 2015). An example of metabolic flexibility is AML with mutated FLT3-ITD, which normally relies on glucose to support central carbon metabolism, but, upon the effect of the tyrosine kinase inhibitor AC220, the glycolytic phenotype is reverted and glutamine becomes the main source to support central carbon metabolism. In addition, glutamine's role in maintenance of redox balance is essential for cell survival. This rational led to the therapeutic targeting of glutamine dependency by combining GLS inhibitors such as CB-839 with FLT3 kinase inhibitors, which have shown efficacy *in vitro* as well as *in vivo* models of AML (Gallipoli et al., 2018). Nevertheless, mechanisms of resistance arise. The expression of pyruvate carboxylase constitutes a mechanism for GLS resistance, since upon GLS suppression, cells are able to use glucose-derived pyruvate rather than glutamine for anaplerosis, by converting pyruvate into oxaloacetate that would fuel the TCA (tricarboxylic acid) cycle (Cheng et al., 2011).

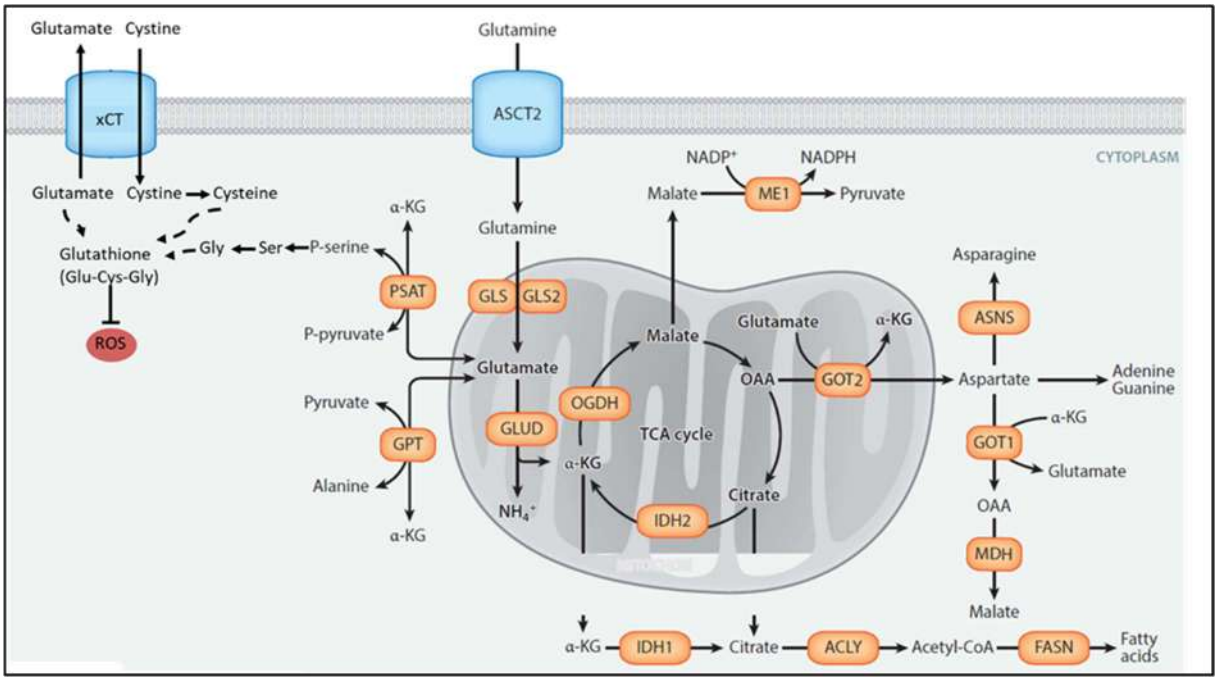

Figure 3. Glutaminolysis and related pathways
Adapted from Yang et al., 2017.

Serine and glycine

Serine is a non-essential amino acid that supports aminoacid and protein synthesis as well as glutathione production. It also takes part in the folate cycle as one-carbon donor, contributing to nucleotide biosynthesis, to NADPH generation and to methylation reactions. Serine can be uptaken by different neutral transporters like ASCT1/SLC1A4 or ASCT2/SLC1A5, or it can be *de novo* synthesized within the cell. Serine synthesis is of particular importance in brain, where presence of serine is essential for its role in neurotransmission and where due to the blood-brain barrier, the quantity of the amino acid that manage to cross the barrier is not sufficient to support normal brain function (Yang et al., 2016). *De novo* serine synthesis is derived from the glycolytic (or gluconeogenic) intermediate 3-PG. Then, D-3-phosphoglycerate dehydrogenase (PHGDH) generates NADH in the oxidation of 3-PG into 3-phosphohydroxypyruvate (3PHP), that will be subsequently converted into 3-phosphoserine by phosphoserine aminotransferase1 (PSAT1) with the generation of α-KG from glutamate, that can further enter the TCA for energy production or for biosynthetic pathways, such as lipid biosynthesis. Phosphoserine phosphatase (PSPH) will transform 3-phosphoserine into serine (Vazquez et al., 2016; Yang et al., 2016). In terms of regulation, 2-phosphoglycerate (2-PG), the resulting product of the reaction catalyzed by the phosphoglycerate mutase 1 (PGAM1) using 3-PG as substrate, activates PHGDH, stimulating the flux of carbons to serine synthesis pathway. Serine regulates the activity of PKM2, activating it when it is abundant, and decreasing its activity when there is a drop in serine levels. Remarkably, slow glycolytic flux allows for the bifurcation of 3-PG for serine synthesis (Yang et al., 2016). **Figure 4** recapitulates serine biosynthesis and its regulation.

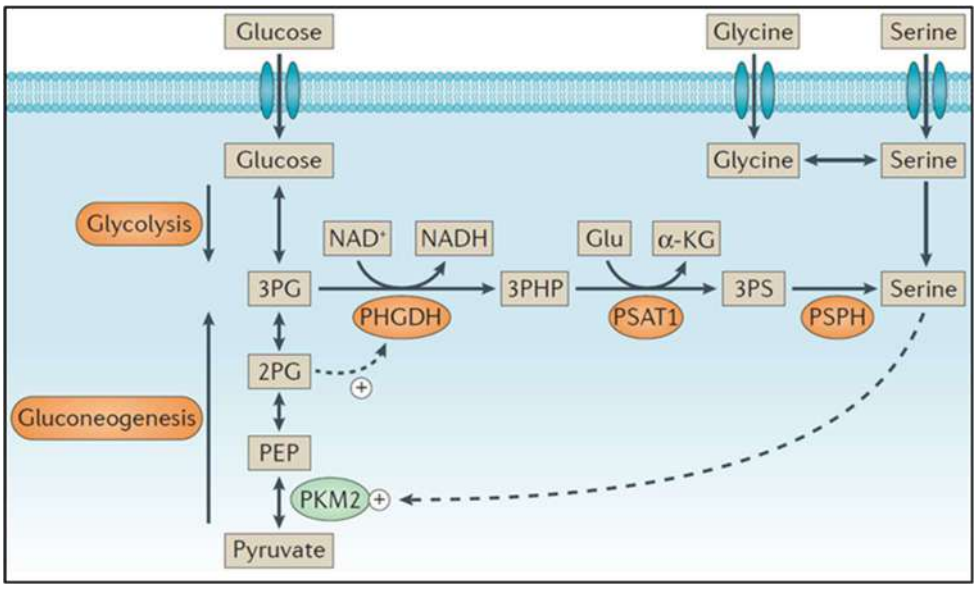

Figure 4. Serine synthetic pathway (SSP) and regulation.
Adapted from Yang and Vousden, 2016.

Serine can have different fates, summarized in **figure 5**. It can be converted into glycine by mitochondrial or cytosolic serine hydroxymethyltransferase (SHMT2 or SHMT1, respectively), releasing one carbon unit that will join the folate cycle, involved in nucleotide biosynthesis and regeneration of NADH and NADPH, as well as of ATP (Vazquez et al., 2016). Folate cycle is also coupled to methionine cycle, which supplies methyl groups for post-translational modifications of DNA, RNA, proteins and lipids. Serine is also involved in the synthesis of cysteine, a component of glutathione, essential for maintaining redox state. Serine also takes part in phospholipids synthesis, such as phosphatidylserine and sphingolipids (Yang et al., 2016). More recently, it has also been highlighted serine's role in ceramide synthesis, since under serine deprivation; a decrease in this mitochondrial membrane component concomitant to increased mitochondrial fragmentation has been described (Gao et al., 2018).

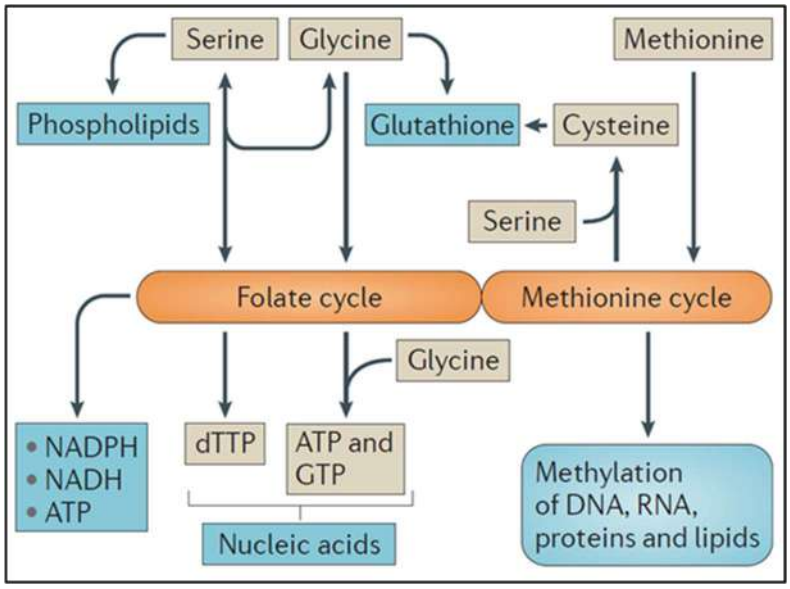

Figure 5. Overview of serine metabolism.
Adapted from Yang and Vousden, 2016.

Serine biosynthesis enzymes PHGDH and PSAT are upregulated upon glutamine withdrawal in AML, which can be a mechanism of resistance to the therapies targeting glutamine (Polet et al., 2015). This study identified that increase in oxidative stress triggered the upregulation of serine metabolism, which highlights the role of serine metabolism in oxidative stress protection. In addition, GLS inhibitors in combination with serine deprivation enhanced the inhibition of leukemic growth *in vivo* (Polet et al., 2015). The first enzyme of the serine synthesis pathways is also amplified in other cancers, such as breast cancer (Possemato et al., 2011) and melanoma (Locasale et al., 2011). PSPH upregulation, induced by the ribosomal mutation RPL10 R98S, is commonly found in T-acute lymphoblastic leukemia (T-ALL) patient samples, where it associates with higher levels of serine and glycine in mouse xenografts, and the abrogation of its expression of results in suppressed proliferation of the leukemic cells (Kampen et al., 2019).

Arginine

Arginine is considered a semi-essential amino acid, because under certain physiological conditions it has to be supplied from diet to meet cellular demands, although it can be *de novo* synthesized from citrulline (Wheatley, 2005). In arginine biosynthesis, cytosolic argininosuccinate synthase (ASS1) generates argininosuccinate from aspartate and citrulline, which is generated by the ornithine transcarbamylase from ornithine and carbamoyl-phosphate. Argininosuccinate is cleaved by argininosuccinate lyase (ASL), releasing arginine and fumarate. Arginine can then be reconverted into citrulline by the arginine deiminase (ADI) releasing its amino group, or by the nitric oxide synthase (NOS) producing also nitric oxide (NO), an important mediator of inflammation. In addition, arginine is substrate for further enzymes, such as arginase II to generate ornithine that will be used for polyamines, glutamate and proline synthesis, or become urea to excrete the excess of ammonia (Miraki-Moud et al., 2015; Keshet et al., 2018). It can also be converted to agmatine by the arginine decarboxylase (ADC) or to guanidinoacetate by guanidinoacetate methyltransferase (GAMT), and participate in creatine synthesis (Morris, 2002; Keshet et al., 2018). Creatine is phosphorylated to phosphocreatine by creatine kinase (CK) to buffer cellular ATP (Fenouille et al., 2017). Mitochondrial isoform CKMT1 is a gatekeeper for the permeability transition pore, since its depletion induces mitochondrial depolarization and apoptotic cell death (Datler et al., 2014). The main metabolic processes in which arginine is involved are summarized in **figure 6**.

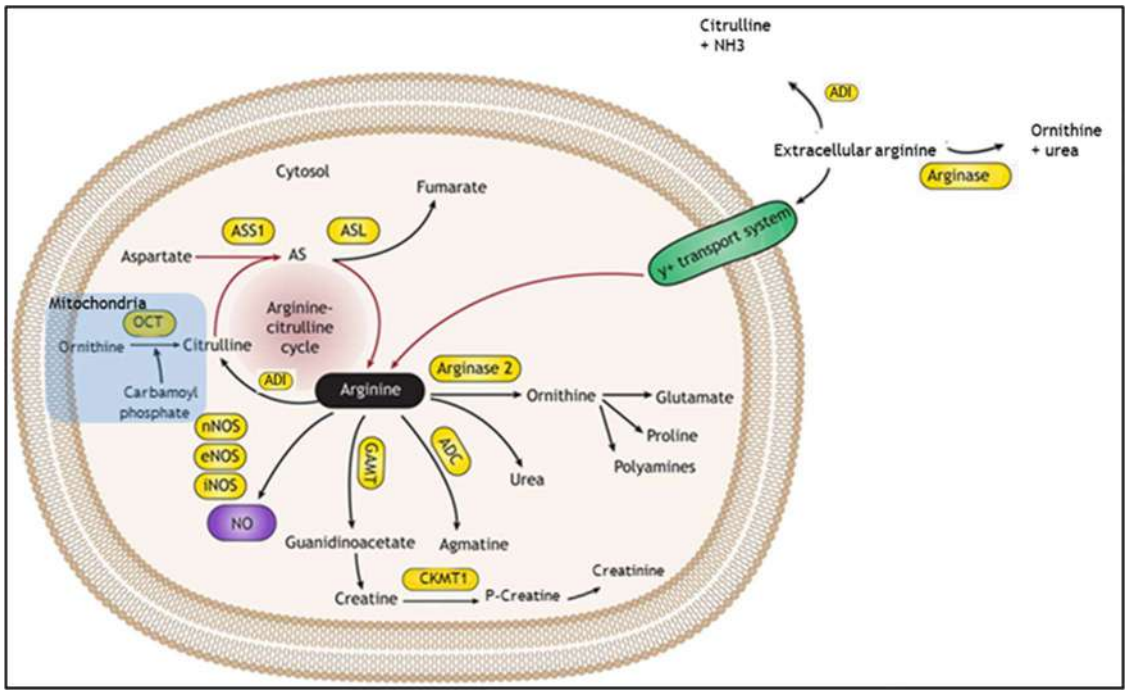

Figure 6. Schematic illustration of arginine metabolism.
Adapted from Keshet and Erez, 2018.

Downregulated expression argininosuccinate synthetase-1 (ASS1) is a common feature among almost all human cancer types, and it leads to arginine auxotrophy (Cheng et al., 2018). Upon arginine starvation concomitant with ASS1 deficiency, aspartate is depleted by the induction of the asparagine synthetase

(ASNS), what leads to the disruption of malate-aspartate shuttle, the activation of the integrated stress response (ISR) and decreased cell viability (Cheng et al., 2018). These authors also showed that dietary arginine restriction reduced tumor growth in a xenograft model of ASS1-deficient breast cancer (Cheng et al., 2018). AML patient's cells are also usually deficient in ASS1, which make leukemic cells auxotrophic for arginine. The use of pegylated arginine deiminase (ADI-PEG 20) depleted arginine pool and resulted in positive response *in vitro*. In addition, the combination of ADI-PEG 20 with AraC was efficient at controlling the disease *in vivo* (Miraki-Moud et al., 2015). In ASS1 deficient bladder cancer, pre-clinical studies also showed anti-cancer effect of arginine deprivation with ADI-PEG 20 both *in vitro* and *in vivo* and identified the activation of the general control nonderepressible 2 (GCN2) kinase, involved also in integrated stress response, as the mechanism mediating therapy efficacy by inducing cell apoptosis and autophagy (Sahu et al., 2017).This results suggest a key role for the integrated stress response in driving anti-cancer effect of arginine deprivation therapies. Arginine dependency of AML cells is also manifested by the constitutive expression of the arginine transporters CAT-1 and CAT-2B, given their deficiency in the argininosuccinate synthase and ornithine transcarbamylase (Mussai et al., 2015). The same group also showed that a pegylated human recombinant arginase, BCT-100, decreased arginine levels, blocked AML proliferation and decreased engraftment *in vivo* (Mussai et al., 2015). However, the outcome of a clinical trial in phase II (NCT01910012) with ADI-PEG 20 in monotherapy for relapsed/ refractory or poor risk AML was less positive than expected, achieving a complete remission rate < 5%, despite the patients having ASS1 deficiency (Tsai et al., 2017). It has also been published that EVI1 overexpression reprograms arginine metabolism, energy metabolism and nucleotide biosynthesis in hematopoietic stem cells. In addition, EVI1-positive AML is dependent on mitochondrial creatine kinase CKMT1, and its inhibition compromises mitochondrial respiration and ATP production, by altering arginine-creatine pathway (Fenouille et al., 2017).

Arginine is involved in immune regulation. Cytosolic or mitochondrial arginase (Arg1 or Arg2, respectively) hydrolyze arginine to ornithine and urea, limiting arginine availability for NO synthesis (Rath et al., 2014). Mechanistically (**figure 7**), arginine depletion blocks the expression of the CD3ζ chain of the T cell receptor (TCR), which prevents its assembly in the membrane and impairs T cell function (Rodriguez et al., 2004). In addition, arginine depletion has been shown to induce cell cycle arrest in T cells by decreasing expression of cyclin D3 and cyclin-dependent kinase 4 (cdk4) via the general control non-derepressible 2 (GCND2) kinase. The lack of cdk4-cyclinD3 complex prevents Rb phosphorylation and the subsequent release and nuclear translocation of E2F transcription factors, blocking cell cycle progression to S phase (Rodriguez et al., 2007). Inhibition of arginase activity has been reported to restore T cell function through increasing available arginine as well as to induce tumor regression in several cancer models (Steggerda et al., 2017; Miret et al., 2019). Recently, it has been proposed that Arginase 1 affects not only arginine catabolism, but also aerobic glycolysis by enhancing maximal glycolytic capacity in group 2 innate lymphoid cells (ILC2s), suggesting a complex network of metabolic intermediates and a

broader role of arginase 1 beyond urea cycle (Monticelli et al., 2016). ILC2s are involved in tissue inflammation regulation and repair (Kabata et al., 2018). Arginase I expression is found in myeloid derived suppressor cells (MDSCs) (Gabrilovichet al., 2012) and M2-like phenotype macrophages (DeNardo et al., 2019; Thapa and Lee, 2019; Viola et al., 2019), whereas leukemic blasts induce an immunosuppressive microenvironment by the expression and release of arginase II (Mussai et al., 2013).

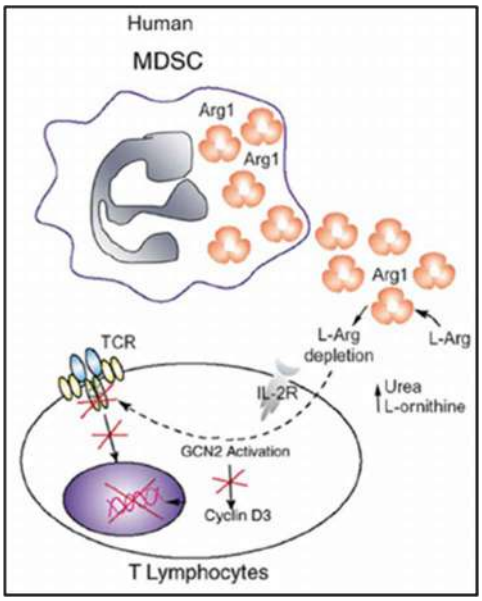

Figure 7. Argininase-mediated T cell dysfunction.
Adapted from Raber et al., 2012

In brief

Amino acids support essential functionalities for cancer cells:

- They can act as anaplerotic substrates and serve as energy source to replenish the TCA cycle (glutamine)
- They protect against oxidative stress by contributing to glutathione generation (glutamine, serine, glycine) and NADPH generation (serine, glycine)
- They can modulate immune function (arginine)
- They contribute to the biosynthesis of macromolecules necessary for proliferation, such as other amino acids, nucleic acids, phospholipids.

1.1.1. Lipids and fatty acids

Lipids are hydrophobic macromolecules with essential and varied functions. They are components of the cellular membranes, they are used for energy supply and storage, they can be a source of NADPH to maintain redox balanced, and they act as signaling molecules. Due to the highly proliferative rate and energy demand of cancer cells, lipids play an important role in cancer metabolism. The roles of lipids in cancer are summarized in **figure 8** (Röhrig and Schulze, 2016).

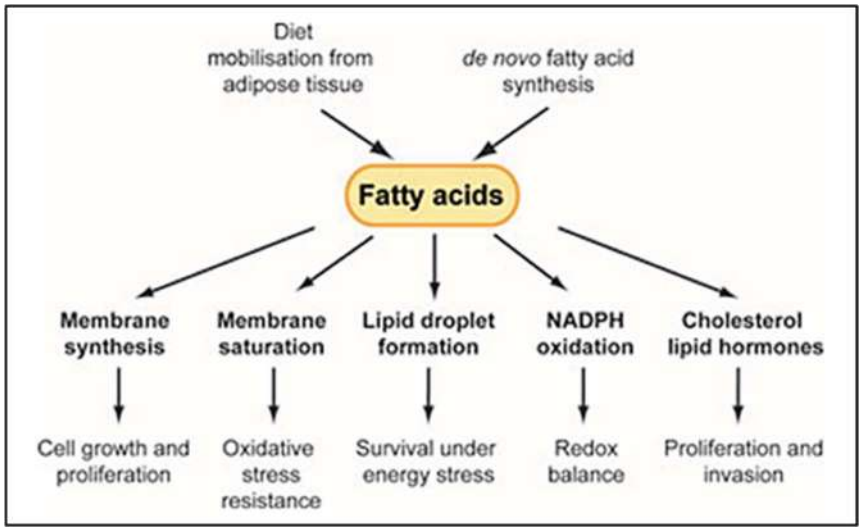

Figure 8. Lipids in cancer.
Adapted from Santos and Schulze, 2012.

Fatty acid synthesis

In human adults, physiologically, *de novo* fatty acid synthesis (FAS) is restricted to specific tissues: liver, adipose tissue and breast during lactation. However, cancer cells are able to perform FAS, connecting lipid biosynthesis to cancer development and progression (Menendez and Lupu, 2007; Röhrig and Schulze, 2016; Chen et al., 2019).

Fatty acid biosynthesis (**figure 9**) begins with the cytosolic conversion of acetyl-CoA into malonyl-CoA by acetyl-CoA-carboxylases 1 and 2 (ACC1, ACC2). Acetyl-CoA originates from the cleavage of citrate by ATP-citrate lyase (ACLY), which is phosphorylated and activated by AKT. Then, fatty acid synthase (FASN) generates palmitate from the serial NADPH-dependent condensation of 7 malonyl-CoA molecules with a priming one of acetyl-CoA. Palmitate (16:0) can then be elongated by FA elongases (elongation of very long fatty acids, ELOVLs) and desaturated at the Δ9 position by stearoyl-CoA desaturases (SCDs) to produce other FAs at the cytoplasmic face of the endoplasmic reticulum membrane. The inhibition of SCD1 reduces monounsaturated fatty acid synthesis as has antileukemic effects (Southam et al., 2015). Other FA desaturases (FADSs) can introduce double bonds at the Δ5 or Δ6 position, or at Δ9 in long-chain FAs (Carracedo et al., 2013; Röhrig et al., 2016; Kreitz et al., 2019). Short

chain FAs can passively cross the membrane and enter the cell, however, essential FAs need to be uptaken from the bloodstream via low-density lipoprotein receptor (LDLR) or other FAs transporters such as FA transport proteins (FATPs) or FA translocase (FAT) together with FA binding proteins (FABPs). Intracellular FAs pool has several and varied roles: they can be used for membrane synthesis (such as glycerophospholipids, cardiolipins and sphingolipids), for signaling processes (eicosanoids) or for the synthesis of triglycerides for energy storage (Santos and Schulze, 2012; Röhrig et al., 2016).

Numerous studies underscore the importance of FA biosynthesis for cancer cell growth and survival. One of the pioneer works in the field was the identification of the fatty acid synthase (FASN) as tumor antigen in breast cancer (Kuhajda et al., 1994). Inactivity of ACC1 (induced either by siRNA or by pharmacological drugs) caused apopotosis in prostate cancer (Brusselmans et al., 2005; Beckers et al., 2007) and in breast cancer (Chajès et al., 2006). The knockdown of ACLY impairs glucose metabolization to lipid in human adenocarcinoma cells (Hatzivassiliou et al., 2005) and murine lymphoid cells (Bauer et al., 2005), and leads to reduced tumor formation in xenografts (Bauer et al., 2005; Hatzivassiliou et al., 2005; Migita et al., 2008). In AML, the mitochondrial transacylase tafazzin, which generates the mature mitochondrial cardiolipin, has been reported to be essential for the growth, viability and stemness of AML cells (Seneviratne et al., 2019).

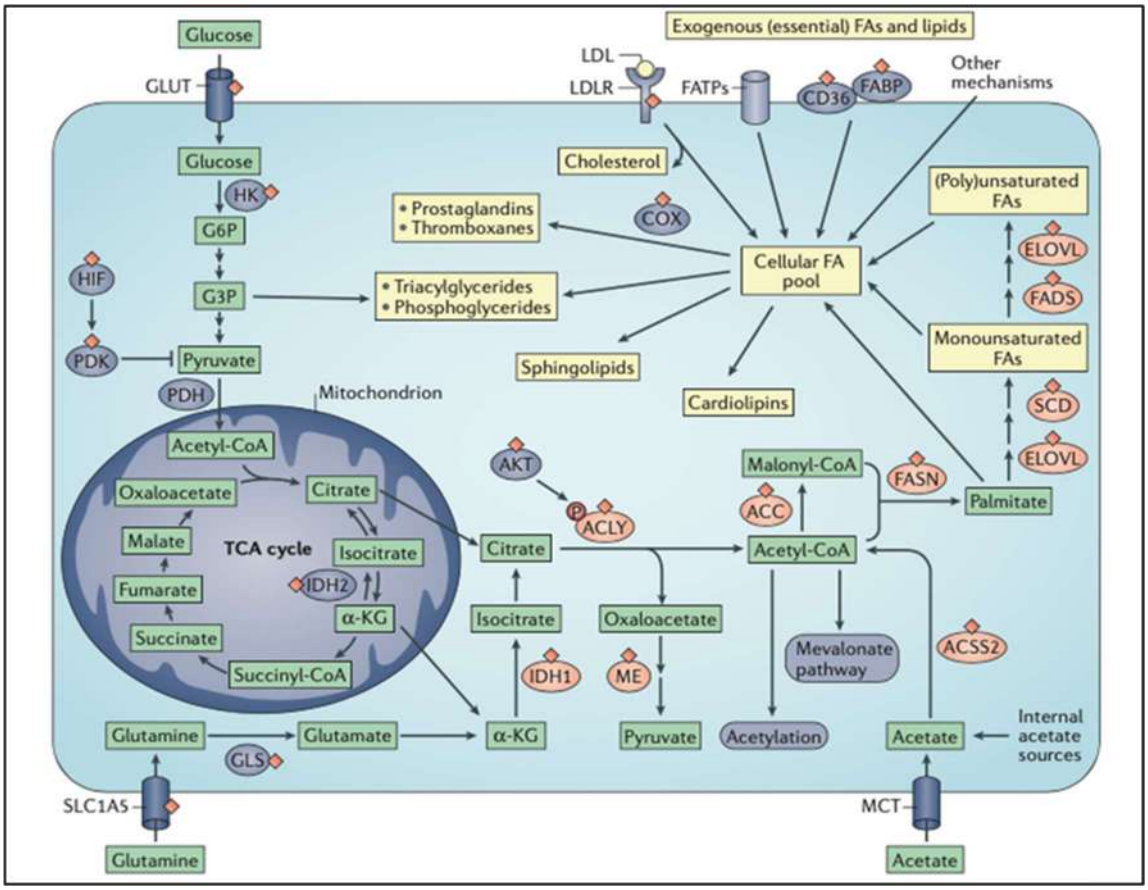

Figure 9. Overview of *de novo* synthesis of fatty acids (FAs).
Adapted from Röhrig et al., 2016.

Fatty acid oxidation

Fatty acids constitute an important energy source via their catabolism by fatty acid oxidation (FAO). In some cancer types, FAO has proven to be an effective therapeutic target (Samudio et al., 2010; Duman et al., 2019).

FAs can be incorporated from the extracellular media, or obtained by hydrolysis of triglycerides in lipid droplets or by lipophagy (Carracedo et al., 2013). They enter the cell by the membrane fatty acid translocase CD36, by fatty acid binding proteins (FABP) or by different fatty acid transport proteins (FATP) (Kreitz et al., 2019). Once inside the cell, FAs are activated to acyl-CoA in a two-step reaction catalyzed by the acyl-CoA-synthetase. Acyl-CoA can enter the mitochondria by conjugating to carnitine, in a reaction catalyzed by carnitine palmitoyl transferase 1 (CPT1), the limiting-step reaction for FAO, which is inhibited by malonyl-CoA. Once the carnitine-conjugated FA is inside the mitochondria, CPT2 will release the carnitine and regenerate the CoA ester, which can undergo FAO. During FAO, each cycle will shorten by two carbons the acyl-CoA, generating NADH, $FADH_2$ and one acetyl-CoA per cycle. NADH and $FADH_2$ will enter the electron transport chain (ETC) to produce ATP (Houten and Wanders, 2010; Carracedo et al., 2013; Kreitz et al., 2019). The process of β-oxidation (FAO) consists of several steps, summarized in **figure 10**. First, an acyl-CoA-ester is dehydrogenated by the acyl-CoA dehydrogenase to yield a trans-2-enoyl-CoA. Then, the double bond is hydrated by the enoyl-CoA hydratase, resulting in L-3-hydroxy-acyl-CoA. In a third step, the 3- hydroxy-acyl-CoA dehydrogenase will generate 3-keto-acyl-CoA. As final step of each cycle, 3-keto-acyl-CoA thiolase will cleave the molecule, resulting in a two-carbon chain-shortened acyl-CoA and acetyl-CoA (Houten and Wanders, 2010). Acetyl-CoA would then enter the Krebs cycle, and with oxaloacetate gives rise to citrate by the action of citrate synthase. Citrate will then be exported to the cytosol to produce NADPH via IDH1 and malic enzyme (ME1) (Carracedo et al., 2013).

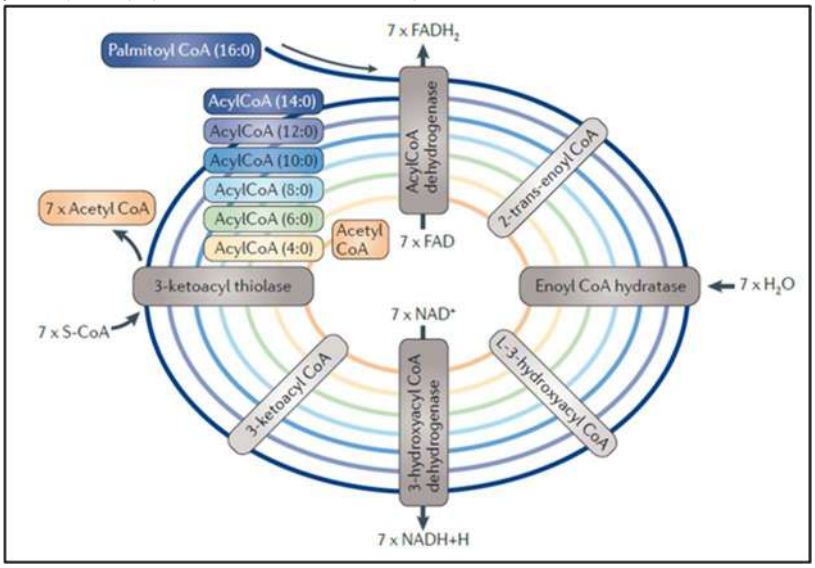

Figure 10. Schematic representation of the β-oxidation of palmitic acid in the mitochondria.
Adapted from Carracedo et al., 2013.

FAO has been idenfitied as metabolic vulnerability in the context of cancer. In AML Inhibition of FAO with the CPT1 inhibitor etomoxir drastically reduced AML cell proliferation, and sensitized human leukemic blasts to apoptosis induction by BLC-2 inhibitors, (Samudio et al., 2010). Another CPT1 inhibitor, ST1326, induced cell cycle arrest and apoptosis in AML cells (Ricciardi et al., 2015). In addition, high CPT1A expression has been associated with negative prognosis in CN-AML (Shi et al., 2016). In the same line, Wu and colleagues showed that the carnitine transporter CT2 is overexpressed in AML and that its shRNA knockdown reduced growth in AML cells while shifting the cells to a glycolytic phenotype by decreasing oxygen consumption (Wu et al., 2015). FAO serves as main energy source in the OxPhos subtype of diffuse large B cell lymphoma (OxPhos-DLBCL, Caro et al., 2012). In glioblastoma, it has been recently reported that FAO allows cells to adapt to dynamic microenvironment, being an important node of metabolic plasticity (Kant et al., 2020). In breast cancer, it's been reported that adipocyte-derived leptin, by stimulating the transcription of CPT1B via JAK/STAT3 in breast cancer stem cells (BCSCs), activates FAO, which was essential for chemoresistance (Wang et al., 2018). Several studies have also shown that lipids can influence leukemic stem cells (LSCs) fate decisions and response to drugs, and that they are important in the tumor microenvironment. Adipocytes are the dominant stromal cells in adult bone marrow, and this increases with age, reaching the 60% of all cells by the age of 65 (Tabe et al., 2020). Cell growth and survival in AML monocytic cells is supported by bone marrow adipocytes by promoting fatty acid β-oxidation via a transcriptional network that includes Peroxisome proliferator-activated receptor gamma (PPARg), FABP4, CD36, and BCL2 (Tabe et al., 2017). It has also been reported that, while LSCs isolated from the novo AML patients rely on amino acids for oxidative phosphorylation, LSCs from relapsed patients are more dependent on FAO, suggesting an association between FAO and chemoresistance and, more importantly, a metabolic vulnerability that can be exploited as a therapeutic opportunity (Jones et al., 2019). Remarkably, the role of the FA receptor CD36 in cancer has also been evidenced by several works. CD36 has been reported to be overexpressed in metastatic tissues of human ovarian tumors, and to be essential for the development of malignant phenotypes, Interestingly, CD36 inhibition in co-culture systems with ovarian cancer cells and adipocytes resulted in attenuated adipocyte-induced cholesterol and lipid droplet accumulation and reduced intracellular reactive oxygen species (ROS) content, as well as tumor burden in mouse xenografts (Ladanyi et al., 2018). In prostate cancer, high CD36 expression and increased lipid uptake is also associated with aggressive disease, and CD36 antibody therapy reduced cancer severity in patient-derived xenografts, identifying it as a target in prostate cancer (Watt et al., 2019). Overall, these studies underscore the importance of lipid uptake in cancer. An interesting study identified a $CD36^{+}$-LSC subpopulation that used gonadal adipose tissue as a niche to support its metabolism as well as to evade chemotherapy, in addition to induce lipolysis in adipocytes (Ye et al., 2016).

All of these findings highlight the important and complex role of lipids in cancer, and remarkably in AML, tumor microenvironment and in chemoresistance.

Regulation of fatty acids metabolism

The equilibrium between NADPH-consuming (FAS) and NADH and NADPH-producing (FAO-derived) reactions is regulated by AMPK. NADPH is essential for the cells, since it provides redox power to neutralize oxidative stress and both NADPH and NADH are co-enzymes for many anabolic enzymes, therefore, essential to sustain cell proliferation. Low energy levels activate AMPK, which promotes FAO by phosphorylation and inhibition of acetyl-CoA carboxylase (ACC) (Mihaylova and Shaw, 2011), and potentially also through CPT1C expression and PPAR signaling (Carracedo et al., 2013). FAO promoted by promyelocytic leukemia (PML)-PPAR-δ has been described to regulate HSCs maintenance, by ensuring a correct asymmetric cell division (Ito et al., 2012). In this work, it was also suggested that the inhibition of FAO could lead to exhaustion of leukemic initiating cells (LICs) reservoirs, and therefore, be a therapeutic option. However, in a context of nutrient abundance, ACC2 is hydroxylated and activated by prolyl-hydroxylase PHD3, α-KG-dependent dioxygenase from the EGLN1 family (Kreitz et al., 2019). AML cells expressed low levels of PHD3, independently of energy status, what made the axis PHD3/ACC2 inactive and favoring FAO over FAS (German et al. 2016). In the same study, they also showed that the re-expression of PHD3 recovered ACC2 activity and inhibited FAO, which limited leukemic growth *in vitro* and extended survival *in vivo* (German et al. 2016). Expression of FAS enzymes is controlled by a family of three basic-helix-loop-helix-leucine zipper (bHLH LZ) transcription factors known as sterol regulatory element binding proteins (SREBPs) (Röhrig et al., 2016). The enzymes involved in the regeneration of NAPDH (enzymes of the PPP, ME and IDH) are also regulated by SREBPs (Düvel et al., 2010, Röhrig et al., 2016). Remarkably, SREBPs is induced by mTOR (Porstmann al., 2008), thus being a downstream mediator for anabolic gene expression programming (Düvel et al., 2010). Other transcription factors contribute as well to the regulation of FAS, such as carbohydrate-responsive element-binding protein (ChREBP) (Tong et al., 2009), or MondoA, which is induced by MYC and regulates glutaminolysis and lipogenesis in cancer cells (Carroll et al., 2015).

In brief

- Lipids can provide large quantities of reducing equivalents to feed the ETC and biomass for new membrane generation in cancer cells.
- Targeting lipid metabolism in the context of tumor microenvironment-cancer cells can be of high interest to reduce chemoresistance.

1.2. Oncogenic signaling pathways involved in the regulation of cancer metabolism

Oncogene and tumor suppressor genetic programs influence cell metabolism, having a great impact on cell proliferation and eventually, in cancer progression. The main oncogenic pathways involved in cancer metabolic regulation are discussed below.

1.2.1. AMP-activated protein kinase

The AMP-activated protein kinase (AMPK) is a highly conserved Ser/Thr protein kinase (Faubert et al., 2013). AMPK is considered to be the energetic sensor of the cell, playing a central role in energy homeostasis since it is the main regulator of the balance between energy demand and energy supply within cells (Carling et al., 2011). In addition, it is considered a tumor suppressor, as evidenced by the work of Faubert and colleages where they showed that inactivation of AMPK signaling accelerated Myc-induced lymphomagenesis, increased biosynthetic pathways to support proliferation and induced a metabolic switch towards aerobic glycolysis through HIF-1α stabilization (Faubert et al., 2013).

As energy sensor, AMPK is activated by an increase in intracellular AMP/ATP or ADP/ATP ratio, caused by a drop in ATP levels (Hardie and Carling, 1997; Carling et al., 2011), such as in muscle under exercise (Winder and Hardie 1996) or upon caloric restriction (Cantó and Auwerx, 2011). AMPK is allosterically activated by AMP (Carling et al., 1987; Hardie and Carling 1997). Hormones such as leptin (Minokoshi et al., 2002) and adiponectin (Yamauchi et al., 2002) can also activate it. AMPK activation can ocurr through phosphorylation of AMPKαThr172 by the upstream kinase LKB1 (Hawley et al., 2003; Woods et al., 2003), and by calcium/calmodulin-dependent protein kinase kinase 2 (CAMKK2/ CAMKKβ) (Hawley et al., 2005; Hurley et al., 2005; Woods et al., 2005). In addition, an increase in intracellular calcium ions induced by CAMKK2 can also activate AMPK (Hawley et al., 2005; Hurley et al., 2005; Woods et al., 2005), which appears to be specially important for the regulation of AMPK in specific tissues in response to hormones, such as ghrelin when binding its receptor in neuronal cells in the hypothalamus (Andersson et al., 2004) or thrombin in endothelial cells (Stahmann et al., 2006), as well as a protective mechanism AMPK dependent upon DNA damage (Vara-Ciruelos et al., 2017). More recently it's been reported that decreased levels of F-1,6-BP and glucose starvation can activate AMPK independently of AMP/ADP (Zhang et al., 2017). When F-1,6-BP is limiting, aldolases promote the assembly of a lysosomal complex containing several proteins (ATPase, ragulator, axin, LKB1 and AMPK) that activate AMPK (Zhang et al., 2017). Since AMPK acts as tumor suppressor, it is not strange that its upstream activator LKB1 is suppressed or diminished in some cancers, such as non-small cell lung cancer (Ji et al., 2007), ovarian cancer (Contreras et al., 2008), breast cancer (Shen et al., 2002), cervical cancer (Wingo et al., 2009; McCabe et al., 2010), and pancreatic cancer (Yang et al., 2015), what results in impaired AMPK activation. Nevertheless, the inability to activate AMPK upon certain energy stress conditions create a metabolic vulnerability in cells harbouring LKB1 mutations.

Once activated, AMPK supports mainly catabolic pathways, such as glycolysis, fatty acid oxidation and autophagy, while it suppresses anabolic processes, including macromolecules biosynthesis and proliferation, as summarized in **figure 11**.

AMPK switches on glucose catabolism by different means. First, it increases glucose uptake through GLUT1 and GLUT4. Mechanistically, AMPK phosphorylates and induce degradation of the arrestin TXNIP, what results in impaired GLUT1 internalization (Wu et al., 2013). Regarding GLUT4, AMPK induces the translocation of GLUT4-containing vesicles to the plasma membrane through phosphorylation of Rab-GAP protein TBC1D1 (Pehmøller et al., 2009) of the histone deacetylase HDAC5 (McGee et al., 2008). In some cell types, AMPK stimulates glycolytic flux by increasing cellular concentration of F-2,6-BP through phosphorylation of the 6-phosphofructo-2- kinases PFKFB2 and PFKFB3 (Marsin et al., 2000). In addition to contributing to glucose catabolism, AMPK promotes mitochondrial oxidative metabolism, more efficient in terms of ATP production and provider of less anabolic precursors. In the short term, AMPK enhances fatty acid uptake into mitochondria through phosphorylation of ACC2, which results in lower levels of malonyl-CoA and reduced FA synthesis, active CPT1 and stimulation of mitochondrial fatty acid oxidation (Merrill et al., 1997). In the longer term, AMPK activation promotes mitochondrial biogenesis through direct phosphorylation of PPARG coactivator 1α (PGC-1α) (Jäger et al., 2007) or by inducing PGC-1α deacetylation and activation by sirtuin 1 (SIRT1), through increased cellular NAD^+ levels (Cantó et al., 2009). AMPK also promotes both autophagy and mitophagy through ULK1 phosphorylation (Egan et al., 2010; Kim et al., 2011) or through phosphorylation and activation of the Ca^{2+}/calmodulin kinase DAPK, that will subsequently phosphorylate the key autophagy protein Beclin-1 (Shiloh et al., 2018). Mitochondrial fission is also promoted by AMPK through the direct phosphorylation of mitochondrial fission factor (MFF) (Toyama et al., 2016).

On the other hand, AMPK activation switches off main anabolic pathways. AMPK phosphorylates and inactivates ACC1 (Carling et al., 1987) and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) (Hardie et al., 1989), dowregulating fatty acid and sterol synthesis. AMPK phosphorylates the transcription factors SREBP1c (Li et al., 2011) and ChREBP (Kawaguchi et al., 2001) as well, which results in the downregulation of the expression of ACC1 and FASN. In addition to fatty acid biosynthesis, AMPK inhibits triglyceride and phospholipid synthesis by inactivating glycerol phosphate acyl transferase (GPAT), the first enzyme of the pathway (Muoio et al., 1999). AMPK also inactivates glycogen synthase and therefore, glycogen synthesis (Bultot et al., 2012). Nucleotide biosynthesis is also impaired by AMPK through direct phosphorylation of the major isoforms of the phosphoribosyl pyrophosphate synthetase PRPS1 and PRPS2 (Qian et al.,2017), as well as ribosomal rRNA synthesis by direct phosphorylation of the transcription factor for RNA polymerase I, TIF-1α (Hoppe et al., 2009). AMPK also switches off protein synthesis through the inactivation of mammalian target of rapamycin complex-1 (mTORC1) by phosphorylation of Raptor (Gwinn et al., 2008) and through activating phosphorylation of TSC2 that maintains the mTORC1-activating G protein Rheb in its inactive GDP- form (Inoki et al., 2003).

Elongation step is also suppressed by AMPK through phosphorylation of the elongation factor-2 kinase (EF2K), which once activated phosphorylates elongation factor-2 at Thr56 (Johanns et al., 2017). Pharmacological AMPK activation has been reported to induce cell cycle arrest through increased expression of the G1 cyclin-dependent kinase inhibitors $p21^{CIP1}$ (CDKN1A) and $p27^{kip1}$ (CDKN1B) promoted by p53 (Imamura et al., 2001; Jones et al., 2005; Rattan et al., 2005). Overall, the regulatory role of AMPK in cell proliferation, energetics and metabolism highlights its importance in cancer therapy.

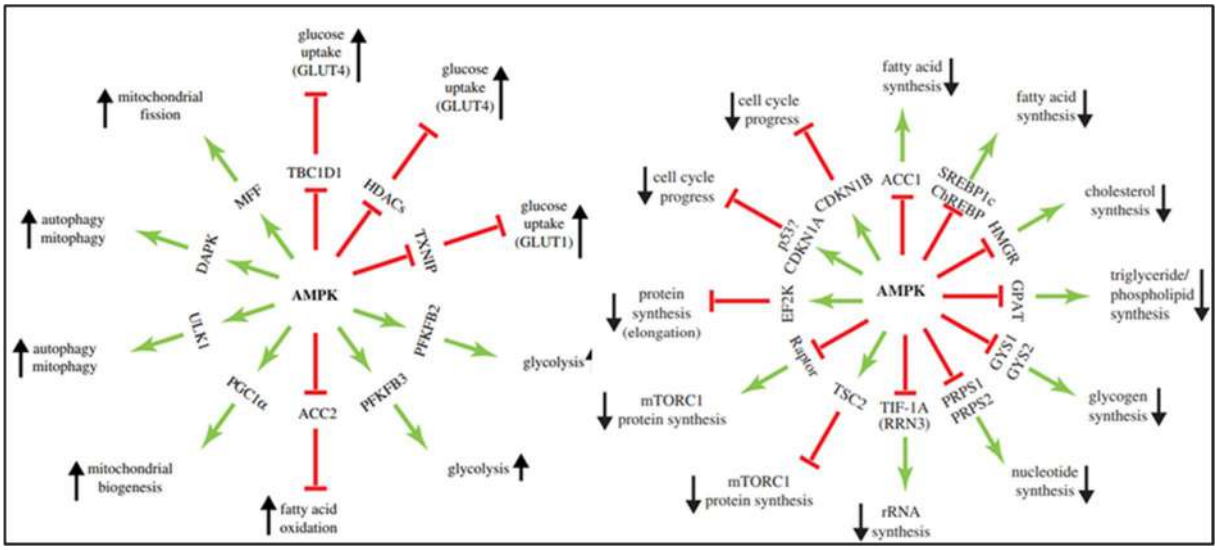

Figure 11. AMPK is a key regulator of energetic metabolism.
On the left, targets and pathways activated by AMPK. On the right, targets and pathways inhibited by AMPK. Adapted from Vara-Ciruelos et al. 2019.

1.2.2. c-Myc

c-Myc is the transcription factor encoded by the oncogene *MYC*, often deregulated in cancer and associated with aggressive, poorly differenciated tumors (Pelengaris et al., 2002). c-Myc integrates cell proliferation, growth and metabolism. The main metabolic pathways regulated by c-Myc are represented in **figure 12**.

c-Myc regulates purine and pyrimidine synthesis, necessary for cell proliferation, through upregulation of the tri-functional enzyme Carbamoyl phosphate synthase II, Aspartate transcarbamoylase and Dihydroorotase (CAD) (Eberhardy et al., 2001), dihydroorotate dehydrogenase (DHODH) and phosphoribosyl pyrophosphate amidotransferase (PPAT) (Liu et al., 2008). In addition, c-Myc also activate positive cell cycle regulators, like CDK4 and CDK6 (Mateyak et al., 1999; Hermeking et al., 2000), and suppresses CDK inhibitors, like p21 (Gartel et al., 2001; Rand et al., 2018), p27 (Yang et al., 2001), and p16 (Rand et al., 2018). Interestingly, c-Myc is able to induce cell cycle even in DNA damaged condition, suggesting selection for p53 loss to fuel clonal evolution and tumor progression (Raimondi et al., 2019), classically by c-Myc induced ARF disruption (Eischen et al., 1999). In this regard, it has

recently been reported that c-Myc can also inactivate p53 through the c-Myc-Inducible Long noncoding RNA Inactivating P53 (MILIP), which promotes p53 polyubiquitination and turnover (Feng et al., 2020).

c-Myc also stimulates glucose uptake and glycolysis through transactivation of genes encoding the glucose transporter GLUT1, and the glycolytic enzymes PFK, enolase (Osthus et al., 2000), HK2 (Kim et al., 2007), LDHA (Shim et al., 1997) as well as the lactate transporter MCT1 (Doherty et al, 2014), which, as discussed later in the chapter "1.3.2.3.2.iii. Lactate", participates in the establishment of a symbiotic relationship between the cancer cells and surrounding normal cells to induce immunosuppression (Allen et al., 2016; Park et al., 2016). c-Myc also regulates PKM2 expression by controlling its splicing (David et al.,2010). Regarding induction of glycolysis, c-Myc metabolism cooperates with hypoxic metabolism mediated by HIF-1, which is evidenced by the fact that even during normoxia, c-Myc promotes HIF expression and inhibits HIF-1α degradation (Kim et al., 2007; Huang, 2008). c-Myc is also involved in glutamine addiction through transcriptional reprogramming of mitochondrial metabolism, in which glutamine sustains TCA cycle anapleurosis and provides high rates of NADPH production to fuel lipid and nucleotide biosynthesis (Wise et al., 2008). c-Myc promotes the expression of the glutamine importers SLC1A5/ ASCT2 and SLC38A5/ SN2 (Wise et al., 2008), as well as it enhances GS expression through DNA demethylation by thymine DNA glycosylase (TDG) (Bott et al., 2015) and mitochondrial glutaminase GLS1 expression, which converts glutamine to glutamate, through miR-23a/b suppression (Gao et al., 2009). Mitochondrial biogenesis is also up-regulated by c-Myc via mitochondrial transcription factor A (TFAM) (Li et al., 2005), to support glutamine catabolism. *De novo* synthesis of fatty acids is also enhanced by c-Myc, through increased expression of ACLY, ACC and FASN (Dejure and Eilers, 2017). c-Myc is also involved in ROS and oxidative stress control, activating pathways that support GSH and NADPH production. c-Myc triggers serine biosynthesis pathway through the regulation of SHMT, both cytosolic and mitochondrial isoforms (Nikiforov et al., 2002). Increased serine biosynthesis leads to higher GSH production, as well as cell cycle progression and nucleic acid synthesis, all processes essential for cell survival and proliferation, specially under stress conditions of limited nutrient availability (Sun et al ., 2015). In addition, c-Myc induces peroxiredoxin 3 (PRDX3), which maintains mitochondrial oxidative stress homeostasis (Wonsey et al., 2002).

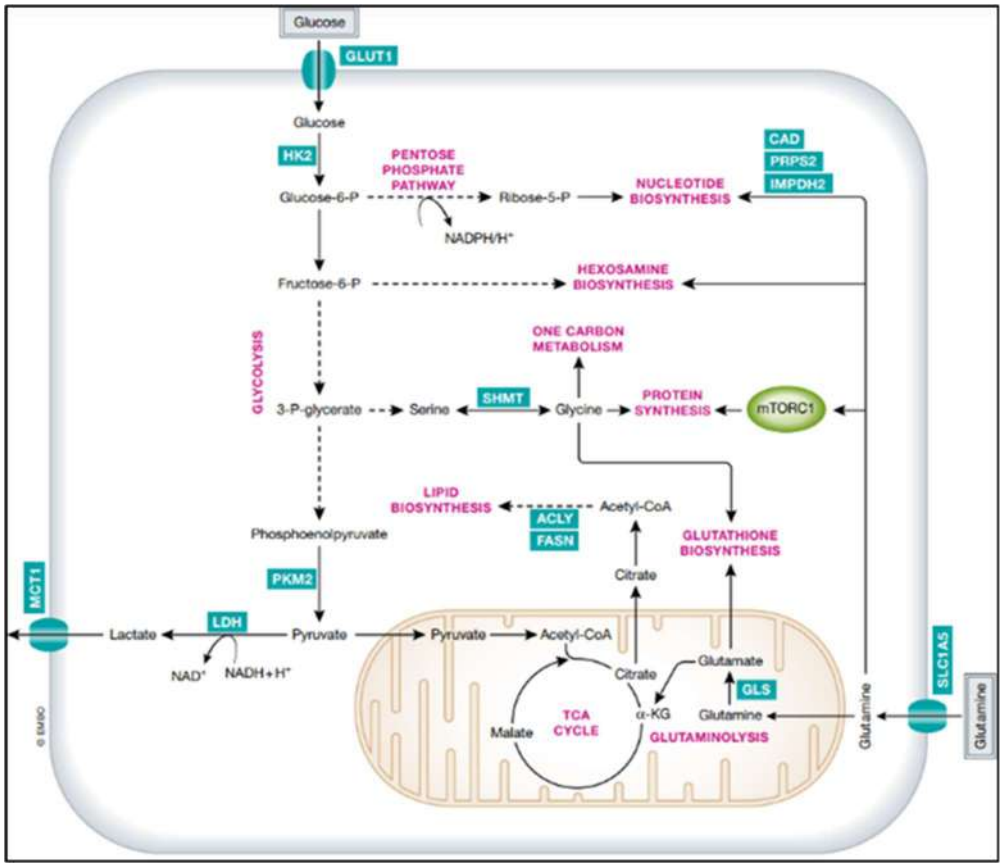

Figure 12. Metabolic changes induced by MYC.
MYC promotes anabolic reactions (magenta) to support proliferatiion. MYC increases glucose and glutamine uptake and uses them as building blocks for several macromolecules, such as lipids, nucleotides by promoting the expression of transporters and metabolic enzymes (turquoise).
Adapted from Dejure and Eilers, 2017.

1.2.3. Hypoxia and hypoxia inducible factors

Hypoxia, reduced oxygen availability, triggers a coordinated an adaptive response to equilibrate O_2 supply with metabolic and bioenergetic demand (Semenza, 2001). In response to low oxygen tension, cells engage multiple evolutionarily conserved molecular responses, such the one mediated by the hypoxia-inducible factor (HIF), mTORC1, autophagy, endoplasmic reticulum (ER) stress response, or the O_2-dependent dioxygenases (Nakazawa et al., 2016).

The transcription factor HIF-1 is the master regulator of oxygen homeostasis in the cell. HIF-1 is a heterodimer, constituted of HIF-1β and HIF-1α subunits, both constitutively expressed, but stability of the α subunit being highly regulated by O_2 levels. (Semenza, 2003). Under O_2 availability, HIF-1α is hydroxylated on proline residues 402 and 564 by prolyl hydroxylase domain (PHD) proteins (mainly PHD2), which use O_2 and α-KG as substrates. (Jaakkola et al., 2001; Kaelin and Ratcliffe, 2008). The von Hippel-Lindau (VHL) protein binds to hydroxylated prolines and recruits an ubiquitin ligase complex through its interaction with Elongin C (Kamura et al., 2000). Ubiquitination tags HIF-1α to degradation by

the proteasome (Salceda and Caro, 1997; Semenza, 2009). Factor inhibiting HIF (FIH-1) binds to HIF-1α and hydroxylates its Asn803 to block HIF-1α transactivation domain interaction with the coactivators p300 or CBP (Mahon et al., 2001; Lando et al., 2002). Therefore, O_2-dependent hydroxylation is a negative regulator both of HIF-1α stability and transcriptional activity (Semenza, 2009). However, hydroxylation reactions are inhibited under hypoxia, and therefore, HIF-1α is stabilized, binds to its coactivators and subsequently increases transcription of target genes involved in angiogenesis, glucose metabolism, cell proliferation/ survival and invasion/metastasis. HIF-1α transcriptional and postranscriptional regulation is represented in **figure 13**.

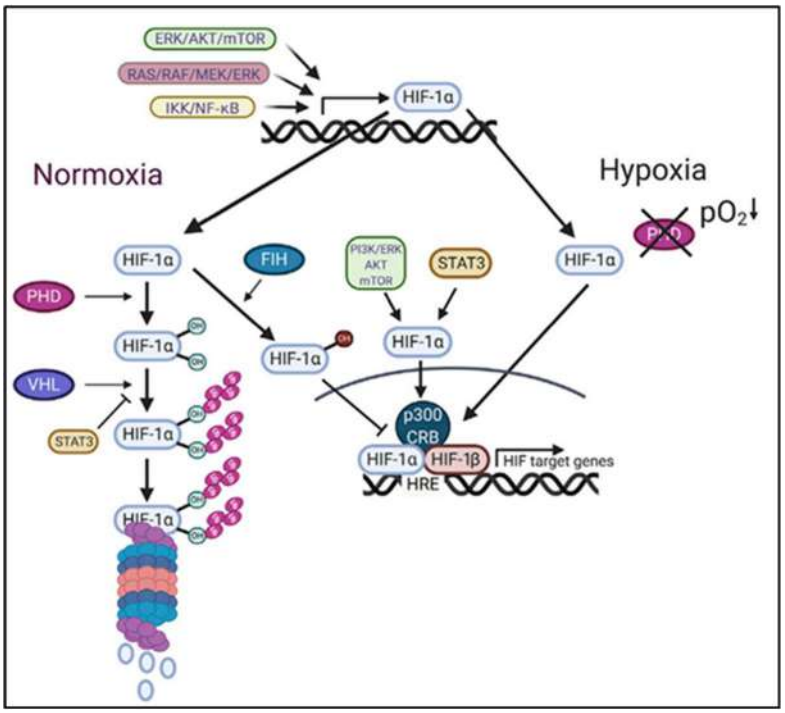

Figure 13. Transcriptional and post-transcriptional regulation of HIF-1α.
Adapted from McGettrick and O'Neill, 2020.

In cancer, HIF-1α can be stabilized under several conditions. The first one is under intratumoral hypoxia, common in solid tumors, where tumor microenvironment tends to be deprived from oxygen and nutrients (Höckel and Vaupel 2001). HIF-1α can also be stabilized due to microenvironment alterations, like ROS, which have been reported to stabilize HIF-1α under normoxia through PHD inhibition (Chandel et al., 2000). ROS derived from ERK axis have also been reported to stabilize HIF-1 (Lamberti et al., 2017). Cancer-specific genetic alterations like mutations in the TCA enzymes SDH and FH impair function of α-KG-dependent dioxygenases due to succinate and fumarate accumulation, and lead to stabilization HIF-1α in an O_2 independent manner (Isaacs et al., 2005; Selak et al., 2005). Oncogenic gain of function (like PI3K/AKT/ mTOR signaling) or tumor suppressor loss (like VHL loss) have also been reported to take part in normoxic HIF-1 stabilization. Interestingly, also therapeutic resistance to both chemotherapy and radiotherapy has been linked to hypoxia (Höckel et al., 1993; Brizel et al., 1996), rendering the

pharmacological impairment of this pathway an attractive therapeutic option (Harada et al., 2009; Agani and Jiang, 2013; Semenza, 2013).

One of the first pathways found to be upregulated by HIF-1α (**figure 14**) is glycolysis. In fact, the aim of HIF-1α signaling is to use glycolysis as main source of ATP, uncoupling it from mitochondrial oxidative phosphorylation (Semenza, 2011). Not surprisingly, HIF-1 is thus one of the main contributors to the Warburg effect (Denko, 2008), since it triggers the expression of glucose transporters and glycolytic enzymes (Semenza et al., 1996; Iyer et al., 1998; Seagroves et al.,2001) as well as PDK1 (Kim et al., 2006; Papandreou et al., 2006) and the lactate transporter MCT4 (Ullah et al., 2006). Thus, through upregulation of both LHDA and PDK1 (that regulates PDH), HIF-1 shunts pyruvate away from mitochondria and protects the cell from oxidative stress. Classically, HIF-1 was though to drive the switch to glycolysis because of a matter of ATP production maintenance; however, different works have shown that it's also a mechanism to reduce ROS production and oxidative stress (Kim et al., 2006, Zhang et al., 2008; Zhao et al., 2014). This was first evidenced in HIF-1 deficient mice embryonic fibroblasts (MEFs), where cell death was induced by toxic ROS due to inhability to switch to glycolytic metabolism and not by ATP depletion (Kim et al., 2006, Zhang et al., 2008). HIF-1 promotes PPP, NADPH generator pathway, through inducing O-GlcNAcylation of G6PD, the first committed step in the PPP (Rao et al., 2015), or through the mitochondrial localization of the p53 target TP53-induced glycolysis and apoptosis regulator (TIGAR) that results in high HK2 activity and also increased PPP flux (Cheung et al., 2012). Regarding mitochondria, HIF-1 triggers mitochondrial autophagy through the upregulation of the expression of BNIP3 and BNIP3L, decreasing their number and therefore, the oxidative metabolism (Zhang et al., 2008; Bellot et al., 2009). HIF-1 directly modulates ETC through the expression of NDUFA4L2, that inhibits ETCI activity (Tello et al., 2011) and through the replacement of the cytochrome c oxidase subunit COX4-1 by the more efficient COX4-2 (Fukuda et al., 2007), both resulting in decreased ROS production. HIF-1 also induces the expression of miR-210, a repressor of several ETC subunits including *NDUFA4* (ETCI), *SDHD* (ETCII), *COX10* (ETCIV) and *ISCU1/2*, involved in iron sulfur center synthesis (Chen et al., 2010; Puisségur et al., 2010).

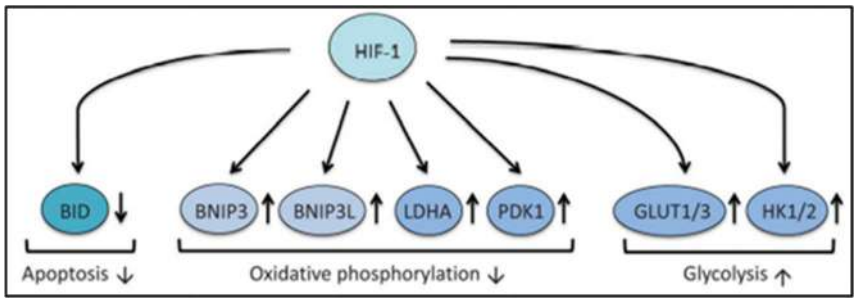

Figure 14. Metabolic target genes of HIF-1.
HIF-1 promotes glycolysis, shuts down OxPhos and can inhibit apoptosis by blocking the expression of BID. Adapted from Matsuura et al., 2016.

There are several HIF-1 inhibitors that are or have been evaluated in clinical trials, such as Apigenin, that reduces HIF-1 protein through impairment of PI3K/AKT signaling (Mirzoeva et al., 2008); PX-478, which is the first HIF-1α inhibitor in clinical stage for the treatment of solid tumors (Tibes et al., 2010); Bortezomib, which is proteasome inhibitor that also represses HIF-1α transcriptional activity and reinforce the FIH-mediated inhibition of p300 recruitment, approved for multiple myeloma and mantle cell lymphoma (O'Connor et al., 2005; Befani et al., 2011); or BAY 87-2243, an ETCI inhibitor that reduced HIF-1α gene expression and protein levels but whose clinical trial (NCT01297530) was terminated due to toxicity (Ellinghaus et al., 2013). One of the approaches that may have higher therapeutic interest is the combination of HIF-1 inhibitors with chemotherapy/radiotherapy, given that chemotherapy and radiotherapy resistance associates with HIF-1 expression, and that it seems an efficient approach in several preclinical models (Frolova et al., 2012; Zhao et al., 2014; Smolarczyk et al., 2018; Khan et al., 2019).

1.2.4. p53

The TP53 gene encodes the transcription factor p53, a tumor suppressor classically denominated as "the guardian of the genome" due to its involvement in genome stability maintenance and cancer protection (Lane, 1992; Lakin and Jackson, 1999). TP53 mutations are the most common ones in sporadic human cancers, suggesting an important selection against p53 function in tumorigenesis (Kandoth et al., 2013). In cells exposed to stress signals, p53 drives apoptosis or senescence, restricting tumor development (Brady and Attardi, 2010). Under physiologic conditions of no cellular stress, E3 ubiquitin ligase MDM2 targets p53 for degradation. However, under a variety of stress signals, including DNA damage, ROS, hypoxia or oncogene expression, p53 binds to DNA specific sequences to reprogram cell expression (Brady et al., 2011). p53 transcription network is summarized in **figure 15**.

One of the most classic actions of p53 is the induction of cell cycle arrest at the G1 phase by stimulating transcription of the gene for the cyclin dependent kinase inhibitory protein p21, which inhibits the cyclin E/cdk2 and cyclin A/cdk2 kinases, preventing cell cycle progression. p53 can also trigger cell-cycle arrest within the G2 phase by inducing the expression of the protein 14-3-3ζ, or alternatively, it can trigger apoptosis through stimulating the transcription of pro-apoptotic factors (Lakin and Jackson, 1999). Regarding cell death, p53 induces apoptosis through direct transcriptional activation of the pro-apoptotic BH3-only proteins PUMA and NOXA (2003; Aubrey et al., 2017). It can also induce ferroptosis, a non-apoptotic, iron-dependent cell death type (Jiang et al., 2015; Kang et al., 2019) by inhibiting the expression of the cystine-glutamate antiporter SLC7A11 (Jiang et al., 2015) or by enhancing that of SAT1 (spermidine/spermine N1-acetyltransferase 1) (Ou et al., 2016) and GLS2 (Kang et al., 2019).

More recently, the regulatory role of p53 in cell metabolism has also being recognized. p53 seems to oppose to Warburg effect by supporting oxidative phosphorylation and downregulating glucose

transporters and glycolytic enzymes (Lacroix et al., 2020). More specifically, it reduces the glucose transporters GLUT1 and GLUT4 transcriptionally (Schwartzenberg-Bar-Yoseph, et al., 2004); GLUT3 through limiting IKK-NF-kB activation (Kawauchi et al., 2008); as well as it regulates the transcription of other genes involved in glycolysis modulation, like MCT1 (Boidot et al., 2011),TP53-inducible glycolysis and apoptosis regulator (TIGAR) (Bensaad et al., 2006), PFKFB3/4 (Franklin et al., 2016; Ros et al., 2017). It also promotes the expression of the tumor suppressor miR-34a, which among other regulatory and anti-tumoral actions, decreases the expression of the glycolytic enzymes HK1, HK2, GPI, and PDK1. (Kim et al., 2013; Zhang et al., 2019).

While decreasing glycolysis, p53 promotes OxPhos by several coordinated actions. p53 downregulates PDK2, allowing PDH to fuel TCA with acetyl-CoA (Contractor and Harris, 2011). p53 enhances also glutaminolysis through increased expression of GLS2, which also contributes to ROS protection (Hu et al., 2010; Suzuki et al., 2010), and fatty acid oxidation, through CPT1 (Sanchez-Macedo et al., 2013), malonyl-CoA descarboxylase (Liu et al., 2014), acyl-CoA dehydrogenase (Jiang et al., 2015),or the phosphatidate phosphatase Lpin1 (Assaily et al., 2011), to maintain TCA cycle. By fueling TCA cycle, p53 guarantees α-KG levels to maintain cell differentiation via chromatin modification of 5-hydroxymethylcytosines (Morris et al., 2019). Another mechanism exerted by p53 to maintain cell differentiation is by inducing the transcription of miR-34, which has also been involved in limiting cellular reprograming and plasticity (Choi et al., 2011). In addition, p53 promotes mitochondrial biogenesis by interacting with and stimulating TFAM (Yoshida et al., 2003; Wen et al., 2016); ROS protection and apoptosis control through Ferredoxin Reductase (FDXR) (Liu and Chen, 2002; Zhang et al., 2017) and Apoptosis Inducing Factor (AIF) (Stambolsky et al., 2006); enhanced activity of the ETC through increasing the enzyme Synthesis of Cytochrome c Oxidase 2 (SCO2) (Matoba et al., 2006); mitochondrial genome integrity through interaction with polγ (Achanta et al., 2005; Bakhanashvili et al., 2008) and mitochondrial quality control through mitophagy by increasing Parkin (Zhang et al., 2011) and Mitochondria Eating Protein (MIEAP) (Kitamura et al., 2011). p53 also enhances the expression of mitochondrial protein import machinery to promote mitochondrial function, like TOM20, TIM23 and mitochondrial heat shock proteins mtHSP70 and mtHSP60 (Saleem et al., 2015), as well as it is involved in mitochondrial dynamics supporting mitochondrial fission via Dynamin-related protein 1 (DRP1) (Li et al, 2010) or fussion by participating in the processing of the long form of Opa1 (L-Opa1) (Kong et al., 2014) according to the situation.

p53 can protect from ROS by downregulating ROS-producing enzymes like nitric oxide synthase 2 (NOS2) (Forrester et al., 1996) and by inducing the expression the ROS-detoxifying enzyme Superoxide Dismutase 2 (SOD2) (Hussain et al., 2004). In addition, as previously mentioned, p53 induces the expression of TIGAR and PFKFB3, which favour NADPH production through PPP to maintain the pool of reduced GSH (Bensaad et al., 2006, Franklin et al., 2016; Ros et al., 2017).

In order to limit proliferation, p53 inhibits nucleotide synthesis by inhibiting inosine-monophosphate dehydrogenase (IMPDH) through the p53-induced microRNA-34a (miR-34a) (Kim et al., 2012) and guanosine 5'-monophosphate synthase (GMPS) (Holzer et al., 2017),while it promotes DNA repair upon DNA damage through the activation of ribonucleotide reductase (RNR) (Tanaka et al., 2000). p53 can promote autophagy by the regulation of proteins involved in the process (Kenzelmann Broz et al., 2013) or through inhibition of mTOR by AMPK s (Feng et al., 2005).

p53 has also been reported to be involved in modulation of the immune populations in the tumor microenvironment (TME). More specifically, loss of p53 increases myeloid infiltration through enhanced cytokine secretion and increase Tregs, resulting in delayed tumor rejection and highlighting the role of p53 in anti-tumor immune response (Bezzi et al., 2018; Blagih et al., 2020).

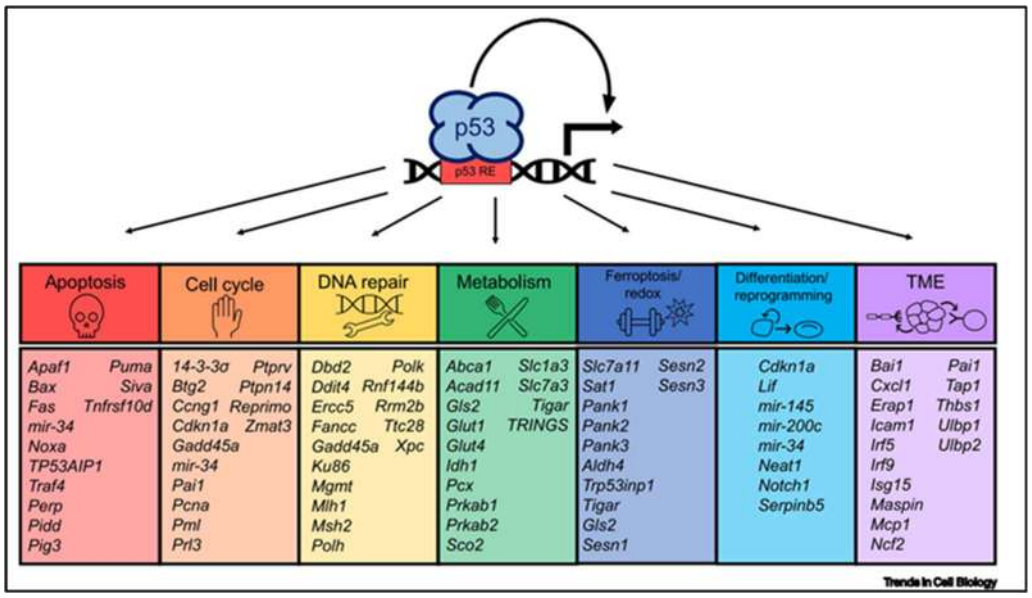

Figure 15. p53 regulatory transcriptional program.
Adapted from Boutelle and Attardi (2021).

1.2.5. PI3K/AKT signaling

The phosphatidylinositol-3-kinase (PI3K)/AKT pathway integrates growth factor, insulin and cytokines signaling, regulating in return several processes such as cell growth and proliferation, survival, metabolism and motility (Thorpe et al., 2014).

Under physiological conditions, the above mentioned signals can activate tyrosine kinases receptors (RTKs), cytokine receptors, integrins and G protein-coupled receptors (GPCRs) that will activate PI3K (**figure 16, left**). Mutations in PI3K-activating RTKs, like epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2), appear amplificated and activated in some cancers (Yuan and Cantley, 2008). There are 3 classes of PI3K, among which PI3K class Ia (heterodimers of a catalytic subunit among p110α, p110β or p110δ, associated with a regulatory subunit, p85α or p85β,

which are activated by RTKs, G protein-coupled receptors and the small G protein RAS) and class Ib (consist of the catalytic subunit p110γ, associated with the regulatory subunit p101, and they are are activated by GPCRs) are the more studied (Xu et al., 2020). Mutations in the gene encoding the p110α catalytic subunit (*PIK3CA*) are frequently found in cancer (Karakas et al., 2006; Samuels and Waldman, 2010). PI3K activation in the plasma membrane leads to the production of phosphatidylinositol 3,4,5-trisphosphate (PIP3) through the phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP2). Phosphatase and tensin homologue (PTEN) opposes to PI3K signaling by dephosphorylating PIP3 into PIP2 (Liu et al., 2009). PTEN is the second most mutated tumor suppressor in cancer, after p53, and its loss of function leads to sustained PI3K signaling activity, which highlights the involvement of the pathway in cancer (Yin and Shen, 2008). PIP3 serve as docking sites for the recruitement of PI3K downstream effector proteins with a pleckstrin homology (PH) domain, amongst which there is the ser/thr kinase AKT, also known as Protein kinase B or PKB (Fruman et al., 2017). Once AKT binds to PIP3, it is subsequently phosphorylated by phosphoinositide-dependent protein kinase 1 (PDPK1), which is essential for its activation (Le Good et al., 1998), and also by mTORC2, which enhances AKT activity (Sarbassov et al., 2005). There are 3 AKT isoforms, however, they all seem to be functionally redundant (Santi and Lee, 2010). AKT amplification and overexpression is also found in cancer (Altomare and Testa, 2005).

In this point it will only be discussed AKT downstream effects on metabolism, although its actions involve also other cellular responses, such as cell survival and proliferation. Among downstream AKT targets there are master metabolic regulators like mTORC1, glycogen synthase kinase 3 (GSK3) and transcription factors members of the forkhead box O (FOXO). Active GSK3 inhibits glycogen synthesis by phosphorylating the glycogen synthase (Cross et al., 1995; Beurel et al., 2015). In addition, GSK3 has other substrates such as MYC, SREBP, nuclear factor erythroid 2-related factor (NRF2) or HIF1, which upon GSK3 phosphorylation are ubiquitinated and degraded by proteasome (Beurel et al., 2015). However, in response to growth factors and insulin, AKT phosphorylates and inhibits GSK3, stabilizing GSK3 targets. AKT also phosphorylates and inhibits TCS2, which results in mTORC1 activation (discussed in the next chapter) and leads to protein synthesis as well as to the processing and activation of sterol regulatory element binding proteins (SREBP) and the mRNA translation of the transcription factors MYC, HIF1 and ATF4 (Hoxhaj and Manning, 2019)(**figure 16**). FOXO is another target of AKT, and its phosphorylation results its nuclear exclusion and in the repression of its target genes, which usually are involved in the suppression of cell proliferation, growth and survival (Greer and Brunet 2005; Zhang et al., 2011). AKT regulates metabolism in the long-term by controlling several transcription factors (**figure 16, right**) or but also in the short term by directly phosphorylating metabolic enzymes (**figure 17**).

In the long term (**figure 16, right**), AKT promotes aerobic glycolysis through the induction of the expression of glucose transporters and glycolytic enzymes via HIF-1 and MYC (as described in the previous chapters) and by relieving FOXO repression of glycolytic genes (Gross et al., 2008). In addition, in order to support proliferation, anabolic programs have to be reinforced. AKT also stimulates *de novo*

lipid synthesis inducing the processing of SREBP through mTORC1 activation (Porstmann et al., 2008) and promoting stability of SREBP by inhibiting GSK3 (Sundqvist et al., 2005). It also stimulates nucleotide biosynthesis through increasing NADPH production via PPP by G6PD AKT-mTOR-SREBP expression enhancement (Düvel et al., 2010). Through MYC, AKT upregulates transcription and translation of phosphoribosyl pyrophosphate synthase 2 (PRPS2), as well as glutamine uptake though SLC1A5 and large amino acid transporter 1 (LAT1) (Wise et al., 2008; Gao et al., 2009). Lastly, PI3K/AKT signaling stimulates pyrimidine synthesis through mTORC1-S6K-mediated phosphorylation and enhancement of CAD activity, the enzyme catalyzing the first three steps of pyrimidine biosynthesis (Ben-Sahra et al., 2013; Robitaille et al., 2013). PI3K/AKT-mTORC1 also induces *de novo* purine biosynthesis through MYC, SREBP and ATF4, stimulating the expression of mTHF and other enzymes in the purine synthesis pathway (Ben-Sahra et al., 2013). PI3K/AKT orchestrate an increase in protein synthesis; mainly driven by mTORC activation (Wang and Proud, 2006), but also complemented by MYC through the stimulation of RNA polymerases I and III, involved in ribososome biogenesis (van Riggelen et al., 2010). Immunosuppressants that target nucleotide synthesis pathways have been used to target cancer cells, due to high proliferation shared by both cancer cells and immune cells upon activation. Interestingly, the vast majority of cellular RNA is rRNA, thus impairing nucleotide biosynthesis with drugs like mizoribine (inosine monophosphate dehydrogenase inhibitor) can be an effective therapy in tumors with upregulated MYC and mTOR, which drastically increase rRNA biogenesis (Valvezan et al., 2017; Huang et al., 2018). Regarding ROS, appart from NADPH production through PPP stimulation, AKT increases NRF2, involved in ROS detoxification by inducing the expression of several antioxidant response enzymes, like glutathione S-transferase, superoxide dismutases, and NAD(P)H:quinone oxidoreductase 1 (Kansanen et al., 2013)

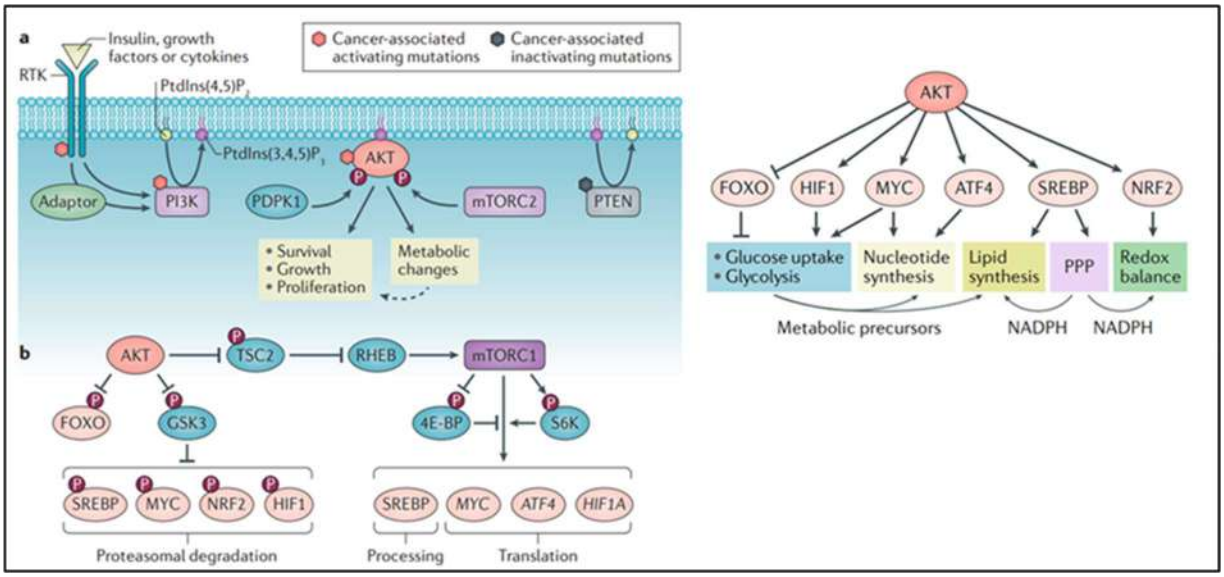

Figure 16. PI3K/AKT pathway.
On the **left**, **(a)** AKT activation and **(b)** AKT phosphorylation of its downstream effectors FOXO, GSK3, and TSC2. On the **right**, transcription factors mediating the metabolic regulation exerted by AKT.
Adapted from Hoxhaj and Manning, 2019

AKT also phosphorylates and acutely regulates several metabolic enzymes (**figure 17**). In fact, AKT activation is sufficient to promote aerobic glycolysis (Rathmell et al., 2003; Elstrom et al., 2004). More in detail, AKT promotes glucose uptake by increasing GLUT1 and GLUT4 membrane expression. Several mechanisms have been proposed, one involving AKT mediated-phosphorylation and inhibition of the GLUT4-trafficker TBC1D4 (Sakamoto and Holman, 2008), and another one involving thioredoxin-interacting protein (TXNIP), which when active, promotes endocytosis of GLUT1 and GLUT4 thus inhibiting glucose uptake, but its phosphorylation by AKT results in its inhibition and in increased glucose uptake (Parikh et al., 2007; Waldhart et al., 2017). In addition, AKT also promotes activity of key glycolytic enzymes, including HK2, partly by increasing its association with voltage-dependent anion channel (VDAC) at the outer mitochondrial membrane which provides mitochondria-derived ATP to sustain glucose phosphorylation by HK2 and promote mitochondrial integrity preservation and cell survival (Gottlob et al., 2001; Pastorino et al., 2002; Majewski et al., 2004). G6P can then enter either the hexosamine pathway, PPP or glycolysis. AKT also indirectly stimulates PFK1 through the direct phosphorylation and activation of the PFKFB2, which increases the PFK1 allosteric activator fructose-2,6-BP resulting in enhanced PFK1-driven glycolytic flux (Hue, and Rider, 1987; Deprez et al., 1997; Mouton et al., 2010). PI3K signaling can also potentiate glycolytic flux independently of AKT by inducing Rac-dependent release of the glycolytic enzyme aldolase A from an actin-bound state (Hu et al., 2016). High aldolase A expression is correlated with poor cancer prognosis (Jiang et al., 2018; Ye et al., 2018), and it has been proposed as therapeutic target (Chang et al., 2019). AKT also activates transketolase (TKT), enhancing the non-oxidative PPP branch to support ribose-5P generation for nucleotide biosynthesis (Saha et al., 2014). In addition, AKT can phosphorylate and increase ACLY activity, boosting cytosolic acetyl-CoA production and *de novo* lipid synthesis as well as protein acetylation (Berwick et al., 2002).

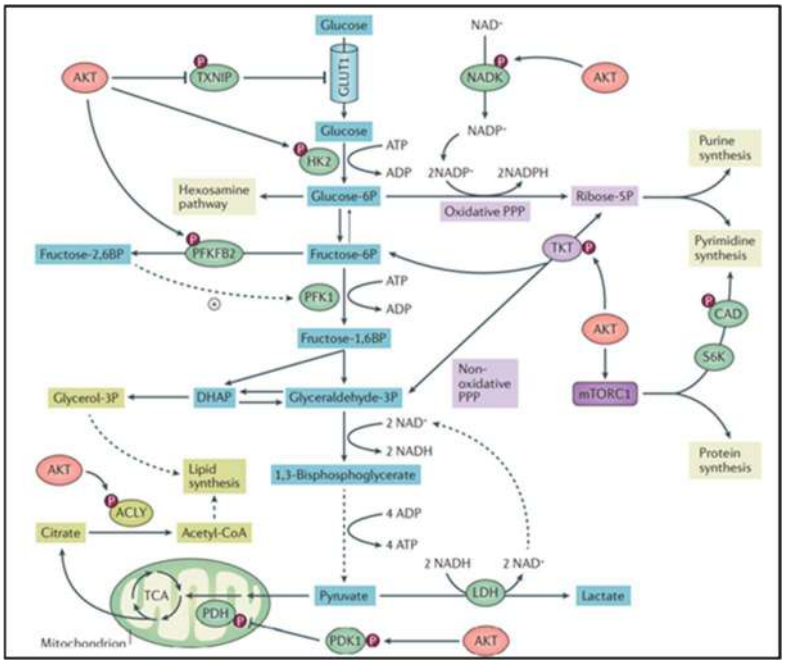

Figure 17. AKT regulates metabolism through post-translational regulation of several metabolic enzymes. Adapted from Hoxhaj and Manning, 2019.

1.2.6. Mammalian target of rapamycin (mTOR)

The serine/threonine kinase mTOR is the catalytic subunit of the multi-protein complexes mTORC1 and mTORC2, both composed by different subunits and divergent in substrate specificities and cellular functions (Saxton and Sabatini, 2017) (**figure 18**). mTOR and PI3K/AKT pathway are communicated, being mTORC2 an upstream AKT regulator which phosphorylates AKT and enhances its activity (Sarbassov et al., 2005); and mTORC1 a downstream AKT effector target, as described in the previous chapter "1.2.5. PI3K/AKT signaling". In response to growth factors, mTORC2 promotes cell survival and cytoskeletal remodeling through phosphorylation of its targets AKT, PKC and glucocorticoid-induced protein kinase 1 SGK1 (Sarbassov et al., 2005; Facchinetti et al., 2008; García-Martínez and Alessi, 2008). However, mTORC1 is activated by nutrients, amino acid, and growth factors and it is mainly involved in cell growth and division, therefore regulating anabolic pathways including lipids and nucleotides biosynthesis while suppressing catabolic pathways like authophagy (Saxton and Sabatini, 2017). Another difference between the two complexes is their sensibility to rapamycin, driven by the differences in their complex composition: mTORC1, containing Raptor, is sensitive to rapamycin whereas mTORC2, that contains rapamycin insensitive companion of mTOR (Rictor), is insensitive to acute rapamycin treatment (Jacinto et al., 2004; Sarbassov et al., 2004; Saxton and Sabatini, 2017). Due to its involvement in metabolic regulation, this chapter will be focus on mTORC1.

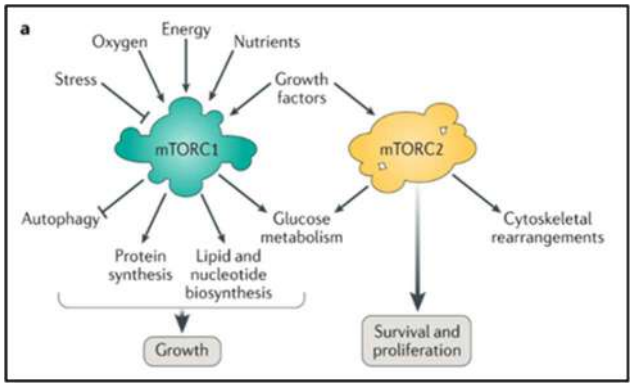

Figure 18. mTOR complex 1 (mTORC1) and 2 (mTORC2) integrate different signals.
Adapted from Liu and Sabatini, 2020.

Several mechanism contribute to mTORC1 activation and regulation (**figure 19**). Firstly, in the presence of cytokines, growth factors and ATP, small G proteins called Rheb (Inoki et al., 2003; Long et al., 2005) and Rag GTPases (Kim et al., 2008; Sancak et al., 2008) integrate information from nutrient availability, and modulate mTOR kinase activity and intracellular localization, respectively, in order to promote cell growth. Rag can exclusively recruit mTORC1 from cytoplasm to the lysosomal membrane in the presence of nutrients, and only then mTORC1 can co-localize with GTP-Rheb (Dibble and Manning, 2013), ensuring that the anabolic program triggered by mTORC1 is only carried out under appropriate conditions to support sustained growth. Another important point of mTORC1 regulation is the heterotrimeric tuberous sclerosis complex (TSC), which regulates lysosomal Rheb by converting the active Rheb-GTP

into the inactive Rheb-GDP state, therefore, braking mTORC1 activation (Inoki et al., 2003; Tee et al., 2003). TSC is regulated at many levels. First, in response to insulin or growth factors, AKT phosphorylates and inhibits the GTPase-activating protein TSC2 (Valvezan and Manning, 2019), allowing the accumulation of Rheb-GTP which binds to and activates mTORC1 (Menon et al., 2014). TSC is inhibited by the mTOR substrate S6K1, which phosphorylates insulin receptor substrate 1 (IRS-1) in a negative feedback loop to brake PI3K/AKT pathway (Harrington et al., 2004); by Wnt (Inoki et al., 2006) and tumour necrosis factor (TNF) (Lee et al., 2007); and by the Ras downstream targets ERK (Ma et al., 2005) and p90 ribosomal S6 kinase (RSK) phosphorylation (Roux et al., 2004). In cancer, TSC function is often loss since PI3K/AKT and Ras pathways are often constituvely activated (Altomare and Testa, 2005; Yin and Shen, 2008; Pylayeva-Gupta et al., 2011; Prior et al., 2020). mTORC1 activity is also regulated by PRAS40, which in basal state associates with Raptor and blocks Rheb-mTORC1 activation, but under insulin signaling, is phosphorylated by AKT, releasing active Rheb-GTP-mTORC1 (Haar et al., 2007; Sancak et al., 2007; Wang et al., 2007). mTOR signaling is repressed upon cellular stress (Demetriades et al., 2016). AMPK indirectly activates TSC2 through Raptor phosphorylation under energy or nutrient scarcy (Gwinn et al., 2008; Inoki et al.,al.,2003), shutting down anabolism and reducing ROS damaged in mitochondria by promoting its autophagy (Vara-Ciruelos et al. 2019). Independently of AMPK, hypoxia induces REDD1 expression, which can inhibit mTORC1 by activating TSC (Brugarolas et al., 2004; DeYoung et al., 2008). In addition, unfold protein response (UPR) in endoplasmic reticulum can inhibit mTORC1 by increasing transcription of the mTORC1 negative regulator Sestrin2 (Park et al., 2014; Saveljeva et al., 2016). Other cellular stresses, like DNA damage, induce the expression of TSC2 (as well as of AMPK and PTEN) to downregulate mTORC1-promoted cell proliferation and protect genome integrity (Feng et al., 2007; Budanov and Karin, 2008). In terms of nutrients, amino acids are essential for mTORC1 activation, especially leucine and arginine (Hara et al., 1998). Lysosomal recruitment of mTORC1 is dampened upon leucine starvation by the inhibitory binding of the cytosolic leucine sensor Sestrin2 to GATOR2 (Wolfson et al., 2015, Saxton et al., 2015). Cytosolic arginine levels are sensed by the cellular arginine sensor for mTORC1 (CASTOR1), which in the absence of arginine also binds to and inhibits GATOR2 (Chantranupong et al., 2016; Saxton et al., 2016). There is another arginine sensor, SLC38A9, which monitors amino acid levels inside the lysosomal lumen and effluxes neutral amino acids resulting from autophagic protein degradation outside the lysosome to activate mTORC1 pathway after prolonged starvation, by promoting GTP loading of RagA/B (Rebsamen et al., 2015; Wang et al., 2015; Shen and Sabatini, 2018). In the presence of amino acids, the folliculin-folliculin-interacting protein 2 (FLCN-FNIP2) complex sustains mTORC1 activation, acting like GTPase activating protein for Rag (Petit et al., 2013; Tsun et al., 2013). Methionine or SAM deprivation are sensed by SAMTOR, which binds to GATOR1 and KICSTOR inhibiting mTORC1 (Gu et al., 2017). Still, it is to determine whether mTORC1 can sense other essential metabolites for cell growth (Liu and Sabatini, 2020).

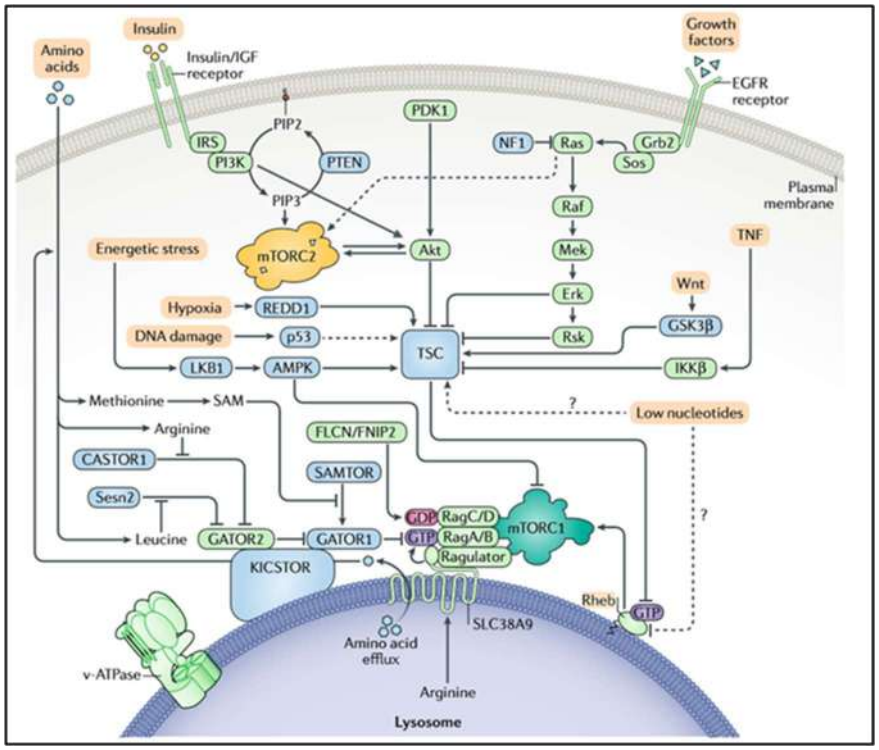

Figure 19. Regulation of the mTOR signaling pathway.
Adapted from Liu and Sabatini, 2020.

Once activated, mTORC1 triggers an anabolic program to sustain cell growth and proliferation (**figure 20**). mTORC1 promotes protein synthesis through the phosphorylation of two targets: the eukaryotic initiation factor 4E-binding proteins (4E-BPs), what leads to enhanced 5′ cap-dependent translation of mRNAs through release of eIF4E (Hara et al., 1997; Gingras et al., 1999); and p70 S6 kinase 1 (S6K1) (Burnett et al., 1998), which phosphorylates ribosomal protein S6, and the regulatory factors upstream binding factor (UBF) (Hannan et al., 2003), transcription initiation factor 1A (TIF-1A)(Mayer et al., 2004) and MAF1 (Michels et al., 2010; Shor et al., 2010) promoting RNA polymerases I and III activity. S6K1 also cooperates to enhance protein synthesis through the activation of the positive regulator of cap-dependent translation eIF4B, and by degrading the the eIF4A inhibitor programmed cell death 4 (PDCD4) (Dorrello et al., 2006). In addition, S6K1 enhances translation elongation of spliced transcripts through SKAR (Ma, et al., 2008). mTORC1 also drives lipid synthesis to sustain membrane biogenesis. For that, mTORC1 phosphorylates the SREBP inhibitor lipin 1 to exclude it from the nucleus (Porstmann et al., 2008; Peterson et al., 2011). In addition, mTORC1 regulates the expression of lipid homeostasis genes through the nuclear receptor PPARγ (Kim and Chen, 2004). To sustain nucleotide demand of proliferative cells, mTORC1 promotes purine biosynthesis through the activation of the transcription factor ATF4 and its downstream MTHFD2 (Ben-Sahra et al., 2016); as well as pyrimidine biosynthesis by S6K1-mediated CAD activation and phosphorylation (Ben-Sahra et al., 2013; Robitaille et al., 2013). mTORC1 upregulates HIF-1α, which increases glycolytic flux and provides energy an carbon sources for macromolecules biosynthesis (Land and Tee, 2007; Düvel et al., 2010; He et al., 2018). In addition,

SREBPs are also activated by mTORC1 to increase PPP flux, rendering NADPH for lipid biosynthesis and carbon precursors for nucleotides (Düvel et al., 2010). mTORC1 also represses catabolic autophagy by phosphorylating the autophagy induction effectors ULK1 and ATG13 (Hosokawa et al., 2009; Kim et al., 2011). mTORC1 also dampens autophagosome maturation by phosphorylating UVRAG and disrupting its interaction with the HOPS complex (Kim et al., 2015). In addition, mTORC1 regulates lysosomal biogenesis by controlling the translocation of the transcription factor EB (TFEB) and the related transcription factor E3 (TFE3) (Martina et al., 2021; Settembre et al., 2012; Roczniak-Ferguson et al., 2012).

To date, several mTOR inhibitors have been developed, however, with limited anti-cancer therapeutic success. The first generation of rapamycin derivatives tested on clinic (Tabernero et al., 2008; Hsieh et al., 2012) could not inhibit the pro-survival pathways regulated by mTORC2- and, in addition, they withdrew mTORC1-brake to autophagy, leading to nourishment of cancer cells under nutrient scarcy (Palm et al., 2015). Second generation inhibitors targeting both mTORC1 and mTORC2 by competing with ATP can lead to resistance through AKT activation without mTORC2 input (Rodrik-Outmezguine et al., 2011; Ghobrial et al). Therefore, new generation therapies need to be developed (Rodrik-Outmezguine et al., 2016; Fan et al., 2017; Xu et al., 2020).

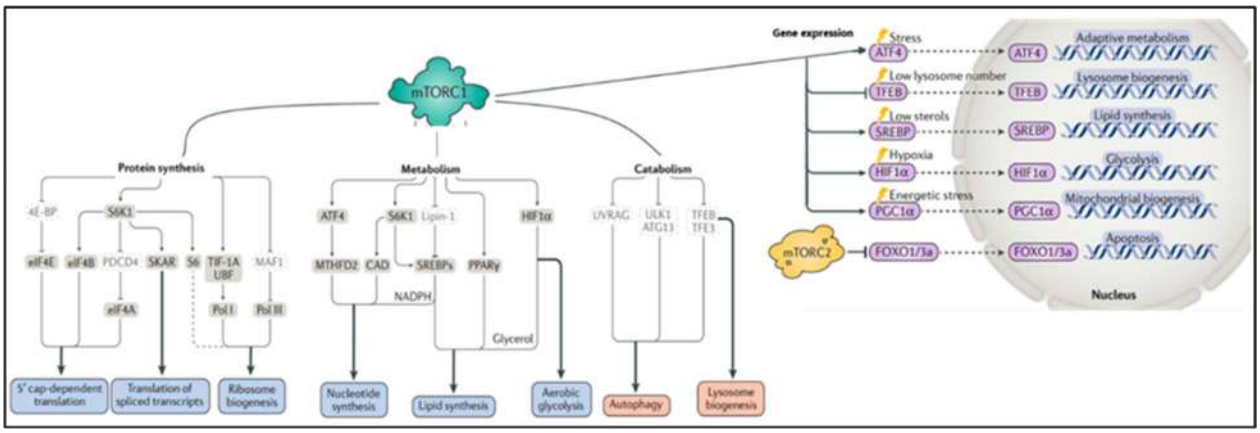

Figure 20. mTORC1 signaling targets to promote cell growth.
On the **left** mTORC1 effectors through phosphorylation; on the **right**, mTORC1 gene expression regulation. Adapted from Liu and Sabatini, 2020.

In brief

Oncogenic signaling determine important aspects of cancer cell metabolism. c-Myc, HIF-1 and the PI3K/AKT/mTORC1 axis cooperate to establish an anabolic program that allows cancer cells generating biomass for proliferation as well as ROS protection, in parallel with a limited mitochondrial catabolism. Tumor suppressors like AMPK or p53 induce a metabolic program opposed to the one exerted by oncogenes, in which mitochondrial catabolic pathways plays a central role.

1.3. Mitochondrial metabolism is essential in cancer

1.3.1. General overview

Mitochondria are essential organelles, classically known as the powerhouse of the cells due to their key role in bioenergetics. However, mitochondria involvement is not limited to ATP production through oxidative phosphorylation. They participate in the biosynthesis of numerous macromolecule precursors, as well as in the sensing and coordination to stressors such as oxidative stress, nutrient deprivation, ER stress or DNA damage, which can lead to apoptosis of damaged cells. In addition, mitochondria regulate reactive oxygen species (ROS) generation, with its implications in signaling (Eisner et al., 2018; Spinelli and Haigis, 2018). Mutations in mitochondrial enzymes, such as succinate dehydrogenase (Baysal et al., 2000; Ricketts et al., 2008; Janeway et al., 2010; Killian et al., 2013; Kim et al., 2013b), fumarate hydratase (Tomlinson et al., 2002; Sudarshan et al., 2011;Castro-Vega et al., 2013) or isocitrate dehydrogenase (Dang et al., 2009; Mardis et al., 2009; Ward et al., 2010), can influence gene expression by altering the activity of chromatin remodeling enzymes (Figueroa et al., 2010; Xu et al., 2011) or by inducing specific gene programs through the establization of HIF-1 even under normoxia via PHDs inhibition (Isaacs et al., 2005; Selak et al., 2005). Thus, mitochondria is a highly important organelle within cells.

1.3.1.1. TCA cycle and oxidative phosphorylation

The tricarboxylic acid (TCA) cycle, also denominated Kreb's cycle or citrate acid cycle (CAC), is a central hub in cell metabolism due to the different substrates that can fuel it, as well as to the diverse functions its intermediates can play, influencing biosynthesis of macromolecules, chromatin modification, DNA methylation and protein post-translational modification.

The TCA cycle (**figure 21**) begins with the condensation of acetyl-CoA with oxaloacetate (OAA), generating citrate in a reaction catalyzed by the citrate synthase (CS). Acetyl-CoA is generated either from glucose-derived pyruvate in the reaction catalized by pyruvate dehydrogenase (PDH), or from fatty acid oxidation (FAO). Then, aconitase (ACO) isomerizes citrate generating isocitrate, which will be transformed into α-ketoglutarate (α-KG) through oxidative decarboxylation catalyzed by isocitrate dehydrogenase (IDH), generating NADH. Subsequently, α-KG is converted into succinyl-CoA in another oxidative decarboxylation reaction, catalyzed this time by α-KG dehydrogenase (α-KGDH), rendering another molecule of NADH. Next, succinyl-CoA is converted into succinate by succinyl-CoA synthase (SCS), coupled to GTP production that can be further converted int o ATP. Succinate dehydrogenase (SDH), the enzyme that takes part both in TCA cycle and in the electron transport chain as complex II (ETC II), oxidizes succinate into fumarate producing $FADH_2$. Fumarate will be coverted into malate by

fumarate hydratase (FH) or fumarase, and then, malate dehydrogenase (MDH) will oxidate generating NADH from NAD^+ and OAA, reaching the origin of the cycle where another acetyl-CoA will join and the cycle will continue (Martínez-Reyes and Chandel, 2020).

Several TCA intermediates serve as building blocks for macromolecule synthesis in the cytosol, therefore, anaplerotic reactions to replenish the cycle are needed. Glutaminolysis is one of these anaplerotic mechanisms, since through the transformation of glutamine into glutamate, it regenerates α-KG by the action of glutamate dehydrogenase (GDH) that will enter into TCA cycle, usually after a decrease in mitochondrial citrate levels due to its transport to cytosol for lipid biosynthesis (DeBerardinis et al., 2007). Another anaplerotic mechanism is the one catalyzed by the pyruvate carboxylase (PC), where pyruvate is converted into OAA that will then join TCA cycle (Cheng et al., 2011). Remarkably, upon ETC function impairment, glutamine-dependent reductive carboxylation can take place to maintain citrate levels and even more strikingly, almost all the intermediates of the TCA cycle (Mullen et al., 2011, Mullen et al., 2014). More in detail, glutamine-derived α-KG is converted into isocitrate by NADPH-dependent IDH2, and by reverse function of ACO, it generates citrate (Mullen et al., 2011, Mullen et al., 2014).

The reducing equivalents produced in the TCA cycle (3 NADH and 1 $FADH_2$ generated per cycle) will fuel the electron transport chain, to lastly produce ATP through oxidative phosphorylation (OxPhos), creating a link between the two processes. Oxidative phosphorylation (**figure 21**) is the largest source of cellular ATP (Spinelli and Haigis, 2018). During this process, electrons from NADH and $FADH_2$ generated in the TCA cycle are transferred through the different complexes of the ETC (I, II, III and IV) to oxygen as final electron acceptor. Along the flow of electrons to O_2, these protein complexes pump protons from the mitochondrial matrix to the intermembrane space, generating a chemiosmotic gradient and a transmembrane electrical potential ($\Delta\psi m$) that creates a proton-motive force. When the protons flow back to the mitochondrial matrix, ETC V (ATP synthase) uses the energy stored in the gradient to phosphorylate ADP into ATP (Stryer et al., 2010). Alternative, ATP synthase can also dissipate the proton gradient when acting in an ATP-consuming way.

In more detail, complex I (NADH-ubiquinone oxidoreductase) oxidizes NADH to NAD^+ and transfers electrons to the ubiquinone (UbQ) carrier, reducing it to ubiquinol (UbQH2) and translocating protons across the inner mitochondrial membrane (IMM). In addition, in the electron transference, ROS can be generated in the ubiquinone binding site of complex I (IQ site), as well as in the flavin site (IF). Mammalian ETCI consists of 44 subunits, seven coded by the mitochondrial genome (ND1, ND2, ND3, ND4, ND4L, ND5 and ND6), and the others by the nuclear genome (Raimondi et al., 2019). Complex II is the succinate dehydrogenase, component also of the Krebs cycle that catalyzes the oxidation of succinate to fumarate, reducing FAD to $FADH_2$ and then transferring the electrons to Fe-S clusters, which will be transferred to ubiquinone, reducing it to generate UbQH2. Importantly, this electron transference does not have an associated translocation of protons (Zhao et al., 2019). Subsequently, complex III (ubiquinol-

cytochrome c oxidoreductase) transfers the electrons carried by UbQH2 (from complex I and II) to cytochrome c, coupling this transference with proton translocation, and sometimes, giving also rise to ROS (Raimondi et al., 2019). Complex IV (cytochrome c oxidase, COX) transfers the electrons from the cytochrome c to O_2, the terminal electron acceptor, generating H_2O and translocating protons to the intermembrane mitochondrial space (Raimondi et al., 2019; Zhao et al., 2019). One consequence of the electron transference along the ETC is the generation of superoxide (O_2^-). It is generated upon proton leakage in the ETC I, II, and III. In addition, other ETC associated enzymes such as proline dehydrogenase (PRODH), mitochondrial glycerol-3-phosphate dehydrogenase (GPDH) and dihydro-orotate dehydrogenase (DHODH) that link the oxidation of their substrates to the reduction of UbQ, are also ROS-producing spots (Raimondi et al., 2019). Superoxide is converted into H_2O_2 by manganese superoxide dismutase (MnSOD), which acts as second messenger and controls cell proliferation and survival (Groeger et al., 2009).

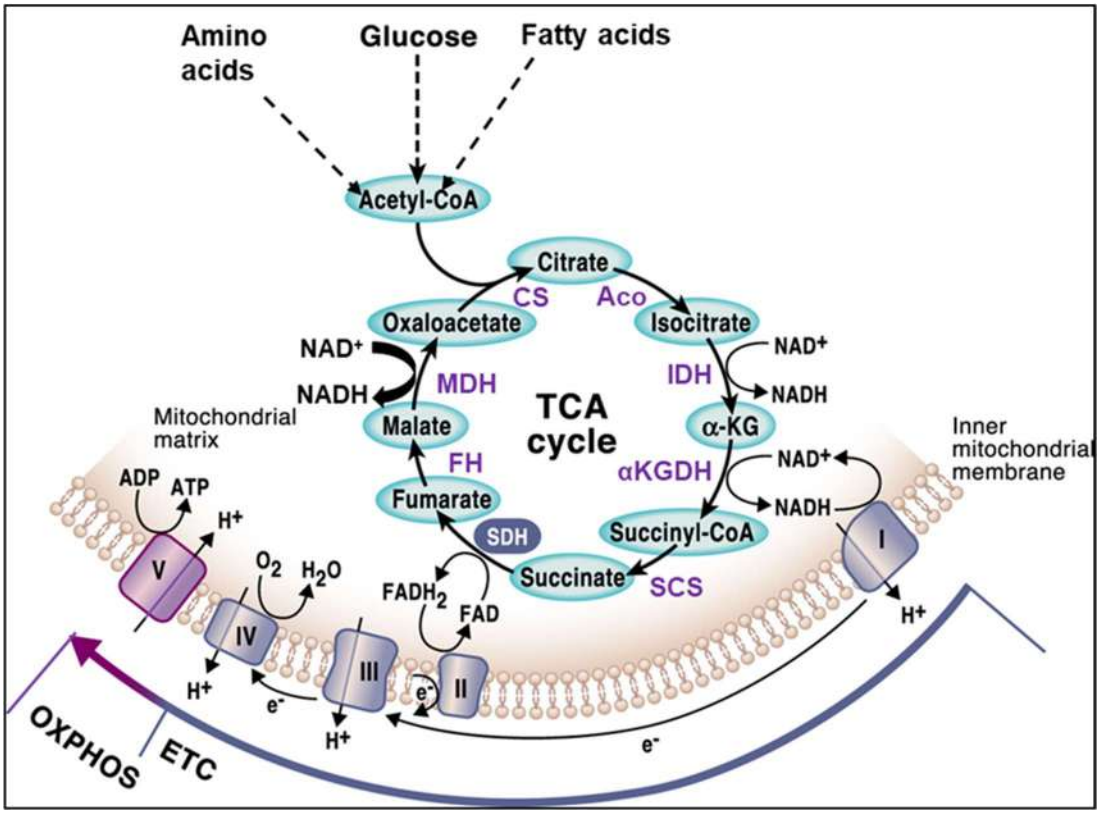

Figure 21. Simplified view of TCA cycle and oxidative phosphorylation.
Adapted from Martínez-Reyes and Chandel, 2020.

Citrate as metabolic regulator

Citrate is an important TCA-derived metabolite, since it connects carbohydrate metabolism, fatty acid metabolism, and epigenetic reprogramming, having also an impact on immune function (**figure 22**). In the context of prostate cancer, decreased citrate levels have been shown to serve as biomarker for

aggressiveness (Giskeødegård et al., 2013). Mitochondrial citrate carrier Slc25a1 exports citrate to the cytosol in exchange of malate. Cytosolic citrate is an allosteric activator of Acetyl-CoA carboxylase (ACC) and of fructose-1,6-BPase, involved in gluconeogenesis. However, it downregulates pathways that produce ATP, therefore, it inhibits phosphofructokinase (PFK) as well as pyruvate kinase (PK) by decreasing levels of the PK activator fructose-1,6-BPase (Williams and O'Neill, 2018). High citrate also decreases TCA cycle, since it inhibits SDH (Hillar and Lennox, 1975) and the acetyl-CoA producer PDH (Taylor and Halperin, 1973). Citrate is generated in the TCA from oxaloacetate and acetyl-CoA by citrate synthase (CS), or alternatively, from α-KG reductive carboxylation via NADPH dependent isocitrate dehydrogenase, in the context of cancer and mitochondrial dysfunction (Mullen et al., 2011; Mullen et al., 2014). There is still no consensus about CS effect in cancer. CS activity is increased in pancreatic and ovarian cancer, which promotes cancer cell proliferation, migration and invasive capacity (Schlichtholz et al., 2005; Chen et al., 2014). However, in cervical and breast cancer CS expression is decreased. This drives the switch from OxPhos to glycolysis, as well as it induces the transformation to epithelial-mesenchymal transition phenotype and therefore accelerates tumor malignancy (Lin et al., 2012).

By ACLY, citrate breakdown provides carbons for cholesterol, ketone bodies, and fatty acid in addition to inducing epigenetic changes through histone acetylation directed by acetyl-CoA. ACLY is a very interesting enzyme. It is involved in immune response modulation as well as in cancer. Preclinical studies have shown that the inhibition of ACLY decreases the expression of the anti-apoptotic MCL-1, arresting tumor growth and increasing sensibility to chemotherapy in gastric cancer (Icard and Lincet, 2016). ACLY also regulates glucose to acetate switch. It was shown that ACLY deficiency induced an increase in acety-CoA from acetate by upregulating ACSS2, providing subtrates for fatty acid synthesis and histone acetylation (Zhao et al., 2016). Not surprisingly, ACLY is found overexpressed and with increased activity in liver, lung, breast, gastric, prostate, colon and bladder cancer and it negatively correlates with the stage, differentiation and prognosis of cancer (Varis et al., 2002; Turyn et al., 2003; Yahagi et al., 2005; Yancy et al., 2007; Migita et al., 2008; Zaidi et al., 2012).

Mitochondrial aconitase (ACO2), mainly involved in the regulation of citrate availability (Gaude and Frezza, 2014), has also been described to be altered in several types of cancer, however resulting in different effects. Decreased levels of ACO2 in gastric cancer have been linked to negative prognosis (Wang et al., 2013). However, ACO2 activity is increased in prostate cancer, which increases citrate oxidation and provides energy for tumor cell proliferation and metastasis, essential for malignancy (Singh et al., 2006; Xue et al., 2019). Regarding immune system, citrate is an important metabolite for macrophage and DC effector function. First, fatty acid synthesis for membrane expansion is essential for antigen presentation. In addition, citrate give rise to cis-aconitate via ACO2, which is subsequently decarboxlylated by immune-responsive gene 1 (IRG1), to produce itaconate. Itaconate has antibacterial effect, in addition to be an anti-inflamatory agent by inhibiting SDH and decreasing the production of NO, IL-1β, IL-18, and HIF-1α (Williams and O'Neill, 2018). Interestingly, citrate oral administration was

shown to reduce tumor growth of several mice xenografts while increasing tumor infiltrating T cells (Ren et al., 2017). In the same study, they showed that citrate can inhibit IGF-1R/PI3K/AKT axis, inducing cell differentiation and activating the immunosuppresive PTEN/eIF2a pathway (Ren et al., 2017).

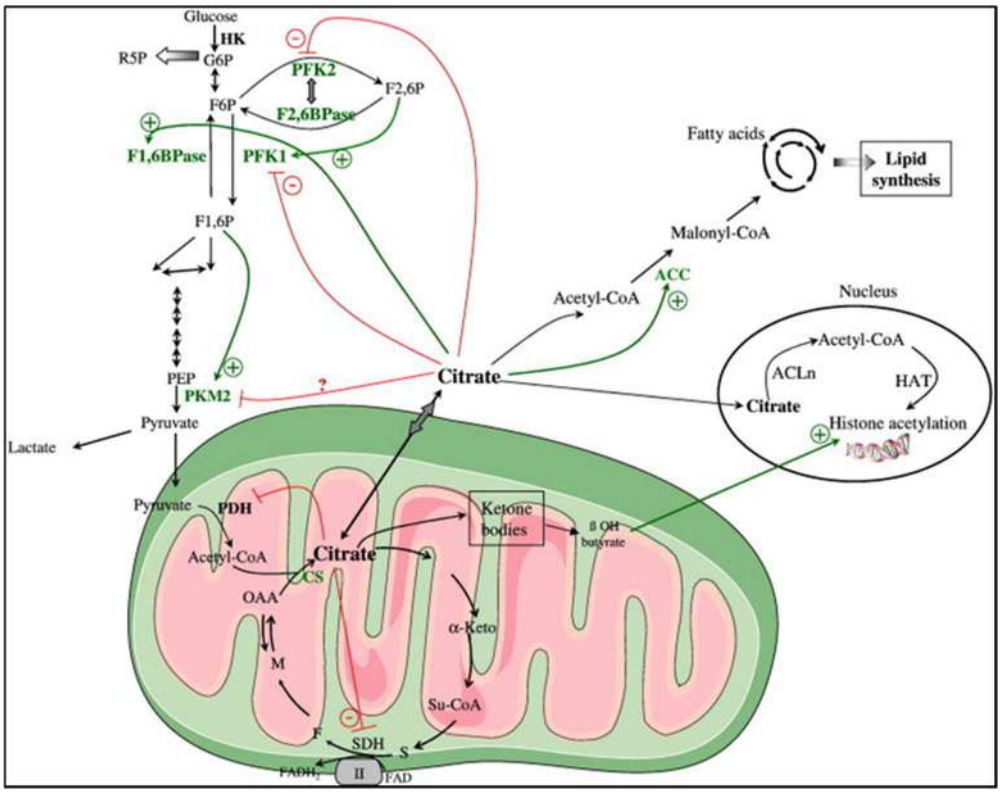

Figure 22. Citrate as metabolic regulator.
Figure adapted from Icard et al., 2012.

Oncogenic regulation of TCA cycle and OxPhos

Several oncogenes increase fueling of carbon sources into the TCA cycle, which results in higher electron flow through the ETC that can leak and generate ROS. Oncogenic KRAS signaling increases glutamine feeding of the TCA cycle (Weinberg et al., 2010). MYC also rewires cancer cell metabolism by stimulating glycolysis and enhancing glutaminolysis to fuel TCA cycle (Wise et al., 2008), and by increasing mitochondrial biogenesis, as denoted by an increase in mitochondrial mass and enhanced oxygen consumption in B-cells ectopically expressing MYC (Li et al., 2005). Mitochondria localized AKT has been pointed out to phosphorylate MICU1, the regulatory subunit of the mitochondrial calcium uniporter (MCU), destabilizing the heterodimer it constitutes with MICU2, leading to increased calcium levels in mitochondria (Marchi et al., 2018), which enhances TCA cycle and oxygen consumption (Brookes et al., 2004). Another key regulator of cell metabolism, mTOR, increases mitochondrial respiration by upregulating the expression of the nucleus-encoded subunits ETCI and V via 4E-BP proteins inactivation (Morita et al., 2013). Another mechanism by which mTOR controls mitochondrial gene expression is through the direct modulation of the transcription factors yin-yang 1 (YY1) and

peroxisome-proliferator-activated receptor coactivator (PGC-1α) (Cunningham et al., 2007). The fusion protein BCR/ABL as well as the activating mutation FLT3-ITD are able to increase oxygen consumption in leukemic cells (Reddy et al., 2010). BCL-2 in leukemia upregulates mitochondrial respiration (Lagadinou et al., 2013), as well as does MCL-1 under BCL-2 expression loss as consequence of venetoclax treatment (Pei et al., 2020).

Respiratory supercomplexes

In the recent years, the existence of respiratory supercomplexes (RSC) it is becoming accepted. Until Acín-Pérez and colleagues showed in 2008 functional evidence of the activity of supercomplexes, they were considered mere structural artifacts of blue native polyacrylamide gel electrophoresis (BN-PAGE) (Acín-Pérez et al., 2008). RSC are functional quaternary structures resulting from the assembly of respiratory chain complexes of varied stechiometries, located in the mitochondria cristae (Milenkovic et al., 2017). Cardiolipin is the most abundant phospholipid of the inner mitochondrial membrane (Paradies et al., 2019). Regarding the formation and stability of these supercomplexes, cardiolipin has been described as essential (Bazán et al., 2013). COX7A2- like protein (COX7A2L) (or supercomplex assembly factor 1 SCAFI) has been identified as crucial for the interaction between complexes III and IV (Lapuente-Brun et al., 2013). Respirasomes are the best characterized RSC. They contain complex I, complex III twice, and complex IV, being therefore usually referred as I/III2/IV (Schägger et al., 2004). Cristae shape and structure, regulated by the IMM protein optic atrophy 1 (OPA1), are essential for the assembly and activity of RCS, as well as for cell growth dependent on the mitochondria (Cogliati et al., 2013). The most intriguing part is the reason for the existence of supercomplexes. It has been accepted that their function was to enhance oxidation catalysis by channeling subtrates (Althoff et al., 2011; Guo et al., 2016; Sousa et al., 2016). However, this theory is challenged by recent structural and biophysical data (Letts et al., 2016; Milenkovic et al., 2017). Supercomplexes have been reported to promote breast and endometrial tumorigenesis by increasing TCA intermediates via the stabilizator factor for the respirasome assembly COX7A2L overexpression and by enhancing hypoxia tolerance (Ikeda et al., 2019). In pancreatic cancer, they allow cancer cells to support an oxidative metabolism even under severe hypoxia (Hollinshead et al., 2020), and they have been found to have enhanced assembly, correlated to neurolysin overexpression, in a subgroup of AML patients as well (Mirali et al., 2020).

1.3.1.2. Biosynthesis of macromolecules

Mitochondria participate in the biosynthesis of nucleotides, FAs, cholesterol, amino acids, glucose and heme group.

1.3.1.2.1. Nucleotides

Mitochondria is involved in the synthesis of nucleotides (**figure 23**). Tetrahydrofolate (THF) carries 1 carbon and transfers it for *de novo* nucleotide biosynthesis (Yang and Vousden, 2016). In addition, activated THF molecules are also involved in the synthesis of amino acids, more specifically in the catabolism of serine into glycine in the mitochondria and in serine synthesis in the cytosol, as well as in the maintenance of redox homeostasis (Yang and Vousden, 2016; Spinelli and Haigis, 2018). This set of reactions; characterized by the generation and transference of activated one-carbon units (1C) is known as the one carbon metabolic pathway or as the folate cycle, previously mentioned in the point "1.1.2.2. Serine and glycine". At a more descriptive level, THF enters the mitochondria through the carrier SLC25A32, and then, it is converted by serine hydroxymethyltransferase-2 (SHMT2) into 5,10-methylene-THF and glycine. Next, methylene-tetrahydrofolate dehydrogenase 2 (MTHFD2) generates the one-carbon-carrying 10 formyl-THF (Yang and Vousden, 2016; Spinelli and Haigis, 2018). The expression of MTHFD2 is regulated by mTORC1 and is essential for cell growth and proliferation (Ben-Sahra et al., 2016). Thus, it is not surprising that highly proliferative cells, such as human cancer cells (Nilsson et al., 2014) or T cells upon activation (Ron-Harel et al., 2016) express high levels of this enzyme. 10 formyl-THF can be used for the generation of formylmethionine tRNA for mitochondrial translation, be cleaved into formate by the MTHFD1-like (MTHFD1L), be exported to the cytosol or alternatively, it can be reconverted into THF by the 10-formyl-THF dehydrogenase. In the cytosol, 10 formyl-THF can be regenerated from formate by the MTHFD1 for *de novo* purine synthesis or 5,10 methylene-THF for pyrimidine synthesis. Then, the cycle is closed by the cytosolic conversion of glycine into serine by the SHMT1 (Yang and Vousden, 2016; Spinelli and Haigis, 2018).

De novo pyrimidine synthesis (**figure 23 right**) begins with the reactions catalyzed by the tri-functional CAD enzyme. The first step of the pathway is the generation of carbamoyl-P and aspartate from CO_2, glutamine and ATP. This reaction is catalyzed by the Carbamoyl-P synthase II activity of CAD.Then, the products of the previous reactions are transformed into carbamoyl-aspartate by the aspartate transcarbamoylase activity. Lastly, the dihydroorotase activity generates dihydroorotate, which is transformed into orotate in the outer side of the inner mitochondrial membrane by the dihydroorate dehydrogenase (DHODH). Orotate gets out of the mitochondria and the bi-functional uridine monophosphate synthase (UMPS) renders uridine-5-P after the two reactions catalyzed by the orotate phosphoribosyl transferase, which generates the intermediate orotidine-5-P, and the orotate decarboxylase (Bajzikova et al., 2019). Remarkably, DHODH transfers the electrons of the oxidation to ubiquinone, thus, its catalytic activity requires available ubiquinone, and therefore, a functional ETC (Raimondi et al., 2019). mTORC1 stimulates the *de novo* pyrimidine synthesis pathway via activation and phosphorylation of CAD by its downstream effector ribosomal protein S6 kinase 1 (S6K1) (Ben-Sahra et al., 2013). Since pyrimidine biosynthesis is essential for proliferation, DHODH is essential for T cell clonal expansion and differentiation into effector cells (Spinelli and Haigis, 2018). In addition, Bajzikova and colleagues

showed that 4T1 cells with deletion of DHODH by CRISPR/Cas9 were not able to proliferate in the absence of uridine. However, the expression of DHODH restored proliferation (Bajzikova et al., 2019). This data suggests that DHODH establishes an essential link between respiration and tumorigenesis, which makes it an interesting therapeutic target. At the gene regulation level, oncogenic MAPK, KRAS and mTOR increase DHODH activity by inducing *de novo* pyrimidine biosynthesis, while MYC increases the expression of DHODH (Raimondi et al., 2019; Mollick and Laín, 2020). DHODH activity is also increased in response to DNA damage and upon chemotherapy DNA repair (Brown et al., 2017).

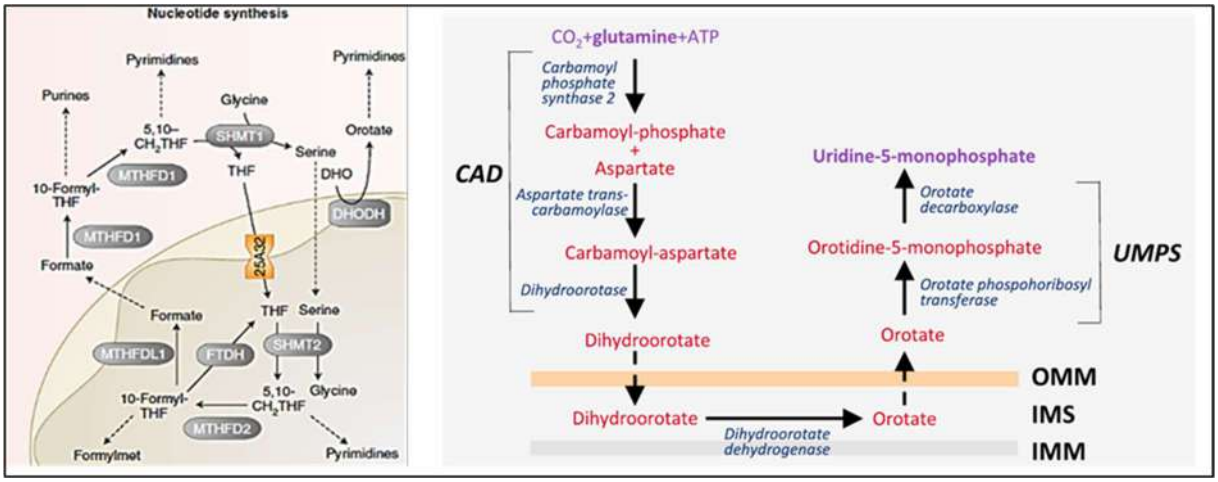

Figure 23. Nucleotide biosynthesis in the mitochondria.
On the **left:** General overview of mitochondrial nucleotide biosynthesis; on the **right**, de *novo* pyrimidine synthesis. Adapted from Spinelli and Haigis, 2018; Bajzikova et al., 2019

1.3.1.2.2. Amino acids

Several amino acids are synthesized in the mitochondria.

Proline

Proline biosynthesis and degradation takes place between cytosol and mitochondria, in a process called "proline cycle" (**figure 24**).

Proline biosynthesis is mediated by Δ1-pyrroline-5-carboxylate reductases (PYCRs) in the cytoplasm, which transform Δ1-pyrroline-5-carboxylate (P5C) into proline consuming NADPH generated in the PPP. Proline biosynthesis is constitutive activated by PI3K in EGFR-mutated non-small-cell lung cancer (Angulo et al., 2007). Proline biosynthesis is also stimulated by MYC, and blockade of its biosynthetic pathway results in decreased tumor growth. (Liu et al., 2015). The same study also highlighted the essential role of proline biosynthesis at mantaining pyridine nucleotide pool, by linking this process to the oxidative arm of PPP and glycolysis (Liu et al., 2015).

Proline oxidation to P5C takes place in the mitochondria, and it is catalized by proline dehydrogenase/oxidase (PRODH/POX), which is considered a tumor suppressor (Raimondi et al., 2019; Burke et al., 2020). In fact, PRODH/POX is downregulated or even absent in many cancers, such as pancreas, liver, kidney, bladder, stomach and colon cancer (Liu et al., 2012). PRODH/POX is located in the inner mitochondrial membrane. It transfers the electrons resulting from proline oxidation to UbQ through $FADH_2$, becoming a point of ROS generation due to electron leak, or of ATP production depending on the context (Raimondi et al., 2019). Indeed, PRODH overexpression increases ROS generation and decreases ETC respiration fitness, due to competition between PRODH and ECT II for UbQ (Hancock et al., 2015). PRODH/POX is upregulated under hypoxia or under low glucose conditions via AMPK activation, where generated ROS can induce autophagy for survival, or apoptosis and block cell proliferation and cell cycle (Liu et al., 2012). In addition, it is also upregulated by p53, being identified at one of the 14 genes most strongly induced by the famous tumor suppressor (Polyak et al., 1997; Liu et al., 2008). Interestingly, c-Myc suppresses PRODH/POX expression through the induction of miR-23b (Liu et al., 2012b). Regarding apoptosis, PRODH/POX can induce both intrinsic and extrinsic pathways: ROS generated by PRODH/POX can induce cytochrome c release from the mitochondria, and caspase-9 activation (intrinsic pathway). Alternatively, it can induce the extrinsic pathway apoptosis by stimulating tumor necrosis factor-related apoptosis inducing ligand (TRAIL) and death receptor 5 (DR5) expression, which will end up with the cleavage of caspase-8 and dead receptor-mediated apoptosis (Liu et al., 2006; Liu et al., 2012; Huynh et al., 2020). Regarding proliferation, MAPK pathway, as well as cyclooxygenase-2 (COX-2)/prostaglandin E_2 (PGE_2) signaling, Wnt/b-catenin signaling and EGFR pathway are inhibited by PRODH/POX (Liu et al., 2006; Liu et al., 2008; Liu et al., 2012). Cell cycle arrest is induced by stimulation of the cell cycle regulators geminin and growth arrest DNA damage inducible proteins (GADDs) by ROS PRODH/POX-derived (Liu et al., 2009; Liu et al., 2012). Proline can act also as an anaplerotic metabolite, since it can be converted to glutamate, which can enter TCA cycle after being converted to α-KG (Liu et al., 2012). By increasing α-KG, PRODH/POX increases activity PHDs, which hydroxylate HIF-1α tagging it for degradation, and thus, inhibiting HIF-1 signaling (Liu et al., 2012; Huynh et al., 2020). In addition, proline can also be converted to ornithine by ornithine aminotransferase (OAT) and enter urea cycle (Liu and Phang 2012).

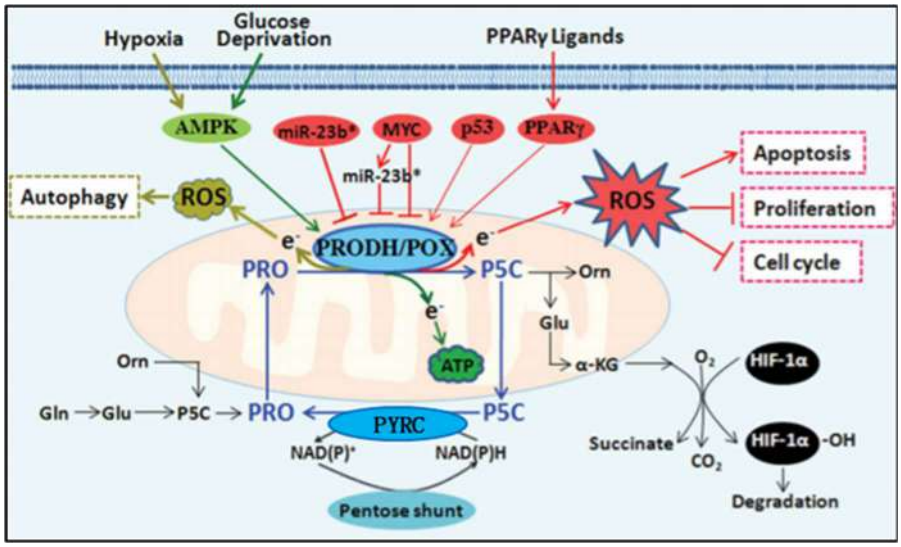

Figure 24. Proline cycle.
Adapted from Liu and Phang 2012.

Glutamine and glutamate

Glutamine and glutamate biosynthesis and metabolism is more extensively reviewed in the point 1.1.2.1. **Figure 25** summarizes glutamine metabolism according to its subcellular localization.

GS is the enzyme which generates glutamine from the ATP dependent condensation of glutamate and ammonia (Matthews et al., 2010; Yang et al., 2016). GS can be located in mitochondria (in liver) or in the cytosol (in astrocytes) accordingly to tissue $\Delta\Psi$, and depending on its localization, it can have a different role (Matthews et al., 2010; Spinelli et al., 2018). GS-derived glutamine can have different fates depending on the pathological context. In glioblastoma, GS-generated glutamine is used for *de novo* purine synthesis (Tardito et al., 2015), while in breast cancer it is used for general biomass generation (Spinelli et al., 2017). Alternatively, glutamine can enter into the cytosol through several plasma membrane glutamine transporters, where it can take part in several processes, such as the biosynthesis of UDP-GlcNAc, asparagine or nucleotides. Glutamine can also enter the mitochondria through SLC1A5, where GLS converts it into glutamate while releasing ammonium. The three GLS isoforms (GLS1, GLS2, and GAC, a splicing isoform of GLS1) are located into the mitochondria (Wang et al., 2010; Lukey et al., 2019; Yoo et al., 2020). Mitochondrial glutamate can be converted into α-KG by GDH1 or by aminotransferases, like glutamic-oxaloacetic transaminase 2 (GOT2) or glutamic-pyruvic transaminase 2 (GPT2) to participate in the biosynthesis of non-essential amino acids (Yoo et al., 2020). Glutamate-dependent transaminases have been reported to be more expressed in proliferative than in quiescent cells (Coloff et al., 2016). Since glutamate-dependent transaminases have mitochondrial and cytosolic isoforms, further research is needed to shed some light on the impact of the subcellular location of glutamate metabolism in proliferation. Glutamine-derived α-KG can fuel TCA, and eventually generate the

oncometabolite 2-HG upon IDH2 mutation or hypoxia, which can modulate demethylathing TET enzymes activity and subsequently regulate epigenetic modifications (Intlekofer et al., 2015).

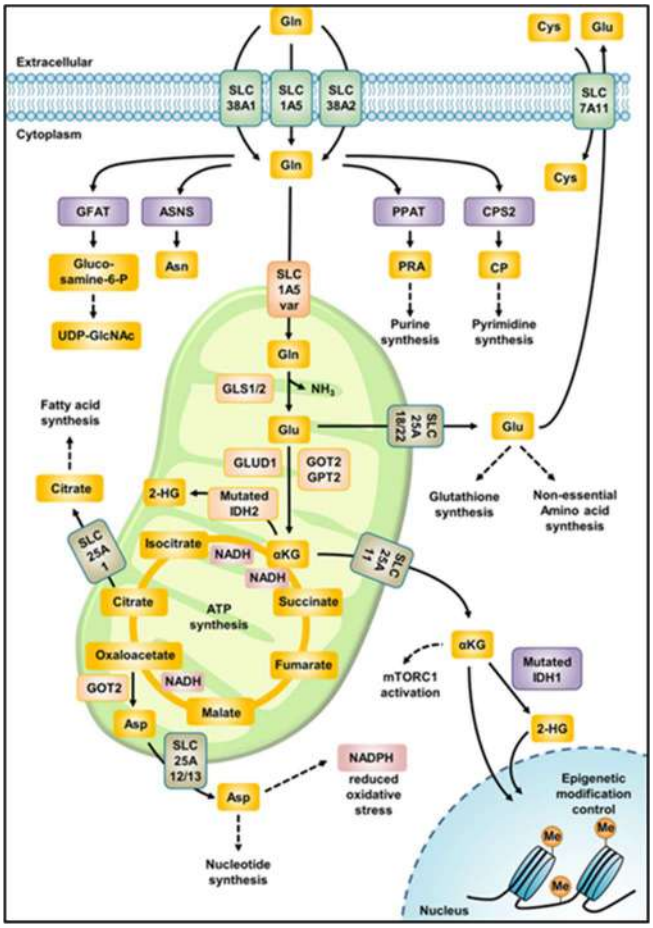

Figure 1. Glutamine metabolic pathways.
Adapted from Yoo et al., 2020.

Aspartate

Interestingly, two studies from 2015 showed that the biosynthesis of aspartate is an essential function of the electron transport chain in proliferating cells (Birsoy et al., 2015; Sullivan et al., 2015). Aspartate biosynthesis takes place in the mitochondria via mitochondrial malate dehydrogenase (MDH2) and its cofactor NAD^+, using TCA-derived oxaloacetate as substrate. Subsequently, GOT2 synthetizes aspartate from oxaloacetate in mitochondria (**figure 26**). The blockage of ETC impairs mitochondrial ATP synthesis but also decreases NAD^+ pool and, eventually, NAD^+/NADH ratio. Therefore, under ETC impairement, citrate derived from reductive carboxylation of glutamine by ATP-citrate lyase is converted to oxaloacetate, further used by the GOT1 for cytosolic aspartate generation. Despite this switch to a reductive biosynthetic aspartate pathway, the levels obtained by this route are not enough to support cell proliferation without external supply of NAD^+, and it leads to aspartate auxotrophy. These works also showed that the addition of pyruvate (Birsoy et al., 2015) or α-ketobutyrate (Sullivan et al., 2015) can restore proliferation under ETC blockade, since these compounds act as electron acceptors and contribute to cytosolic NAD^+ regeneration by the LDH, required for the MDH1.

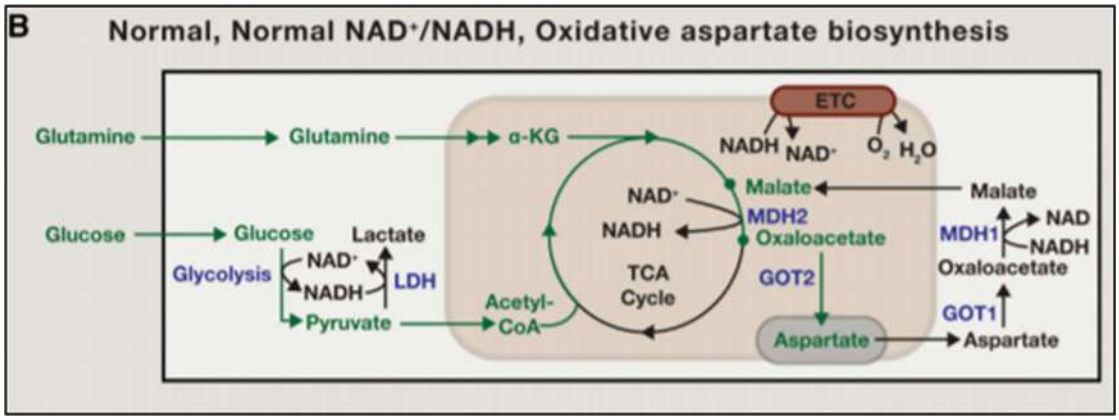

Figure 26. Aspartate biosynthesis requires a functional ETC.
Adapted from Van Vranken and Rutter, 2015.

1.3.1.2.3. Heme biosynthesis

Heme is a prosthetic group involved in multiple biological processes, such as cellular respiration, photosynthesis, O_2 transport in hemoglobin, NO and CO sensing in soluble guanylyl cyclase (sGC), oxygenase catalyzed reactions, peroxide production and destruction, enzyme activity regulation and xenobiotic detoxification. (Kim et al., 2012). Heme group synthesis is a highly compartimentalized process, summarized in **figure 27**.

Mitochondrial aminolevulinate synthase (ALAS) catalyses aminolevulinic acid (ALA) generation from glycine and succinyl CoA. ALAS expression, and therefore heme synthesis, has been reported to be increased in fasted liver by PGC-1α, while glucose intake had the opposite effect, suggesting a nutritional regulation of heme synthesis (Handschin et al., 2005). Then, SLC25A38 exports ALA to cytosol, where it is converted into copro-porphyrinogen III (CPGIII) after four reactions. Subsequently, CPGIII enters mitochondrial intermembrane space through ABCB6, an ATP-dependent transporter. ABCB6 can also export coproporphyrinogen III outside the cell in order to avoid accumulation of heme intermediates. Once in the intermembrane mitochondrial space, coproporphyrinogen oxidase (CPOX) generates protoporphyrinogen IX by catalysing the oxidative decarboxylation of coproporphyrinogen III. Then, protoporphyrinogen oxidase (PPOX) catalyzes the oxidation of protoporphyrinogen-IX to protoporphyrin-IX. The final reaction is the insertion of the ferrous iron in protoporphyrin-IX by ferrochelatase (FECH), to form the heme group. This reaction takes place in the mitochondrial matrix. FECH expression has been reported to be reduced in glioblastoma (Teng et al., 2011), colorectal cancer (Kemmner et al., 2007) and bladder cancer (Hayashi et al., 2015), leading to protoporphyrin IX accumulation. Heme group is degraded to bilirubin by the NADPH consuming enzymes haem oxygenase and bilirubin reductase (Spinelli and Haigis, 2018; McNicholas et al., 2019).

Heme biosynthesis has been reported to be upregulated in high MYCN-AML, correlating with worst survival (Fukuda et al., 2017). Recent work have also shown that heme metabolism is increased in AML patients who fail to achieve remission after chemotherapy. In addition, interruption of heme biosynthesis

sensitized leukemic cells to apoptosis through ETC activity modulation, highlighting a new vulnerability of leukemic cells (Lin et al., 2019).

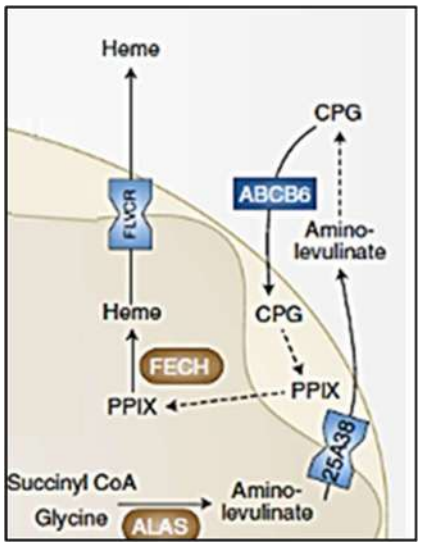

Figure 27. Heme biosynthesis.
Adapted from Spinelli and Haigis, 2018.

1.3.1.3. Redox balance and NAD^+

NAD^+ is an essential metabolite, involved in a wide range of cellular processes, such as redox reactions, epigenetic modifications or DNA repair (**figure 28**). Despite not reaching still a consensus, some studies have shown that mammalian cell types are able to uptake NAD^+. First evidences were provided by Bruzzone and colleagues, identifiying connexin 43 as hemichannels that mediated Ca^{2+}-regulated transmembrane NAD^+ in intact cells (Bruzzone et al., 2001), relevant in neurons. Later on, Billington and colleagues showed that exogenous NAD^+ could rescue NIH-3T3 fibroblasts from death upon NAD depletion induced by the nicotinamide phosphoribosyltransferase (NAMPT) inhibitor FK866 (Hasmann. and Schemainda, 2003; Billington et al.; 2008). In addition, it has been recently shown that NAD^+ supplementation was able to rescue MCF-7 breast cancer cells from the block in DNA repair resulting from PARP1 inactivation induced by FK866 NAD-depletion (Wilk et al., 2020). Nevertheless, mammalian cells have several pathways to provide NAD^+. NAD can be *de novo* synthetized from tryptophan or from nicotinic acid, or, as mainly happens in mammalian cells, recycled via the salvage pathway from nicotinamide (NAM)(Rajman et al., 2018). NAM phosphoribosyltrasferase (NAMPT) is the rate limiting enzyme in NAD^+ salvage pathway, involved in converting NAM to nicotinamide mononucleotide (NMN), and it has been reported to be overexpressed in some cancers and to induce cancer stemmness in others (Wang et al., 2010b; Shackelford et al., 2013b, Lucena-Cacace et al., 2017). NAD^+ is used by several enzymes, including sirtuins, Poly(ADP-ribosyl) polymerases (PARPs), the

ectoenzymes CD38, CD157 and SARM1 and Nicotinamide N-methyltransferase (NNMT), and by modulating NAD^+ content these enzymes and several cellular processes (**figure 28**) are affected.

Sirtuins are class III histone deacetylase (HDAC) enzymes that depend on NAD^+ for their activity (Etchegaray and Mostoslavsky, 2016). In mammalians, this family is constituted by seven sirtuins (SIRT1-7) that control different cellular processes and vary in subcellular localizacion and enzymatic activity. SIRT1 can be located either in the nucleus or in the cysotosol, SIRT2 is located in the cytosol, SIRT3 and SIRT4 in the mitochondria, SIRT5 either in the mitochondria or in the cytosol, SIRT6 is nuclear and SIRT7 found at the nucleolus (Covarrubias et al., 2020; Xie et al., 2020). Sirtuins remove acetyl groups from lysine residues of target proteins using NAD^+: they cleave NAD^+ into NAM and ADP-ribose to transfer the acetyl group, and Acetyl-ADP-ribose, where NAM and the deacetylated protein are relased as products of the reaction (Sauve, 2010). Nevertheless, some sirtuins like SIRT5 or SIRT6 have desuccinylation and defatty-acylation activities (Du et al., 2011; Jiang et al., 2013) while SIRT6 and SIRT4 can act just like ADP-ribosyltransferases liberating NAM (Liszt et al., 2005; Haigis et al., 2006), whose functions require further study. Exercise and fasting result in rised NAD^+ levels induced by AMPK activation, what turns-on sirtuins and exerts transcriptional modulation through SIRT1-dependent deacetylation of PGC-1α and FOXO1 to boost mitochondrial biogenesis and lipid metabolism (Cantó et al., 2010). Sirtuns are also involved in the loop that modulates circadian NAD^+ levels: SIRT1 and SIRT6 regulate circadian clock transcription factors, while SIRT1 regulates NAD^+ synthesis via salvage pathway through regulation of NAM levels, and, in addition, NAMP is under control of circadian clock (Nakahata et al., 2009; Ramsey et al., 2009; Masri et al., 2014). SIRT1, SIRT6 and SIRT7 participate in also DNA repair (Lagunas-Rangel, 2019), while SIRT3, SIRT4 and SIRT5 and nuclear SIRT1 regulate mitochondrial homeostasis by inducing mitophagy to remove defective mitochondria and mitochondrial biogenesis trough PGC-1α (Jang et al., 2012; Sack and Finkel, 2012; Meng et al., 2019). Sirtuins have attracted great attention for their involvement in ageing, since ageing is accompanied by a gradual decline in NAD^+ levels and it is expected to impact sirtuins activity (Covarrubias et al., 2020). However, further research needs to be done to completely understand how NAD^+ influences the natural process of ageing.

Poly(ADP-ribosyl) polymerases (PARPs) are a family of enzymes characterized by its enzymatic activity, involved in DNA damage response and repair. Mechanistically, they mediate the cleavage of NAD^+ into NAM yielding ADP-ribose, that will be added to PARP and to other proteins, what is known as 'poly(ADP-ribosyl)ation' (PARylation) (Covarrubias et al., 2020). In humans, there are 18 PARPs, however, PARP1 is the best characterized and the main responsible for PARP activity upon DNA damage response, thus, the main NAD^+ consumer (Ray-Chaudhuri and Nussenzweig, 2017). Upon activation, PARP1 recruits the repair complexes to the damaged point by PARylation of histones, proteins and even itself, to elicit DNA repair (Covarrubias et al., 2020). Due to its function, PARP1 is one of the main NAD^+ consumers (Liu et al., 2018b), antagonizing SIRT1 since PARP K_m for NAD^+ is lower, and its kinetics

faster (Cantó et al., 2013). An evidence of this is provided by SIRT1 increased activity and improved mitochondrial function in mice fed with a high fat diet treated with PARP inhibitors (Bai et al., 2011; Pirinen et al, 2014), highlighting the NAD^+ consumer role of PARP1 not only upon DNA damage, but also in normal conditions. Remarkably, supplementation with NAD^+ precursors decreased the accumulation of endogenous DNA damage and improved DNA repair capacity in several neurodegenerative diseases (Scheibye-Knudsen et al., 2014; Fei Fang et al, 2016; Hou et al., 2018).

CD38, CD157, SARM1 are ectoenzymes with glycohydrolase activity and also ADP-ribosyl cyclase activity. They cleave NAD^+ generating NAM and ADP-ribose, while they can also generate cyclic ADP-ribose (Essuman et al., 2017; Covarrubias et al., 2020; Xie et al., 2020).
In addition to cyclic ADP-ribose and ADP-ribose, CD38, can also generate the nicotinic acid adenine dinucleotide phosphate (NAAD(P)) by catalizing a base exchange from NAM to NA (Cosker et al., 2010), what places it in a strategic position to activate Ca^{2+} signaling, with its implications on immune cell activation and cell metabolism (Ernst et al., 2013; Yu et al., 2019). Remarkably, it's been recently reported that CD38 can use NMN as substrate as well (Camacho-Pereira et al., 2016). Interestingly, CD157 can use NR as alternative substrate (Preugschat et al., 2014). In addition, both CD38 and CD157 are upregulated in in ageing tissues (Covarrubias et al., 2020b). Thus, several inhibitors targeting CD38 and CD157 aim to improve the efficiency of NAD^+ precursor supplementation for restoring NAD^+ levels in aging and neurodegenerative pathologies (Tarragó et al., 2018). SARM1 relies on Toll/interleukin receptor (TIR) domain for its activity (Carty et al., 2006). It is expressed in neurons, as well as in immune cells (Carty et al., 2006; Essuman et al., 2017). NAD^+ degradation exterted by SARM1 triggers axonal degeneration upon axonal injury (Gerdts et al., 2015), making the inhibition of this enzyme an interesting therapeutic opportunity for neurodegenerative diseases. Regarding its role in the immune compartment, it is still controversial and further research is needed to characterize its involvement in this field, as well as upon physiologic conditions.

Nicotinamide N-methyltransferase (NNMT) constitutes the main NAM clearance pathway, by catalyzing the methylation of NAM using as donor the methyl group from S-adenosylmethionine (SAM) (Pissios, 2017). NAM methylation decreases the index of methylation capacity in tissues, mechanism that has been observed in cancer cells by enhanced NNMT expression (Ulanovskaya et al., 2013). In addition, by methylating NAM, NNMT regulates NAD^+ biosynthesis as well as sirtuin activity, since NAM inhibits it as its resulting product (Bitterman et al., 2002; Covarrubias et al., 2020). NNMT is predominantly expressed in the liver, and it has been recently identified as a therapeutic target for treating fatty liver and fibrosis by diminishing both NAD^+ content and FAO gene expression by inhibiting Sirt3 (Komatsu et al., 2018). Thus, NNMT connects metabolism with epigenetic regulation.

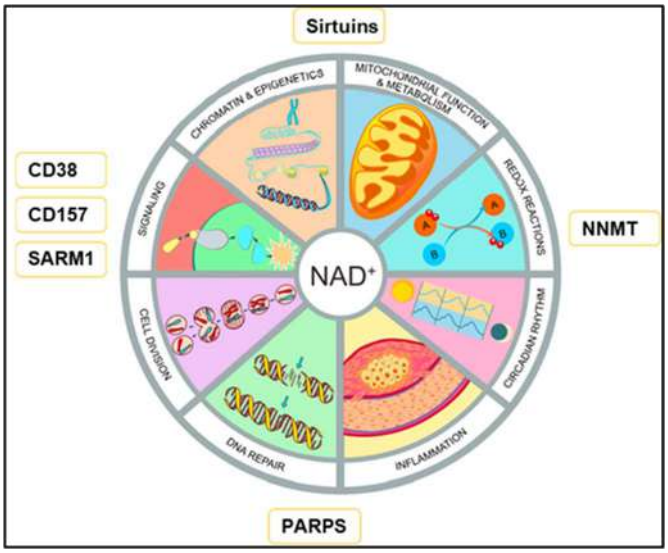

Figure 28. Cellular processes affected by NAD$^+$.
Adapted from Rajman et al., 2018.

Balance and compartimentalization of redox equivalents is essential for cellular homeostasis and for cell survival (Spinelli and Haigis, 2018). Due to the different nature of the reactions that take place within the mitochondria or the cytosol, both compartments have different needs for NAD. A bigger number of reductive metabolic reactions take place it mitochondria in comparison to cytosol. Thus, mitochondria has a more reductive environment than cytosol. (Stein and Imai, 2012). To support the imbalanced distribution and needs of NAD$^+$ between cytosol and mitochondria, yeast have direct NAD transporters, Ntd1 and Ndt2. These two transporters exchange across the inner mitochondrial membrane NAD with AMP and GMP (Todisco et al., 2006). It's been thought for a longtime that mammalian cells lacked a direct NAD transport system and that mitochondrial membrane was impermeable to NAD (Stein and Imai, 2012; Kane, 2014; Spinelli and Haigis, 2018). However, this vision has been challenged, first by the observation of Davila and colleagues that mitochondria could uptake NAD (Davila et al. 2018), and more recently by other works that identified SLC25A51 (also known as MCART1) as a mammalian mitochondrial NAD$^+$ transporter (Kory et al., 2020; Luongo et al., 2020). What has been acknowledge for longer to contribute to the exchange of reductive power by reduction-oxidation reactions between cytosol and mitochondria is the existence of redox shuttles in mammalian cells (Kane, 2014; Spinelli and Haigis, 2018). There are 4 main shuttles (**figure 29**), described below.

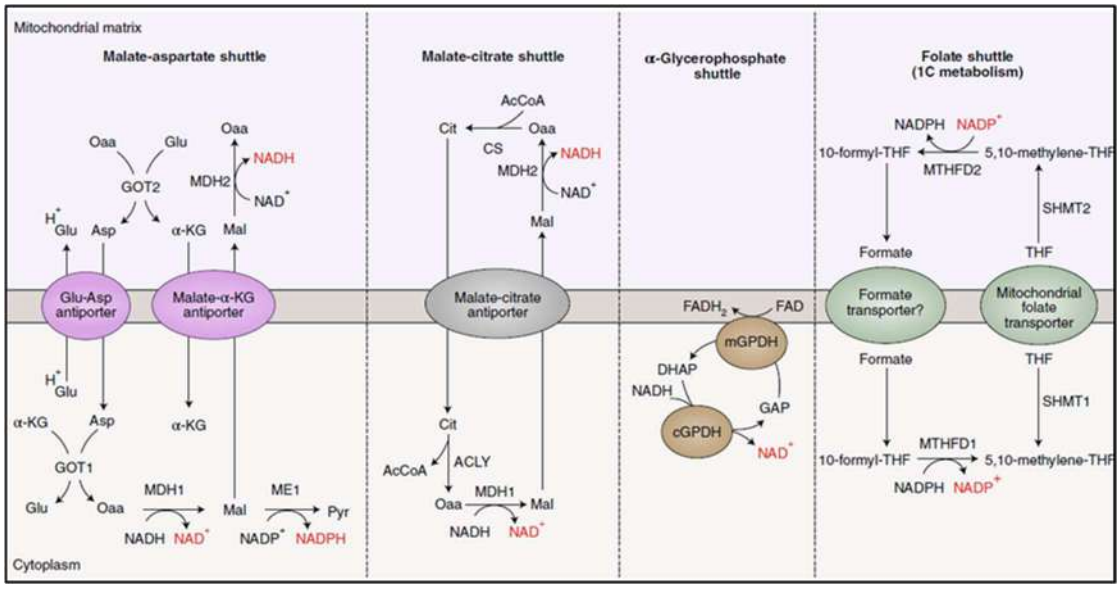

Figure 29. Redox equivalents are balanced in the mitochondria through several shuttles.
Adapted from Spinelli and Haigis, 2018.

One of these shuttles is the malate-aspartate shuttle. It is the predominant shuttle in oxidative tissues (Kane, 2014). It provides cytosolic NAD^+ and mitochondrial NADH (Schantz et al., 1986). In general, reduction of NAD^+ to NADH transfers energy derived from catabolism to drive OxPhos-derived ATP generation (Lewis et al., 2014). This shuttle is constituted by two isoforms of the malate dehydrogenase (MDH1 is cytosolic and MDH2 mitochondrial) which catalyze the reduction or the oxidation of malate to OAA; two isoforms of the glutamate-oxaloacetate transaminase (GOT1 is cytosolic and GOT2 mitochondrial) that catalyze the transamination, the aspartate-glutamate antiporter and the malate-α-KG antiporter (Spinelli and Haigis, 2018). The malate-aspartate shuttle in essential under increased energy demand, increased glycolytic flux or hypoxia, where LDH will increase lactate and cytosolic NAD^+ generation, and this would stimulate glucose catabolism and mitochondrial NADH for ATP production (Robergs et al., 2004). The malate-aspartate shuttle can also affect cellular amino acid compartimentalization as mentioned above with aspartate, when upon ETC blockade, cells reverse GOT1 flux to generate cytosolic aspartate (Birsoy et al., 2015; Sullivan et al., 2015). In pancreatic ductal adenocarcinoma with oncogenic KRAS, malate-aspartate shuttles contribute to increase NADPH/$NADP^+$ ratio using glutamine, which results in oxidative stress protection by for glutathione biosynthesis (Son et al., 2013).

The citrate-malate shuttle is also important, mainly relying on MDH1 and MDH2, while CS, ACLY and the malate-citrate antiporter (CIC) take also part in it (Korla et al., 2015). This shuttle favors an increase in cytosolic citrate levels, promoting FAS (Spinelli and Haigis, 2018). A recent study shows the implication in hepatocarcinogenesis of the citrate-malate shuttle by promoting lactic acid detoxification, and enhancement of lipid biosynthesis and mitochondrial integrity (Lei et al., 2020). It is possible that citrate-

malate shuttle may impact not only FAS but also epigenetic modifications, through ACLY acetyl-CoA-producer activity (Zhao et al., 2016).

α-Glycerophosphate shuttle does not directly impact NAD^+/NADH. It is formed by cytosolic and mitochondrial α -glycerophosphate dehydrogenase (cGPDH and mGPDH). Briefly, DHAP is reduced to GAP by the NADH consuming reaction catalyzed by cGPDH, rendering cytosolic NAD^+. Then, mGPDH oxidizes GAP to DHAP, generating $FADH_2$ that will transfer its electrons to the ETC (Spinelli and Haigis, 2018). The metabolic roles of the shuttle are the regeneration of cytosolic NADH in glycolytic cells, the bypass of ETC during NADH oxidation involved in thermogenesis, and the regulation G3P, conecting and modulating glycolysis, oxidative phosphorylation and lipid biosynthesis. (Mráček et al., 2013). mGPDH has been reported to regulate skeletal muscle regeneration by modulation of mitochondrial biogenesis via CaMKKβ/AMPK (Liu et al., 2018). These authors reported also that deletion of mGPDH attenuated muscle regeneration, while mGPDH rescued expression improved muscle regeneration.

In terms of NADPH, the major contributors are the PPP, the malic enzyme (ME), IDH1 and MTHFD (Altman et al., 2016; Yang et al., 2017). NADPH is involved in the maintenance of reduced glutathione (GSH) and in the reductive biosynthesis, such as lipids, and it is important for proliferation and survival in response to cell stress (Lewis et al., 2014). All the isoforms of MTHFD are bi-directional enzymes, but $NADP^+$ production is favoured in cytosol, whereas the mitochondrial isoform predominantly generates NADPH (Lewis et al., 2014). Upon mitochondrial DNA depletion, serine synthesis and 1C metabolism are activated by ATF4 to maintain NADPH/$NADP^+$ balance (Bao et al., 2016). Also, mTOR promotes via ATF4 1C metabolism mediated by the expression of MTHFD2, in order to support cancer cell proliferation (Ben-Sahra et al., 2016; Zeng et al., 2019).

1.3.1.4. Regulation of apoptosis by energetic and mitochondrial metabolism

Deregulation of apoptosis is one of the hallmarks of cancer (Hanahan et al., 2000). Once again, the involvement of the mitochondria in cancer is undeniable, since it controls cell fate and survival in different ways: it can trigger apoptosis trough mitochondrial outer membrane permeabilization (MOMP); it can spur regulated necrosis through mitochondrial permeability transition (MPT), as well as it modulates energy suppliance and metabolites levels. Apoptosis is a programmed cell death, morphologically characterized by the nuclear and cytoplasmic condensation and breaking up of the cell into a number of membrane-bound, ultrastructurally well-preserved fragments (Kerr et al., 1972). There are two apoptotic pathways depending on the cellular stress that trigeers it: the intrinsic and the extrinsic pathways, summarized and described in **figure 30**. In the intrinsic pathway, cellular stress activates proapoptotic BH3-only proteins that will inhibit antiapoptotic BCL-2 family proteins or directly activate BAX and BAK that will induce MOMP. Upon mitochondrial outer membrane permeabilization (MOMP) formation, the “no return” point for apoptosis regulated by the balance of pro- (BCL-2 family) and anti-apoptotic (BAK, BAX) proteins, cytochrome c is released, and initiator caspases (caspases 8 and 9) dimerize and

become active (Boatright et al., 2003), cleaving and activating executioner caspases (caspases 3, 6 and 7) (Riedl and Shi 2004) that will subsequently cleave protein substrates to execute apoptosis and demolish cellular components (McIlwain et al., 2013). Death receptor binding to its ligands triggers the extrinsic pathway, which induces the assembly of DISC (Death Inducing Signaling Complex) with FADD and caspase-8. Cleaved caspase-8 will activate effector caspases. (Matsuura et al., 2016)

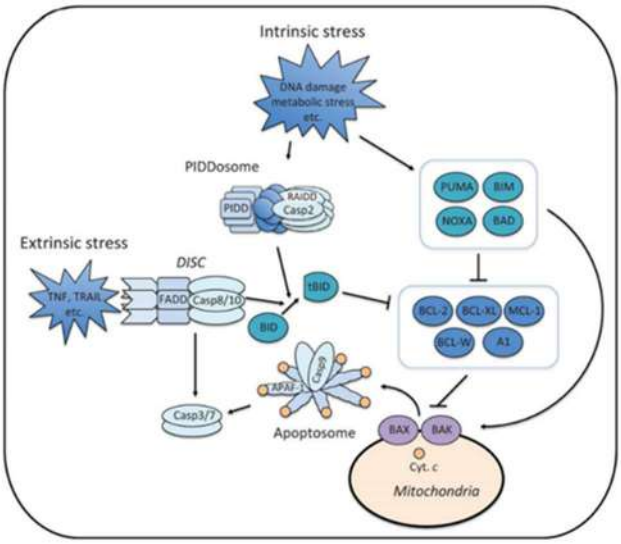

Figure 30. Apoptosis intrinsic and extrinsic pathways.
Adapted from Matsuura et al., 2016.

Metabolism is emerging as a mechanism used by cancer cells to evade apoptosis. Metabolites can regulate pro and antiapoptotic proteins, and apoptosis regulators can modulate cell metabolism. An example of metabolite that regulates apoptosis are ceramides. Ceramides, the neutral lipid block of sphingolipids, are key structural components of eukaryotic membranes, as well as regulators of apoptosis and cell proliferation, being therefore considered tumor suppresors (Morad and Cabot 2012). Ceramide production is induced under several (apoptotic) stimuli, such as TNF-α, growth factor withdrawal, Fas ligand and DNA-damaging reagents, like for example ionizing radiations and chemotherapy (Morad and Cabot 2012; Dadsena et al., 2019). In addition, overexpression of BCL-2/ BCL-xL inhibits ceramide-induced apoptosis (Ganesan and Colombini, 2010), while ceramide can bind protein phosphatase 2A (PP2A) and induce BCL-2 dephosphorylation and inactivation (Ruvolo et al., 2002). Ceramides enhance BAX MOMP through direct interaction with BAX (Ganesan and Colombini, 2010). It has recently also been reported that the voltage-dependent anion channel VDAC2 is a direct ceramide-induced cell death effector, that acts by blocking Bax retrotranslocation into cytosol (Dadsena et al., 2019). Another mechanism by which ceramides induce apoptosis is through the inhibition of AKT (Schubert et al., 2000) and subsequent activation of BAD, a member of the proapoptotic BCL-2-family (Dadsena et al., 2019).

On the other hand, apoptotic regulators influence cell metabolism. Many of the members of the BCL-2 family reside or translocate into the mitochondria and some of them have been directly reported to

modulate mitochondrial metabolism. For example, BCL-2 was already reported in 2007 to regulate mitochondrial respiration at the level of COX to overcome oxidative stress (Manfredi et al., 2003; Chen and Pervaiz, 2007). In AML, ROS-low LSC have been reported overexpress BCL-2, and the inhibition of BCL-2 with Venetoclax impairs oxidative phosphorylation and redox control, erradicating chemotherapy resistant LSCs (Lagadinou et al., 2013). In neurons, IMM localised BCL-xL interacts with F1F0 ATP synthase, enhancing its activity and stabilizying mitochondrial membrane potential ($\Delta\Psi m$) (Alavian et al., 2011; Chen et al., 2011). In breast cancer, both the expression of BCL-xL and BCL-2 have been recently described to enhance the efficiency of the mitochondrial proton motive force-coupling with ATP production, contributing to resistance of tumor cells to metabolic stress (Lucantoni et al.,2020), whereas their inhibition decreases ATP production (Lucantoni et al., 2018). MCL-1, another antiapoptotic protein member of the BCL-2 family when in the outer mitochondrial membrane (OMM), has been also identified as modulator of several mitochondrial functions when in the matrix in an amino-terminally truncated isoform (Perciavalle et al., 2012). These mitochondrial functionalities in which MCL-1 is involved are cristae ultrastructure, maintenance of oligomeric ATP synthase, ATP production, respiration and membrane potential (Perciavalle et al., 2012). MYC and MCL-1 amplification has been described to contribute to chemotherapy resistance in triple negative breast cancer (TNBC) by increasing OxPhos, ROS production and HIF-1 expression on TNBC cancer stem cells (Lee et al., 2017b). In addition, a proposed mechanism of resistance to venetoclax (BCL-2 inhibitor) for leukemic cells is the upregulation of MCL-1. Recent work has identified reliance on different BCL-2 anti-apoptotic proteins in base to differentiation state of AML cells. More specifically, phenotypically primitive AML relies on BCL-2 to induce OxPhos and survival, and therefore, it is sensitive to Venetoclax. However, monocytic AML switches from BCL-2 to MCL-1 to maintain OxPhos and cell survival (Pei et al., 2020). Considering the role of BCL-2 family members in cancer cell survival and therapy resistance, inhibitors of BCL-2 such as venetoclax (Souers et al., 2013; Dinardo, et al., 2019) or of MCL-1 (Kotschy et al., 2016; Caenepeel et al., 2018; Tron et al., 2018) are considered promising therapeutic tools to improve cancer treatment.

It is now accepted that the Warburg effect not only supports uncontrolled proliferation but it also provides cancer cells with a tool to evade apoptosis (Matsuura et al., 2016). From almost 2 decades PI3K/Akt pathway has been known to play a major role in cell survival by cell surface receptors (Datta et al., 1997; Brunet et al., 1999). Through AKT activation, HK2 binds to the voltage-dependent anion channel (VDAC) in the OMM to regulate permeability transition pore (PTP) and couples oxidative phosphorylation to glucose metabolism. More specifically, AKT increases mitochondria-associated hexokinase activity. Interestingly, this step is enough for AKT antiapoptotic activity, since it only requires only the first committed step of glucose metabolism catalyzed by hexokinase (Gottlob et al., 2001). HK2 has also been shown to inhibit the mitochondrial binding of BAX to VDAC, interfering with Bax ability to bind to the mitochondria and induce cytochrome c release (Pastorino et al., 2002). In addition, AKT, like BCL-2, inhibits BAX activation and BAK oligomerization after BID cleavage in a glucose and HK2

depedent manner (Majewski et al., 2004). Phosphorylation of BAD (BH3-only pro-apoptotic BCL-2 family protein) by the c-Jun N-terminal protein kinase 1 (JNK1) inhibits its activity while it activates PFK1, promoting glycolysis and supporting cell survival (Deng et al., 2008). Another mechanism by which glycolysis promotes cell survival is via CDK5 activation. In the presence of glucose, active CDK5 phosphorylates and inhibits the BH3-only protein NOXA, avoiding its pro-apoptotic function (Lowman et al., 2010). Another link between glucose metabolism and apoptosis is established by NADPH and its producing pathway, PPP, which contribute to redox balance of cytochrome c and suppresses apoptosis (Bensaad et al., 2006). Glucose abundance also influence the activation of caspase-2. Under glucose abundance, G6P, NADPH (Nutt et al., 2005) or CoA (McCoy et al., 2013) activate calcium-calmodulin-dependent kinase CaMK2, which phosphorylates and inactivates caspase-2 (Nutt et al., 2005). In addition, G6P also promotes phospho-serine binding protein 14-3-3ζ deacetylation by Sirtuin1, while caspase-2 phosphorylation by CaMK2 creates the binding site 14-3-3ζ (Andersen et al., 2011). However, when the glycolytic flux is limited, 14-3-3ζ is acetylated, releasing caspase-2 that can be dephosphorylated by PP1 and be recruited to the PIDDsome (Andersen et al., 2011). GAPDH, another glycolytic enzyme has also been reported to play a role in cell survival and apoptosis. However, its role is more debated, since in some studies it is considered pro-apoptotic while in others pro-survival (Colell et al., 2009; Zhang et al., 2015). In addition, post-translational modifications of GAPDH can also influence its activity as pro- or anti-apopototic action (Colell et al., 2009; Zhang et al., 2015). HIF-1 involvement in cell death has also been controversial. On the one hand, it is stabished that HIF-1 can stabilize p53 under hypoxia and induce cell cycle arrest and apoptosis (An et al., 1998; Chandel et al., 2000). HIF-1 can also induce apoptosis by enhancing BNIP3 and NIX protein expression, which will bind to BCL-2 anti-apoptotic proteins, moving the balance to the apoptotic process (Sowter et al., 2001; Guo,. 2017). On the other hand, it is known that HIF-1 launches a transcriptional program that switches cell metabolism to glycolysis (Iyer et al., 1998) that, as mentioned above, can protect from cell death. In addition, HIF-1 can induce the expression of anti-apoptotic proteins from the BCL-2 family or of inhibitor of apoptosis (IAPS) (Greijer et al., 2004; Kilic et al., 2006). Overall, there is a body of evidence that metabolism and cell death are interconnected, and that they can influence one another.

In brief

Mitochondria functions go far beyond its essential role in bioenergetics. They are metabolic hubs where the biosynthesis of numerous macromolecule precursors takes place, as well as they regulate cellular processes essential for cancer cells, including redox balance, apoptosis and even gene expression.

1.3.2. Mitochondrial and ETCI dependency in cancer and leukemia

Mitochondrial function is essential for tumor progression. In 2010, it was demonstrated that mitochondrial metabolism and ROS generation were essential for KRAS tumorigenesis, since TFAM loss resulted in reduced tumorigenesis in oncogenic KRAS -driven mouse lung cancer model (Weinberg et al., 2010). Another evidence supporting the essential role of mitochondria in cancer progression was provided by Tan and colleagues. They showed by using metastatic murine melanoma ρ^0B16 and breast cancer $\rho^0$4T1 models that cancer cells lacking mitochondrial DNA could only lead to metastasis *in vivo* if they integrated host mtDNA, which meant recovery of mitochondrial function, functional respirasome and ECTII assembly (Tan et al., 2015).

OxPhos, mitochondrial function and ETC genes were identified to be overexpressed in a set samples from newly diagnosed difuse large B cell lymphoma (DLBCL) (Monti et al., 2005), what is known nowadays as OxPhos-DLBCL. This phenotypes accounts for 30% of DLBCLs, and it is characterized by a great dependence on FAO and GSH pool maintenance for survival (Caro et al., 2012), what puts in evidence its mitochondrial dependency. Metabolically, NSCLCs tumors have been reported to have increased glycolysis and TCA cycle activity compared to non cancerous lung tissues (Hensley et al., 2016), as denoted by the enrichment in labelled lactate, alanine, succinate, glutamate, aspartate and citrate (Fan et al., 2009). Leukemic cells have also shown increased mitochondrial dependency when compared to their normal counterparts. One study of Samudio and colleagues underlined the reliance of AML cells on fatty acid oxidation (FAO) and how its targeting by blocking fatty acids entrance in the mitochondria with etomoxir (CPT1inhibitor) proved therapeutic benefit in leukemia, synergizing with AraC or BCL2 inhibition (Samudio et al., 2010). Other study has shown the dependency for mitochondria in AML by inhibiting mitochondrial protein translation by two different approaches: with the antibiotic tigecycline as well as by silencing the mitochondrial elongation factor Tu (EF-Tu). Both approaches resulted in decreased mitochondrial membrane potential and oxygen consumption rate, as well as in impaired cellular growth and viability selectively on the leukemic cells, not affecting normal hematopoietic cells (Skrtic et al. 2011). Later on, work from the same group showed that the mitochondrial protease CLPP was overexpressed in AML and that its inhibition decreased oxygen consumption by abrogating ETCII activity due to the accumulation of defective ETC proteins and showed anti-leukemic activity in mouse xenografts (Cole et al., 2015). Interestingly, AML cells are also sensitive to the hyperactivation of the CLPP by pharmacological compounds such as ONC201 and ONC212 (Ishizawa et al., 2019). CLPP locked in the active conformation resulted in increased degradation of ETC substrates and impaired OxPhos, and therefore ONC201 is under clinical trial for hematological (NCT03932643) and solid malignancies (Stein et al., 2017; Egan et al., 2021). Liyanage and colleagues showed that AML cells upregulated genes of the mtDNA biosynthetic pathway in comparison to normal hematopoietic cells. Treatment with the nucleotide

analogue 2'3'-dideoxycytidine (ddC) selectively inhibited mitochondrial DNA polymerase γ and resulted in the inhibition of mtDNA replication and oxidative phosphorylation, inducing cytotoxicity in AML cell lines (Liyanage et al., 2017).

In addition, several specific contexts where susceptibility to OxPhos inhibition by using ETCI inhibitors is increased have been reported. Mutations in mtDNA affecting ETCI subunits boosted sensitivity to ETCI inhibitors by 5 to 20 fold (Birsoy et al., 2014). Loss of the tumor suppressor LKB1 in KRAS-driven NSCLC showed an enhanced sensitivity to phenformin, resulting in prolonged survival upon treatment (Shackelford et al., 2013). This highlights a specific vulnerability upon LKB1 loss of function, which leads to energetic collapse upon ETCI inhibition, due to its incapacity to activate AMPK and enhance glycolysis as compensatory mechanism. Also in NSCLC, inactivating mutations in the SWI/SNF chromatin remodeling complex component SMARCA4 resulted in increased OxPhos supported by the activation of PGC-1-α, and enhanced sensitivity to ETCI inhibition, in this case assessed with IACS-010759 (Lissanu Deribe et al., 2018). They suggested that SMARCA4 abrogated compensatory glycolysis and transcriptional adaptation in response to energy stress upon ETCI inhibition, resulting in unmet energy to support anabolic demand, thus creating a metabolic vulnerability.

1.3.2.1. Mitochondria in cancer stem cell maintenance

Cancer stem cells (CSCs) are constituted by specific poorly differenciated cell populations in cancer, and they are responsible for tumor growth, metastasis as well as for tumor recurrence after an apparent successful chemotherapy (Batlle and Clevers, 2017). Therefore, the development of therapies targeting CSCs is of extreme importance, since it would eradicate them and allow sustained responses in cancer patients.

OxPhos involvement in cancer stem cell maintenance has been reported in several cancer types. In chronic myeloid leukemia cancer stem cells, OxPhos is upregulated and involved in resistance to therapy (Kuntz et al., 2017). Pancreatic cancer stem cells have also been shown to rely on OxPhos: Sancho and colleagues showed that pancreatic CSCs were OxPhos, and identified the balance between MYC and PGC-1-α as determinant in the response to mitochondrial inhibition, by the ability of MYC to induce of compensatory glycolysis. MYC suppression and subsequent increase of PGC-1-α resulted in energetic crisis and apoptosis upon metformin treatment (Sancho et al., 2015). In addition, recent work showed that by using galactose as carbon source, pancreatic ductal adenocarcinoma cells were forced to use OxPhos as main energy source, resulting in enrichment of CSC as determined by increased pluripotency gene expression and biomarkers, and greater tumorigenic as well as immune-evasive potential (Valle et al., 2020). Depletion of insulin-like growth factor 2 mRNA-binding protein 2 (IMP2) in gliomaspheres decreased oxygen consumption by affecting ETCI and ETCIV, what resulted in impaired clonogenicity *in vitro* and

decreased tumorigenicity *in vivo* (Janiszewska et al., 2012). Glioma stem cells have been identified also to have greater reliance on OxPhos than differentiated glioma cells, since they had higher ATP levels concomitant with reduced glucose consumption and lactate production (Vlashi et al., 2011). In lung cancer there is also some work suggesting a more OxPhos phenotype of stem cells (Gao et al., 2016). In acute myeloid leukemia (AML), numerous studies unrevealed the dependency of the leukemic stem cells for OxPhos, by targeting it with several approaches. One of the first studies to demonstrate it, was the one performed by Lagadinou and colleagues, where they showed that leukemic stem cells are characterized by low ROS levels and OxPhos dependency lead by BCL-2, given that BCL-2 inhibition impaired OxPhos and eradicate this population in pre-clinical models (Lagadinou et al., 2013). More evidence was provided by Raffel and colleagues, who demonstrated that branched chain α-amino transferase BCAT1, which transfers α-amino groups from leucine, valine and isoleucine to α-KG, appeared overexpressed in AML LSCs, which altered α-KG homeostasis and deregulated DNA methylation, mimicking the effect of IDH or TET mutations (Raffel et al., 2017). Jones and colleagues pointed out that LSC at diagnosis rely on amino acids for OxPhos, whereas relapsed LSCs increase the contribution of fatty acids oxidation to maintain OxPhos activity (Jones et al., 2018). Targeting the electron transport chain has proven to be also an effective mechanism to eradicate leukemic stem cells in preclinical AML models. For example, the combination of the BLC-2 inhibitor venetoclax with the hypomethylating agent azacitidine results in decreased oxidative phosphorylation by blocking ETCII (succinate dehydrogenase), and this induces durable remissions in patients by selective targeting of LSCs (Pollyea et al., 2018). Molina and colleagues described a new ETCI inhibitor, IACS-010759, which blocks oxidative phosphorylation and decreases pyrimidine biosynthesis by decreasing aspartate levels, restricting AML proliferation and showing efficacy *in vivo* (Molina et al., 2018). The use of mubritinib, ERBB2 inhibitor (receptor tyrosine kinase from the EGFR family, absent in the leukemic cell line assessed in the paper, OCI-AML3) targets AML by the inhibition of the ETCI in a ubiquinone dependent manner, delaying mouse AML development *in vivo* without affecting normal hematopoiesis (Baccelli et al., 2019). The inhibition of the RSC modulator neurolysin (NLN) has been recently reported to decrease OxPhos in AML cells and LSCs, as well as to induce selective death of leukemic cells (Mirali et al., 2020). Another attractive target in AML is the DHODH, the flavoprotein located in the inner mitochondrial membrane, which catalyzes the fourth step in *de novo* pyrimidine (Evans et al., 2004). It oxidizes dihydroorotate to orotate, while the electrons are directly transferred to the respiratory chain at the level of cytochrome oxidase c (ETCIII) via coenzyme Q for further oxidation (Vélez et al., 2013). Inhibition of ETCIII causes depletion of pyrimidines through the inhibition of coupled DHODH, since ubiquinone in its reduced state (ubiquinol) is unable to accept the electrons from dihydroorotate (Khutornenko et al., 2010). Therefore, it is an interesting enzyme, since it directly links mitochondrial oxygen consumption and electron transport chain to proliferation. In AML, different DHODH inhibitors blocked proliferation by depletion of the pyrimidine pool. In addition, the inhibition of the enzyme bypasses the differentiation blockade of leukemic initiating cells, inducing a HoxA9-mediated differentiation (Sykes et al., 2016; Christian et al., 2019). A recent study in B-progenitor

acute lymphoblastic leukemia (B-ALL) shows that also in this type of leukemia, the clones present and diagnosis that initiate the relapse, referred in the paper as diagnosis Relapse Initiating clones (dRI), are transcriptomically enriched for mitochondrial metabolism (Dobson et al., 2020).

Interestingly, while many types of cancer stem cells rely on OxPhos, non-cancerous stem cells, like hematopoietic stem cells (HSC)(Takubo et al., 2013), mesenchymal stem cells or neural stem cells rely on glycolysis (Ito and Suda, 2014), what makes OxPhos targeting a selective tool to eradicate CSCs. More importantly, it's been suggested that, in parallel to present increased OxPhos and mitochondrial metabolism, cancer stem cells are metabolically inflexible, what makes them more susceptible to the inhibition of their main energy source, OxPhos (Skrtic et al. 2011; Molina et al., 2018; Pollyea et al., 2018; Baccelli et al., 2019; Panina et al., 2019, Egan et al., 2021).

1.3.2.2. Mitochondria in resistance to anti-cancer therapies.

Increased mitochondrial OxPhos has been identified as a common denominator for a wide range of anti-cancer therapies, both chemoresistance or targeted therapy. Interestingly, this phenotype has been observed in a variety of cancer types, with mechanisms that differ, highlighting the essential role of OxPhos in supporting resistance to cancer therapy.

In NSCLC, resistance both to cisplatin and to irinotecan has been shown have increased reliance of OxPhos through ROS and PGC-α, and therefore, targeting it with metformin or phenformin eradicated resistant cells in pre-clinical studies (Wangpaichitr et al., 2017; Cruz-Bermúdez et al., 2019). Amplification of MYC and MCL-1 has been identified to drive increased mitochondrial OxPhos and chemotherapy resistance in breast cancer (Lee et al., 2017b). Docetaxel resistance in prostate cancer also correlates with an increase in mitochondrial respiration and OxPhos (Ippolito et al., 2016). Other examples are colorectal cancer resistant to 5-fluorouracil (Denise et al., 2015) and gemcitabine resistance in a subset of high OxPhos pancreas tumors (Masoud et al., 2020). Farge and colleagues showed that leukemic cells that resisted chemotherapy in mouse xenografts displayed high levels of reactive oxygen species (ROS), increased mitochondrial mass, and had an active polarized mitochondria (Farge et al., 2017). In addition, superoxide accumulation induced by inhibition of myeloperoxidase (MPO) in chemo-resistant AML resulted in impaired mitochondrial redox balance and OxPhos, as well as in increased oxidative DNA damage, apoptosis and eradication of leukemic AraC resistant cells (Hosseini et al., 2019). MCL-1 has been involved in relapsed AML after Azacytidine + Venetoclax treatment, where cells that escape to the treatment lose BCL-2 expression as a consequence of differentiation and adopt a more monocytic phenotype, becoming reliant on MCL-1 to maintain OxPhos and survival (Pei et al., 2020).

Therapies targeting tyrosine kinase receptors also result in resistance with concomitant OxPhos upregulation. For example, resistance to epidermal growth factor receptor (EGFR) inhibitors in EGFR-driven lung adenocarcinoma is characterized by a switch to OxPhos and it is overcome with ETCI

inhibitors in pre-clinical models (De Rosa et al., 2015; Martin et al., 2016). In melanoma with activating BRAF mutations, it has also been observed enhanced OxPhos dependency supported by PGC-1α upon BRAF inhibition treatment (Haq et al., 2013). In AML, the mechanisms of resistance to FLT3-inhibition involve other FLT3 mutations, NRAS/KRAS mutations and the upregulation of antiapoptotic proteins, such as BCL-2 and MCL-1, which results in mitochondrial OxPhos depedency (Short et al., 2020).

In brief

Mitochondrial metabolism seems to be indispensable for cancer initiation, progression and for the adaptative response to evade chemotherapy and targeted therapies, leading to treatment resistance. Therapies targeting mitochondria show promising anti-cancer activity in preclinical models, and may achieve some selectivity towards cancer cells or CSCs.

1.3.2.3. Mitochondria in immunometabolism of tumors

In the recent years, the complex interplay between metabolic reprogramming and immunity, also called immunometabolism, has emerged as an interesting field to shed some light on immune system physiology and pathology (Mathis and Shoelson, 2011; O'Neill et al., 2016; Bader et al., 2020). Just as an example of the interconnection of immune system and metabolism, effector T cells rely on glycolysis for energy production and survival (Gubser et al., 2013) whereas memory T cells depend on oxidative metabolism (van der Windt et al., 2012) or macrophages, which adapt their metabolism depending on their activating signal: they upregulate glycolysis and fatty acid synthesis when activated by lipopolysaccharide (LPS) whereas when activated by IL-4, they rely on oxidative phosphorylation and fatty acid oxidation for energy production (Rodríguez-Prados et al., 2010). Modulation of metabolic pathways can therefore provide new therapeutic opportunities through the alteration of the immune phenotype, to boost immune system in immunosuppressive contexts like cancer or to dampen it in autoimmune diseases. Maybe, one example of this is provided by metformin. The observation from several studies that diabetic patients treated with this drug had reduced risk for several cancers (liver, pancreatic, colorectal and breast cancer) (Evans et al., 2005; Zhang et al., 2013) led to the hypothesis that metformin had a beneficial effect on the immune system. In addition to retrospectives studies, a body of evidence shows that the antitumor effect of metformin is closely related to the immune system. Pereira et al. showed that in the murine melanoma model B16F10, metformin reduced the number of lung metastasis in immunocompetent mice (C57/Bl6) whereas it proved no therapeutic effect in NSG mice (Pereira et al., 2018). Similar results were obtained with 4T1 breast tumor model, where metformin exerted tumor regression in immunocompetent mice but not in severe combined immune deficiency (SCID) mice (Cha et al., 2018). Metformin enabled C57/Bl6

mice but not T-cell-deficient SCID mice to reject solid tumors both in an intradermal model of X-ray-induced RLmale1 leukemia as well as in a melanoma model (Eikawa et al., 2015)**.** These data highligth the essential role of the immune system in the antitumor activity of metformin in these *in vivo* models, and can arise some questions regarding lactate immunosuppressive effect.

In the next pages, the effects of metformin (molecularly described in chapter "1.3.2.4.1. Metformin: a pleiotropic ETCI inhibitor") have been studied on different immune populations and on different immune checkpoint molecules, and they will be summarized in the upcoming pages.

1.3.2.3.1. Mitochondria in the immune compartment

i. T effector and memory cells

T cells engage different metabolic states along their life cycle to support different functions (**figure 31**). When quiescent, peripheral naïve T cell rely on TCA cycle and OxPhos for ATP generation (MacIver et al., 2013). However, upon activation naïve T cells initiate a specific transcriptional program to drive their differentiation and effector function that goes along with a shift to a pro-growth metabolic state dependent on PI3K/AKT/mTOR signaling (Powell et al., 2012), engaging increased glycolysis (Frauwirth et al., 2002) and glutamine metabolism (Carr et al., 2010; Nakaya et al., 2014) to support clonal expansion. This metabolic reprograming upon TCR signaling is regulated by MYC (Wang et al., 2011) and HIF-1 (Doedens et al., 2013; Palazon et al., 2017). At the end of immune response, only memory cells reliant on FAO and OxPhos for energy production will survive (Pearce et al., 2009; MacIver et al., 2013; Field et al., 2020).

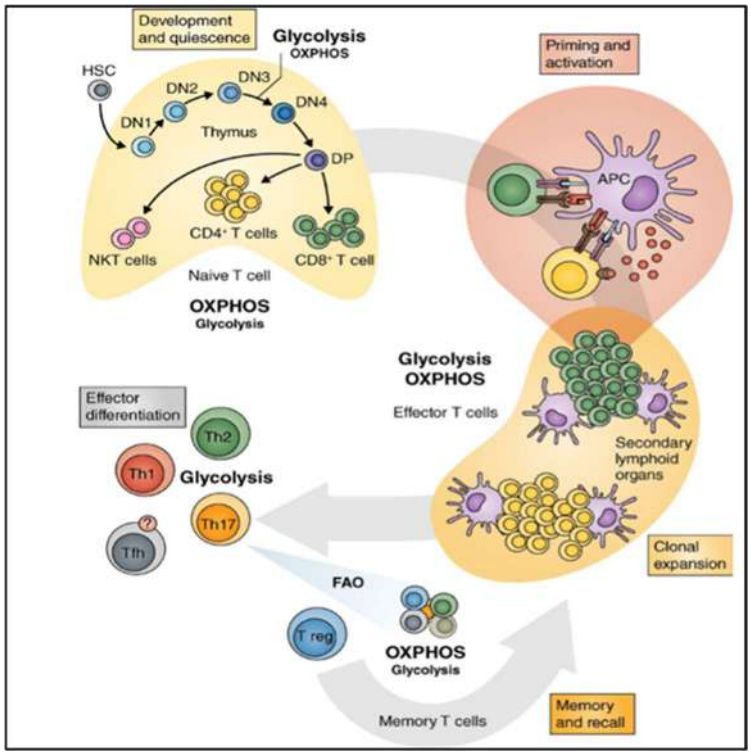

Figure 31. Metabolic changes along T cell life.
Adapted from Buck et al., 2015.

Persistent antigen exposure causes repeated TCR stimulation, which leads to immune exhaustion, characterized by a decreased ability of $CD8^+$ T cells to secrete IL-2, TNFα, and IFNγ, increased expression of inhibitory markers (PD-1, Tim-3, CTLA-4), altered transcriptional program and metabolic insufficiencies (**figure 32**). These events lead to compromised $CD8^+$T cell effector function (Eikawa et al., 2015; Russell et al., 2019).

Metformin has been described to reverse this exhaustion phenotype by shifting the cells to a T effector memory cell phenotype, and therefore, increasing the number of $CD8^+$ effector memory cells in tumors as well as in persistent infections with AMPK activation (Eikawa et al., 2015; Russell et al., 2019). Recently, Böhme and colleagues showed that metformin specifically expanded a memory-like $CD8^+$ $CXCR3^+$ T cell population both in mice and in human, and that it induced FAO and enhanced spare respiratory capacity in these cells (Böhme et al., 2020). In line with these results, AMPK has been reported to regulate T cell metabolism in order to maintain cellular bioenergetics and survival (Blagih et al., 2015), as well as healthy mitochondria in this immune population (Adams et al., 2016). Metformin also potentiates $CD8^+$ cytotoxic activity by increasing the Granzyme B (GB) secretion in tumors (Cha et al., 2018; Pereira et al., 2018). Another way by which metformin has been reported to stimulate T cell immunosurveillance is by inducing PD-L1 degradation (ligand of the exhaustion marker PD-1 and therefore a negative regulation of T cell function) in breast cancer cells via ER accumulation and ER-associated protein degradation (ERAD) in AMPK dependent manner (Cha et al., 2018). In addition, they showed that combination therapy consisting on metformin and α-CTLA-4 significantly increased survival proportion in mice models of breast cancer and colon carcinoma, by boosting CD8 T cell tumor infiltration (Cha et al., 2018).

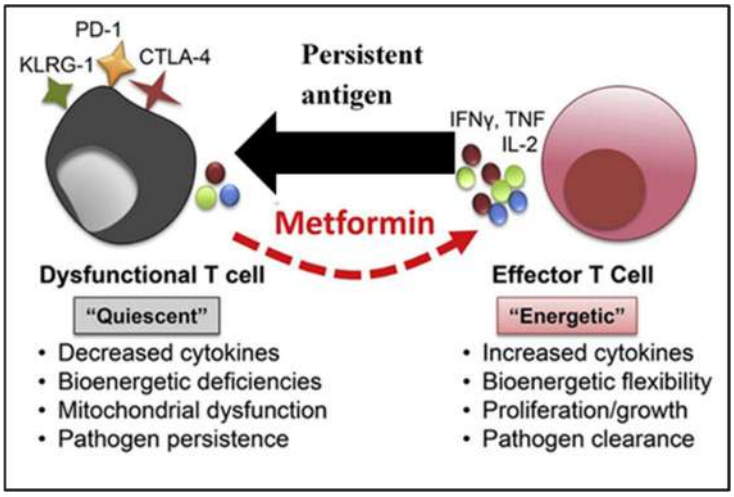

Figure 32. T cell dysfunction contributes to the development of many chronic infections and cancer.
Adapted from Russell et al., 2019.

Pearce and colleagues highlighted that metformin should be administered after T effector cell expansion, in order to not to alter the T effector cell response (Pearce et al., 2009). This is because metformin blocks

metabolic changes (increased glucose consumption induced by c-Myc, HIF-1α and Glut1) necessary for T cell proliferation after T cell antigen receptor engagement (Zarrouk et al., 2014). However, in another work it has been described that when administering metformin in *in vivo* models, it is preferentially uptaken by tumor cells, since their expression of the metformin organic cation transporter (OCT1) is higher in cancer cells than in T cells. Thus, after *in vivo* treatment with metformin OCR of T cells was not affected (contrarily to what they observed *in vitro*), neither the levels of HIF-1α protein, which were increased and directly linked to T cell activation (Scharping et al., 2016). These results may suggest that the boosting effect of metformin in T cell activity is independent from ETCI inhibition.

ii. Regulatory T cells

$CD4^+$ $CD25^+$ regulatory T cells (Treg) are inhibitors of the T-cell mediated response. In contrast to effector T cells, Tregs rely on OxPhos and FAO as sources of energy (Michalek et al., 2011; Beier et al., 2015; Bader et al., 2020). Kunisada et al published in 2017 that metformin downregulated Treg in number and function in tumors, linking this effect to Tregs dependency on oxidative phosphorylation (OxPhos) through fatty acid oxidation (FAO) for their survival. They also showed that the decrease in $CD4^+$ $CD25^+$ cells also increased the number of $CD8^+$ effector cells, which enhanced the anti-tumor immune response (Kunisada et al., 2017). However, in other publications, T regs were increased after metformin treatment (Pereira et al., 2018). They tried to explain this by showing that metformin treatment also decreased Th17 cells, and that the Th17/ Treg ratio is the responsible for the attenuation of the immune response in the case of inflammatory and autoimmune diseases (Son et al., 2014; Yang et al., 2017).

To add more complexity, a recent study showed that OxPhos is essential for Th17 cell pathogenicity in a mouse model of multiple sclerosis (MS) and that by inhibiting OxPhos in Th17 cells, the $CD4^+$ cells will become suppressive Treg cells by expressing Foxp3 preferentially to Th17 genes (Shin et al., 2020). They also suggested the possibility that Treg cells generated with OxPhos inhibitors under Th17 conditions may be different functionally and transcriptionally from classical Treg cells, which are known to depend on mitochondrial metabolism for their function (Angelin et al., 2017). Thus, the effect of ETCI inhibition in T regs remains still imperfectly understood.

iii. Myeloid derived suppressor cells (MDSCs)

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of myeloid origin cells described 2 decades ago in cancer patients that gathers myeloid progenitor cells and immature macrophages, immature granulocytes and immature dendritic cells (Gabrilovich et al., 2007). MDSCs are divided into two subtypes: monocytic (M-MDSC), which are phenotypically and morphologically similar to monocytes; and granulocytic or polymorphonuclear (PMN-MDSC), phenotypically and morphologically similar to neutrophils and the majoritary in most cancer types (Gabrilovich, 2017).

MDSCs are potent suppressors of T-cell function, and they have also been reported to regulate innate immune responses through cytokine production that induce macrophages polarization to M2 phenotype (Gabrilovich et al., 2009; Veglia et al., 2018). MDSCs mediate immunosuppression by expressing inducible nitric oxide synthase (iNOS), IDO and arginase 1; as well by producing reactive oxygen species (ROS), TGFβ and IL-10 (Gabrilovich, 2017; Veglia et al., 2018). The ectonucleotidases CD39 and CD73, responsible for the balance between proinflammatory ATP and immunosuppressive adenosine in the tumor microenvironment (TME), are also expressed by MDSCs in cancer context and contribute to immune scape (Ryzhov et al., 2011; Zhang et al., 2013).

Regarding metabolism, MDSCs are characterized by accumulation of lipids and FAO to promote their suppressive activity (Bader et al 2020; Veglia et al., 2021). MDSCs upregulate CD36 to promote lipid uptake and are reliant on FAO as main energy source (Hossain et al., 2015; Al-Khami et al., 2017). In fact, CD36 or FAO inhibition resulted in decreased immunosuppressive capacity of MDSCs as well as in delayed tumor growth and enhanced therapy response (Hossain et al., 2015; Al-Khami et al., 2017). The fatty acid transport protein 2 (FATP2) has recently been found to regulate immunosuppressive function of MDSCs, being involved in the uptake of arachidonic acid and in the subsequent synthesis of Prostaglandin E_2 (PGE_2) (Veglia et al., 2019). Increased glycolysis has also been proposed as a metabolic feature of MDSCs (Baumann et al., 2020), however its link with immunosuppression is still to be elucidated (Bader et al 2020; Veglia et al., 2021).

Metformin has shown to reduce the number MDSCs in vivo models of ovarian cancer (Li et al., 2018), melanoma (Pereira et al., 2018) and colon cancer (Xu et al., 2019). One described mechanism by which metformin exerts its suppressor effect on MDSCs is decreasing the expression of CD39 and CD73 via AMPKα activation and inhibition of HIF-1α (Li et al., 2018).

iv. Macrophages

Macrophages are a heterogeneous group of cells that are critical mediators of tissue homeostasis. They are components of the TME, where they are called tumor-associated macrophages (TAMs). They are involved in angiogenesis, pro-fibrotic activities, as well as in tumor progession and metastasis (Mantovani et al., 2017). Their activities vary according to developmental origin, tissue of residence and microenvironment conditions. Traditionally, they are divided into M1 or M2 phenotype (**figure 33**). The M1 phenotype, or the classic activated macrophages, are considered more inflammatory and antitumorigenic, while they express high levels of tumor necrosis factor (TNF), inducible nitric oxide synthase (iNOS; also known as NOS2) or MHC class II molecules (Yan and Wan 2021). Metabolically speaking, M1 macrophages rely on aerobic glycolysis, FAS and amino acid metabolism to survive (Williams and O'Neill, 2018; Bader et al., 2020). In addition, upon activation, TCA cycle of M1 macrophages have 2 breakpoints (Jha et al., 2015, Ryan and O'Neill, 2020). The first one is at IDH (Tannahill et al., 2013; Jha et al., 2015; Ryan and

O'Neill, 2020), whose downregulation leads to the production of the microbicide itaconate from aconitate (Jha et al., 2015), and promotes lipid biosynthesis from citrate (Tannahill et al., 2013). The second one is at SDH (Jha et al., 2015, Ryan and O'Neill, 2020), whose activity has been proposed to be inhibited by NO (Jha et al., 2015) as well as by itaconate (Lampropoulou et al., 2016, Seim et al., 2019). However, the M2 phenotype is considered pro-tumorigenic and anti-inflammatory, it expresses high levels of arginase 1 (ARG1), the immunosuppressive IL-10, CD163, CD204 or CD206 (DeNardo et al., 2019; Thapa and Lee, 2019; Viola et al., 2019) and they make use of OxPhos, TCA cycle and FAO (Netea-Maier et al., 2018; Williams and O'Neill, 2018). Opposite to M1 phenotype, M2 macrophages present intact TCA cycle (Viola et al., 2019).

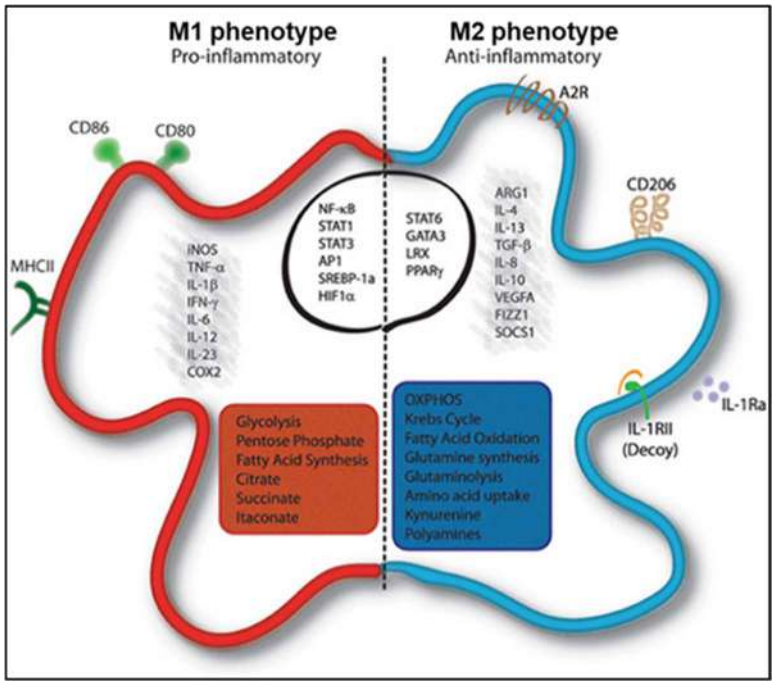

Figure 33. M1 vs M2 macrophage phenotypes.
Adapted from Viola et al., 2019.

In literature, it's been reported that metformin suppressed the polarization of macrophages towards M2 phenotype via activation of AMPK in a Lewis lung cancer model (Ding et al., 2019), which can potentially be due to the decrease in OxPhos induced by the drug. Other study shows that metformin via AMPK activation and STAT3 decrease, inhibits the monocyte to macrophage differentiation and the proinflammatory signals associated to the process (Vasamsetti et al., 2015). More recently, it's been shown that metformin can boost anti-PD-1 therapy when administered within mannose-modified macrophages-derived microparticles (Man-PMs) (Wei et al., 2021). In their work, they showed that these Man-PMs loading metformin were able to repolarized M2-like phenotype macrophages into M1 phenotype, while increasing the TME infiltration of $CD8^+$ T cells and decreasing MDSCs and Tregs infiltration. They described therapeutic benefit in H22 (hepatocellular carninoma), 4T1 (breast cancer) and in azoxymethane (AOM)/dextran sodium sulfate (DSS)-induced colitis-associated colorectal cancer (CAC) murine models (Wei et al., 2021).

1.3.2.3.2. Immuno-modulation of tumor compartment: immune checkpoints and lactate

i. Classic immune checkpoints: PD-L1 and CTLA-4

Immune checkpoints are critical molecules for physiologic immune function. They generate a negative feedback loop essential to establish self-tolerance and to prevent hyperactivated immune response that could lead to excessive tissue damage (Leone et al., 2015). Physiologically, to avoid autoimmunity, naive T cells need two signals to be activated: one is the binding of the antigen dependent T cell receptor (TCR) to the major histocompatibility complex (MHC) on the antigen presenting cells (APCs), and the other is a co-stimulatory signal, normally the interaction of CD28 on T cells with CD80 (also known as B7-1) and CD86 (also known as B7-2) expressed by APCs (Lanzavecchia et al., 1999; Esensten et al., 2016). The cytotoxic T-lymphocyte antigen-4 (CTLA-4) receptor and the programmed cell death protein (PD-1) are the immune checkpoints most deeply studied and therapeutically exploited (**figure 34**).

CTLA-4 inhibits T cell activation by antagonizing CD28 and competing for binding to the costimulatory ligands CD80/B7-1 and CD86/B7-2, by preventing immune conjugate formation and by recruiting Tregs (Intlekofer and Thompson, 2013). First studies showing that CTLA-4 inhibition boosted anti-tumor immune response date to 1996 (Leach et al., 1996), and since then, it has been observed that anti-CTLA-4 treatment increased T cell effector function while depleting intratumoral immunosuppressive Tregs (Peggs et al., 2009; Sharma et al., 2019), placing the intratumoral ratio T regs/T cells as critical factor in predicting anti-CTLA-4 response (Grosso and Jure-Kunkel, 2013, Waldman et al., 2020). Several anti-CTLA-4 antibodies, like Ipilimumab, have been approved by FDA, being especially promising in melanoma (Lee et al., 2016b; Hodi et al., 2010; Schadendorf et al., 2015).

PD-1 axis controls sustained activation and proliferation of effector cells. The interaction of PD-1 with its ligand PD-L1 induces exhaustion and subsequent apoptosis of antigen-specific T cells (Blank et al., 2004; Barber et al., 2005; Ostrand-Rosenberg et al., 2014) while it promotes Treg development and function (Francisco et al., 2009). PD-L1 expression is induced by IFN-γ produced by activated T cells and NK cells, acting as brake in order to stop immune activation (Garcia-Diaz et al., 2017). Thus, PD-1 axis is involved in the central-tolerance establishment within peripheral tissues (Waldman et al., 2020). Due to its involvement in the attenuation of T cell cell response, it is understandable that PD-1 axis is exploited by cancer cells to evade anti-tumor response (Ju et al., 2020). PD-1 axis has been an important target for immunotherapies in order to potentiate anti-tumor immune response (**figure 34, right**), with several antibodies like pembrolizumab, nivolumab or avelumab approved by the FDA (Hargadon et al., 2018). However, despite the initial promising effect of immunotherapy, the majority of the patients does not benefit from treatment and some patients develop acquired resistance (Sharma et al., 2017; Zhang et al., 2018).

AML, like other cancer types, also takes advantage of these immunomodulatory mechanisms and express

the ligands of these molecules to escape from the immune system (Lamble et al., 2018; Wei et al., 2018). It's been described that AML patients have increased Tregulatory cells (Dulphy et al., 2014), with increased expression of regulatory co-receptors on $CD8^+$ T cells, such as PD-1, TIM and LAG3 (Williams et al., 2018). Interestingly, PD-1 is more expressed in relapse than in diagnosis (Schnorfeil et al., 2015; Williams et al., 2018). In addition, leukemic blast express PD-L1 (Zhang et al., 2009), and there is a study associating PD-L1 expression in AML with lower relapse free survival (Chen et al., 2008). Regarding CTLA-4, it's been published that the polymorphism CT60, located in the 3'-untranslated region (UTR) of the CTLA-4 gene was associated with shorter overall survival and a higher risk of relapse (Perez et al., 2008). In addition, a clinical trial with single agent ipilimumab, an anti-CTLA-4 with relapsed patients after allogeneic HSCT showed a good response, with 5/22 patients that achieved complete remission and 4/22 patients had a durable remission for 1 year. The response was associated with decreased activation of Tregs, expansion of T effector cells and infiltration of $CD8^+$ cytotoxic T cells (Davis et al., 2016). Up to date, the only antibody approved for use in AML is the anti-CD33 antibody drug conjugate gemtuzumab ozogamicin (Liu et al., 2019).

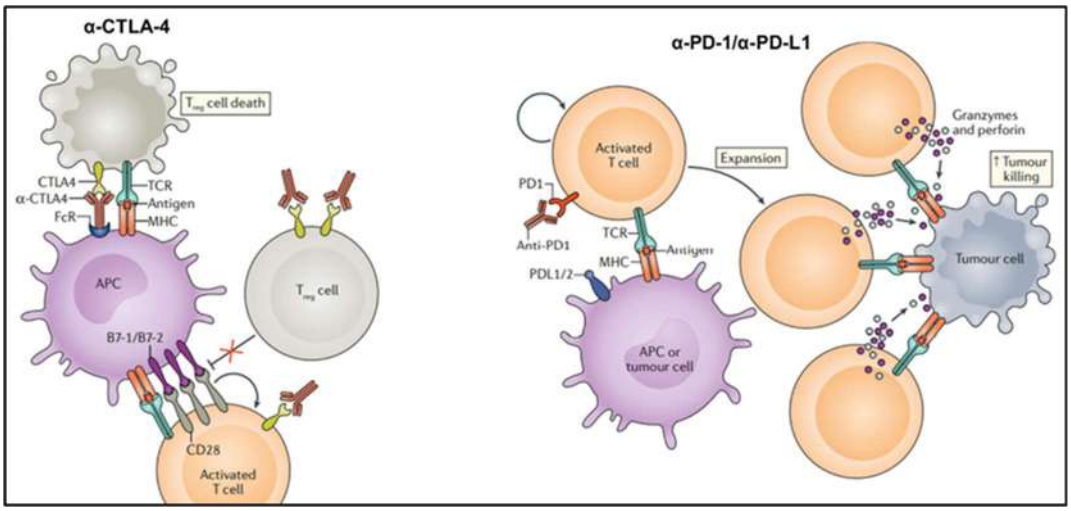

Figure 34. CTLA- 4 and PD-1/PD-L1 blocking antibodies.
Adapted from Waldman et al., 2020

ii. ATP, adenosine and ectonucleotidases

Nucleotides and their receptors begin to emerge as immune modulators (**figure 35**). ATP can be actively secreted, passively released or generated in the extracellular microenvironment. When in extracellular space, ATP can interact with purinergic receptors P2R or be hydrolyzed to the immunosuppressive adenosine by CD39 and CD73. There is a huge variety of P2R, that have different affinities and nucleotide selectivity that contribute to several intracellular transduction pathways and that can result in different responses. In addition, the responses can be different depending on the concentrations of extracellular ATP. Purinergic receptors regulate chemotaxis in monocytes, dendritic cells and eosinophils, as well as migration of NK cells (Junger et al., 2011). T cell activation is also regulated through the activation of

purinergic receptors P2Xs by the ATP released by Pannexin 1 (Schenk et al., 2008; Woehrle et al., 2010). However, to add some complexity, the chronic presence of extracellular ATP at low concentrations can have anti-inflammatory effects, which have been observed in dendritic cells, monocytes, $CD4^+$ T cells and neutrophils (Di Virgilio et al., 2009; Junger et al., 2011; Di Virgilio et al., 2018). In addition, purinergic receptors are also involved in metabolism. The stimulation of P2X receptors (purinergic receptors that acts as ATP-gated ion channels to facilitate the influx of extracellular cations) increases oxidative phosphorylation and ATP synthesis to buffer intracellular Ca^{2+} (Di Virgilio et al., 2018). This has been described also in the context of AML (Ledderose et al., 2016), and it could be a mechanism contributing to chemoresistance.

Alternatively, several membrane ectonucleotidases can generate adenosine from the degradation of ATP or ADP, such as CD39 (also known as NTPDase1), CD39L (NTPDase2) and CD39L3 (NTPDase3), CD203a (also known as NPP1). AMP is hydrolyzed by CD73 (also known as 5′-NT) to generate adenosine. Adenosine in the microenvironment, derived from extracellular ATP, is well known by its immunosuppressive effects through its interaction with purinergic receptors P1 (also known as adenosinergic receptors) (Vijayan et al., 2017). Adenosine regulates, through its generation by ectonucleotidases and by its interaction with its receptors, the Treg population, and it suppresses T and natural killer (NK) cell functions (Young et al., 2016; Vijayan et al., 2017; Virgilio et al., 2018).

One of the most attractive and promising targets to potentiate anti-tumor response through adenosine signaling inhibition is the adenosine P1 receptor A2AR (Ohta et al., 2006; Beavis et al., 2013; Cekic et al., 2014; Young et al., 2016; Vijayan et al., 2017). A2AR deletion or pharmacologic inhibition in mice increased tumor rejection mainly by increasing $CD8^+$ T cell activity in several mouse cancer models (Ohta et al., 2006; Waickman et al., 2011) and also in human refractory renal cell cancer (Fong et al., 2019). A recent study has also shown that targeting A2AR in CAR T cells improves their function and can potentiate anti-cancer response (Masoumi et al., 2020). Several A2AR antagonists or CD73 inhibitors are already in clinical phase, as single agent or in combination with immunotherapy, and clinical trials have proven that they are safe and well tolerated (Steingold and Hatfield, 2020; Willingham et al., 2020). CD39 and CD73 have been reported to be induced by hypoxia (Eltzschig et al., 2003; Eltzschig et al., 2009), and indeed, it's been suggested that adenosynergic signaling is a mechanism by which HIF-1α suppresses anti-tumor response (Steingold and Hatfield, 2020; Willingham et al., 2020). CD39, and to a lesser extent CD73, expression in human AML blasts at diagnosis has been proposed to collaborate with Tregs in the production of extracellular adenosine, contributing to the regulation of the immune response in AML (Dulphy et al., 2014). In addition, CD39 has also been linked to resistance and poor response in AML, and its expression on leukemic blasts supports oxidative phosphorylation by regulation of AMP levels and activation of the cAMPK pathway, that may enhance PGC-1-mediated mitochondrial biogenesis, contributing to chemoresistance (Aroua et al., 2020).

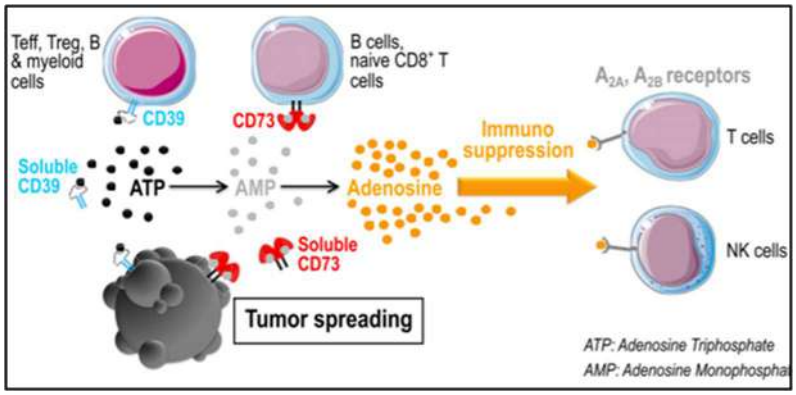

Figure 35. Ectonucleotidases create an immunosuppressive tumor microenvironment.
Adapted from Perrot et al., 2019.

iii. Lactate

Lactic acid and low pH began to be acknowledged as a tumor characteristic already in 1989 (Tannock and Rotin, 1989). Lactate production is enhanced upon OxPhos inhibition, given that cells exclusively rely on pyruvate fermentation to obtain energy (Pasteur effect). Nowadays, lactate has a recognized immunossupressive function, as summarized in **figure 36**.

In macrophages, lactate has been described to induce polarization to the M2-like phenotype through HIF-1α (Colegio et al., 2014). G-protein-coupled receptor 132 (Gpr132) senses lactate and promotes M2 phenotype polarization, and its deletion impairs breast cancer lung metastasis in mice (Chen et al., 2017). M2 macrophages have an anti-inflamatory phenotype, and they are involved in cancer immunosuppression, but also in tissue regeneration after ischemia. Indeed, lactate (produced by endothelial cells, EC) oxidation by M2 macrophages is critical for revascularization and muscle recovery after ischemia, whereas loss of PFKFB3 in EC impairs muscle recovery (Zhang et al., 2020). Regarding T cells, both glucose depletion and lactic acid impair cytotoxic and effector T cell functionality. Tumor-derived lactate has been reported to suppress cytotoxic T cell proliferation and cytokine production, and therefore to dampen its cytotoxic activity, by blocking lactate export via MCT-1 in T cells through increasing extracellular lactate, which results in T cells disrupted ability to maintain glycolysis (Fischer et al., 2007). Tumor-derived lactic acid decreases NFAT, which results in impaired activation of TILs and NK cells (Brand et al., 2016). Another mechanism, pH independent, by which lactate suppresses effector T cells is through lactate-induced reductive stress, which is, the decrease in the NAD^+/NADH ratio through NAD^+ reduction (Quinn et al.,2020). Increased lactate leads to inhibition of GAPDH and PGDH, which depletes glycolytic intermediates beyond GAPDH, including 3-PG and therefore serine, crucial for T cell proliferation (Ma et al., 2017). Indeed, supplementation with serine restores T cell proliferation under lactate-induced reductive stress (Quinn et al., 2020). Tumor-derived lactate has been described to induce PD-L1 expression through GPR81 in lung cancer (Feng et al., 2017). Mechanistically, GPR81 inhibited PKA activity through decreasing cAMP levels; which led to the activation of the transcriptional

coactivator TAZ, which resulted in PD-L1/ PD-1 immune checkpoint establishment and impaired T cell function (Feng et al., 2017). Contrarily to effector T cells, Tregs adapt to low-glucose high-lactate environment through metabolic adjustments induced by Foxp3, which downregulates Myc and glycolysis while upregulating OxPhos and increasing NAD^+/NADH ratio (Angelin et al., 2017). Thus, lactate favors immunosuppressive environment by suppressing cytotoxic T cells while allowing activity of immunoregulatory Tregs. Glycolysis is essential for complete T cell effector function. Without glucose, cytokine production is impaired in T cells (Chang et al., 2013). In TME, glucose competition by the tumor and tumor infiltrating cells (TILs) can impair effector T cell function even in the presence of tumor antigens robustly recognized by T cells, which resulted in tumor progression and highlighted the importance of glucose availability for T cells (Chang et al., 2015). When comparing CD4 T cells grown in glucose or in galactose, those galactose-cultured cells lost effector function and expressed higher levels of PD-1 as marker of exhaustion. This is an interesting remark that could lead to think that PD-1 expression is regulated by metabolic constraints and not only by chronic antigen stimulation (Chang et al., 2013). Interestingly, checkpoint blockade antibodies against CTLA-4, PD-1 and PD-L1 restored glucose availability in tumor microenvironment and rescued cytokine production in T cells (Chang et al., 2015).

High-LDHA melanoma is extremely metastatic, due to glucose competition and TME acidification, which have been reported to impair NK cell activation (Brand et al., 2016). In addition, tumor-secreted lactate stimulates MDSC recruitment, which dampens NK cell cytotoxic activity, marked by decreased expression of perforin and granzyme and reduced tumor progression control (Husain et al., 2013). Lactate induces NKG2D ligand expression on monocytes, which induces downregulation of NKG2D on NK cells (Crane et al., 2014). Autologus tolerogenic dendritic cells, a subtype of monocyte derived cells, produce high levels of lactate, which directs T cell response toward immunogenic tolerance by suppression of their proliferation and expansion (Marin et al., 2019). These cells can be a promising therapeutic tool for graft-versus host disease (GVHD) after transplantation, inflammatory or autoimmune diseases, as proposed by Marin and colleagues.

Metabolic symbiosis involving the cooperation of different parts of a tumor to produce and oxidate lactate has been reported to contribute to drug resistance (Allen et al., 2016; Park et al., 2016; Hayes et al., 2021). For example, metabolic symbiosis has been reported to take place upon angiogenesis inhibition is overcome by mTOR signaling inhibition (Allen et al., 2016). In more detail, hypoxic tumor cells distal to blood vessels are glycolytic, as they express high levels of GLUT1 and MCT4 induced by HIF-1α. However, normoxic proximal tumor cells express the lactate transporter MCT1, and due to mTOR signaling, they can metabolize lactate thanks to an enhanced glutamine metabolism (Allen et al., 2016). It has also been reported that resistance to PI3K/mTOR inhibitors is associated with lactate oxidation by breast cancer cells, and the distruption of mitochondrial function using ERRα restores drug efficacy (Park et al., 2016).

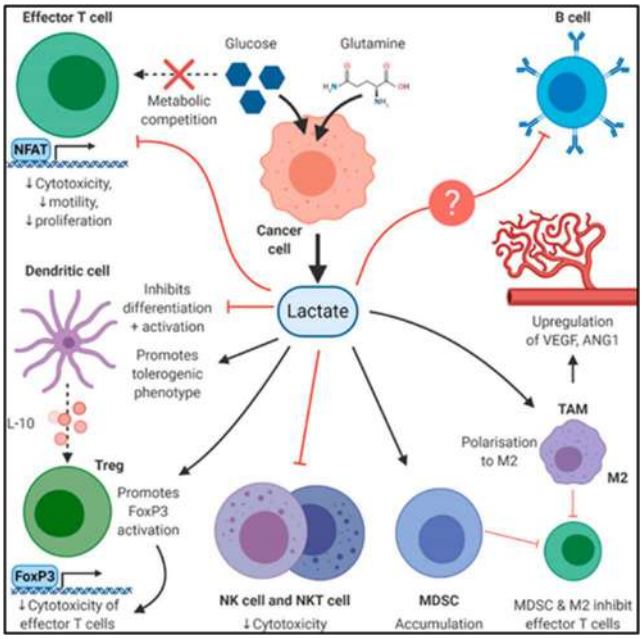

Figure 36. Immunosuppressive effects of lactate.
Adapted from Hayes et al., 2021.

A huge effort has been invested in developing therapies targeting lactate transport and synthesis. Regarding MCTs, several inhibitors have been described, such as stilbene disulphonates (Poole and Halestrap, 1990) and organomercurials (Wilson et al., 2005). More recently, specific MCT1 targeting compounds have been developed, such as SR13800 (Doherty et al., 2014) and AZD3965 (Polański et al., 2014), which is in phase I clinical trial for adult solid tumors, diffuse large B cell lymphoma and Burkitt Lymphoma (NCT01791595). Overall, targeting lactate efflux can be an interesting therapeutic approach to reactivate immune system upon immunosuppresive lactate overload.

In brief

- Metabolism regulates functionality of immune cell populations, characterizing even immune phenotypes, like for example in the case of M1 or M2 macrophages. Importantly, metabolic modulation of the immune compartment can provide new therapeutic opportunities through the regulation of the immune function, boosting immune system in immunosuppressive contexts like cancer or dampening it in autoimmune diseases.
- Immunosuppressive populations like Tregs, M2 macrophages or MDSCs rely on OxPhos.
- Anti-tumor immune response can be dampened by immune-modulatory molecules, such as the immune checkpoints PD-L1 and CD39 or by immunossupressive lactate generated by the tumor microenvironment.
- Antitumor effect of metformin seems closely related to the immune system, since in several models its effect is lost in immunodefient preclinical models, which may question lactate immunosuppressive effect. Metformin has been shown to downregulate PD-L1 in breast cancer cells and CD39 in MDSCs, providing a mechanism by which it could boost immune system.

1.3.2.4. Targeting mitochondrial dependency of cancer cells with ETCI inhibitors

Given all the above mentioned, OxPhos inhibition has emerged in the recent years as an approach with incredible potential to target cancer initiation, progression and relapse, due to the targeting of specific vulnerabilities of some cancer types and cancer stem cells. Another complementary mechanism by which ETCI inhibitors are thought to exert their anticancer effect is by alleviating tumor hypoxia, since they reduce oxygen consumption by cells, thus increasing O_2 availability in the tissue (Ashton et al., 2018). The upcoming pages with briefly describe the different ETCI inhibitors that currently are or have been into clinical trials.

1.3.2.4.1. Metformin: a pleiotropic ETCI inhibitor

Metformin is a cationic biguanide used for the treatment of type 2 diabetes (T2D) due to its ability to modulate metabolism (Inzucchi et al., 1998; Nathan et al., 2008; Rena et al., 2017). Retrospective studies have identified that diabetic patients treated with the antidiabetic-pleiotropic drug metformin had reduced risk for several cancers (liver, pancreatic, colorectal and breast cancer) (Evans et al., 2005; Noto et al., 2012; Zhang et al., 2013), what positioned metformin as an interesting agent for cancer treatment and prevention (Saraei et al., 2019), and led to its evaluation in several clinical trials (**table 1**).

At organism level, it reduces glycemia by opposing to glucagon signaling, stimulating glucose uptake in muscle and adipocytes while decreasing hepatic gluconeogenesis, boosting insulin sensitivity and glucose utilization in extrahepatic tissues. (Rena et al., 2017; He, 2020). At the molecular level, it exerts a wide range of effects (**figure 37**), whose mediator mechanisms are still imperfectly understood. One of the most studied effects of metformin is the inhibition of the electron transport chain complex I (El-Mir et al., 2000; Owen et al., 2000). By inhibiting the oxidative phosphorylation, it induces a decrease in the ATP/AMP ratio in the cell and activates AMPK (Musi et al., 2002), the sensor of the energetic status of the cell (described in the chapter "1.2.1. AMP-activated protein kinase"). However, it has been also reported that metformin can activate AMPK independently of the adenine nucleotides ATP, ADP, AMP ratios (Fryer et al., 2002; Hawley et al., 2002). Classically, metformin effects on cancer cells can be divided into two categories: AMPK-dependent or AMPK-independent.

Through AMPK activation, metformin inhibits mTORC1 by TSC2 phosphorylation, AKT signaling, cell cyle, HIF-1α and MYC while it promotes ATM signaling and autophagy (Pernicova and Korbonits, 2014; Vancura et al., 2018; Saraei et al., 2019; Marcucci et al., 2020). Also in an AMPK dependent manner, metformin inhibits insulin-IGF-1/PI3K/AKT pathway through AMPK phosphorylation of IRS-1 (Zakikhani et al., 2010).

However, the effect of metformin can be also AMPK independent, as proven by many studies. First evidences came up in 2010, when Foretz and colleagues showed that metformin inhibited gluconeogenesis in hepatocytes independently of AMPK and LKB1 through hepatic energy state. They also showed that

the inhibition of the gluconeogenesis by metformin was preserved even under expression of gluconeogenic genes through PPARγ coactivator 1α (PGC-1α) overexpression, evidencing that metformin suppressed gluconeogenesis by a mechanism independent from transcription (Foretz et al., 2010). Then, Ben Sahra and colleagues showed that metformin inhibited mTOR and induced cell cycle arrest through REDD1 by an AMPK-independent mechanism in prostate cancer (Ben Sahra et al., 2011). REDD1 (RTP801/Dig2/DDIT4) is a target of HIF-1 involved in a wide variety of functions, such as regulation of cell survival, response to DNA damage, nutrient depletion, glucocorticoid and insulin. REDD1 is an mTOR inhibitor, and it is regulated by p53 (Ben Sahra et al., 2011). Through ETCI inhibition, metformin has also been reported to reduce ROS production and related DNA damage, in an AMPK-independent manner (Algire et al., 2012). In addition, metformin can activate ataxia telangiectasia mutated (ATM), to maintain genome integrity (Vazquez-Martin et al., 2011). Interestingly, polymorphisms adjacent to the ATM locus impair glycemic response to metformin in type 2 diabetes, suggesting that ATM activation is required for the glucose-lowering effect mediated by metformin (Zhou et al., 2010). AMPK-independent metformin mechanism of action has also been observed in AML. By silencing the AMPK-α1 and 2 subunits, Scotland and colleagues showed that cell cycle arrest and inhibition of proliferation induced by metformin in AML cells was independent of AMPK (Scotland et al., 2013). The accumulation of AMP induced by OxPhos blockade inhibits adenylate cyclase, what subsequently reduces cAMP levels and protein kinase A (PKA) activity (Miller et al., 2013). Indeed, Miller and colleagues found this mechanism to be responsible of the glucagon signaling blockade exerted by metformin.

Overall, metformin effects are wide, can be AMPK-dependent or independent, and are not exclusively a consequence of ETCI inhibition (**figure 37**).

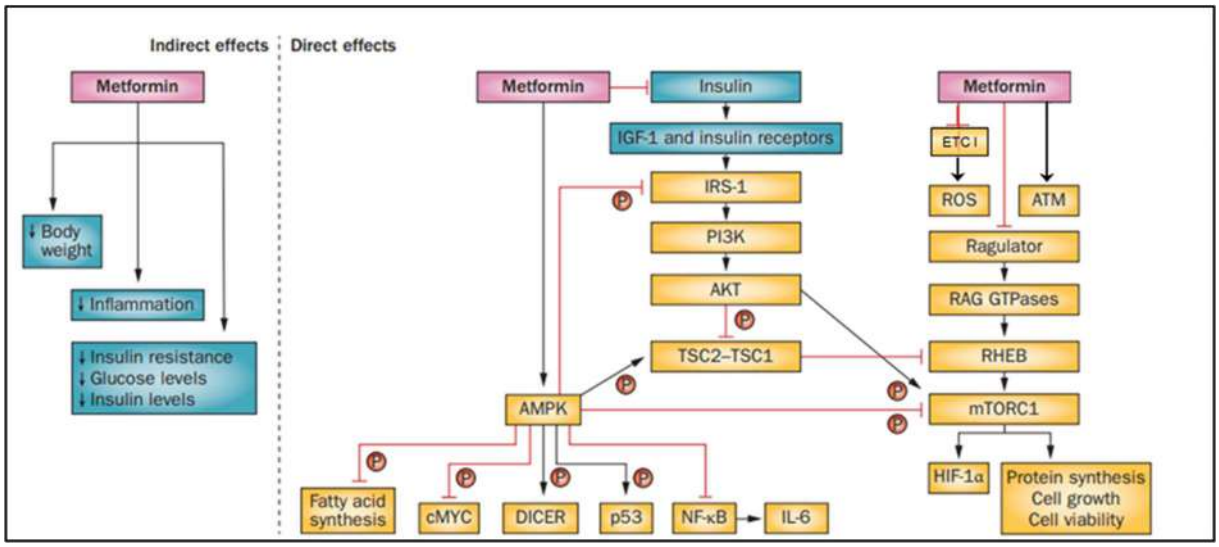

Figure 37. Metformin's pleiotropic effects.
Adapted from Pernicova and Korbonits, 2014.

1.3.2.4.2. Other ETCI inhibitors

Metformin is used in the majority of the cancer pre-clinical studies with the aim to target ETC I. Nevertheless, since it is a pleiotropic ETCI inhibitor its anticancer effect is not merely attributable to ETCI inhibition, as it exerts actions on many other cellular targets. This motivated the research and development of more specific OxPhos inhibitors, targeting selectively ETCI in order to block the electron transference from the beginning. ETCI inhibitors evaluated in clinical trials are summarized in **Table 1.**

Phenformin is another biguanide, however, contrarily to metformin, it does not need any transport protein to enter the cells (Rubiño et al., 2019). This results in higher concentrations of phenformin within cells, as well as a more global effect, being not limited to cells overexpressing OCT. However, phenformin was removed from the market due to the induction of lactic acidosis (Kwong and Brubacher, 1998).

Carboxyamidotriazole (CAI) was initially discovered as an inhibitor of calcium-mediated signal transduction that inhibits tumor angiogenesis within metastases (Luzzi et al., 1998). More recently, it was found to inhibit ETCI, and to have synergistic effect with 2-DG in lung cancer models (Ju et al., 2016). It has been reportet that CAI in combination with platinum-based chemotherapy prolonged progression free survival (PFS) and could be a useful therapeutic option to treat NSCLC (Si et al., 2020), despite previous clinical trial failing at demonstrating clinical benefit following chemotherapy in NSCLC (Johnson et al., 2008). The clinical trial NCT01107522 raised the conclusion that CAI combination with chemoradiation is safe in GBM and anaplastic gliomas, since it displayed favorable brain penetration and promising signals of activity in this difficult-to-treat population (Omuro et al., 2018).

BAY87-2243 is a very potent ETCI inhibitor that abrogates HIF-1α and HIF-2α protein accumulation, showing promising pre-clinical efficacy in lung cancer xenografts (Ellinghaus et al., 2013). Unfortunately, and despite apparent lack of toxicity in mice (Ellinghaus et al., 2013), the initial phase I trial in human (NCT01297530) had to be terminated due to unexpected safety issues resulting from massive emesis (Ashton et al., 2018).

IACS-010759 exerts selective inhibition of ETCI and has shown anti-cancer efficacy in several preclinical models, including glioblastoma and AML by inducing energy depletion and reducing aspartate levels, resulting in decreased nucleotide biosynthesis and proliferation of cancer cells (Molina et al., 2018). Interim results of the phase I clinical trial of IACS-010759 in solid tumors suggested acceptable toxicity and early signs of response (Yap et al., 2019). Nevertheless, 1/3 patients experienced emesis (Yap et al., 2019).

ME-344 is an isoflavone that induces cell apoptosis by enhancing ROS levels through heme oxygenase 1 (HO-1) inhibition (Zhang et al., 2019c). HO-1 inhibition reduced oxygen consumption by impairing ETCI and ETCII functionality (Carr et al., 2020). ME-344 It has shown significant antitumor activity in HER2-

negative breast cancer after treatment with bevacizumab and induced vascular normalization and tissue reoxygenation (Quintela-Fandino et al., 2019).

Table 1. Recent Clinical trials with ETCI inhibitors. Adapted from Sica et al., 2020.

Drug	Clinical trial	Indication	Phase of the trial
Metformin	NCT03137186	Advanced Prostate cancer	2
	NCT03685409	Oral cancer chemoprevention	3
	NCT03017833	Solid tumor and advanced cancer in combination with TAK-228 (MLN0128)	1
	NCT03017833	Thoracic neioplasm	1
	NCT03684707	Oral cancer	4
	NCT03238495	HER2-positive breast cancer with chemotherapy	2
	NCT02176161	Prostate cancer as adjuvant	2
	NCT02945813	Prostate cancer in combo with radiotherapy	2
	NCT01980823	Combination of Metformin and Atorvastatin in newly diagnosed operable Breast cancer	1
	NCT02640534	Castration resistant prostate cancer in combination with Enzalutamide	2
Phenformin	NCT03026517	BRAF mutated Melanoma, in Combination With Dabrafenib and Trametinib	1
Carboxyamidotriazole	NCT01107522	Solid tumors, glioblastoma, Recurrent malignant gliomas	1
IACS-010759	NCT03291938	Advanced cancers	1
	NCT02882321	Refractory or Relapsed AML	1
ME-344	NCT02806817	HER2 negative Breast cancer	1

1.3.2.4.3. EVT-701: a new selective ETCI inhibitor

EVT-701 (**figure 38**) is a new selective inhibitor of mitochondrial complex 1, derived from manassantin A. Manassantin A is a natural compound extracted from Saururus cernuus and Saururus chinensis (Hossain et al., 2005). The extraction process is not very efficient, since from 172 g of leaves, only 11,7 mg of Manassantin can be extracted (Hodges et al., 2004). Alternatively, it can be synthetized in 19 steps (Hanessian et al., 2006). Manassantin has recognized role as HIF-1 inhibitor, since it avoids HIF-1 protein accumulation (Hossain et al., 2005). Manassantin A also affects activity of other essential signaling proteins, such as NF-κB or MAPK (Hwang et al., 2003; Lu et al., 2013). Remarkably, it is an inhibitor of the mitochondrial electron transport chain, targeting complex I (Lai et al., 2013; Ma et al., 2017b). EVT-701 is a drug-like compound, resulting from extensive medicinal chemistry campaign to facilitate synthesis path and scalability, improve ADME profile while maintaining potency of Manassantin A. More

than 300 molecules were synthesized and evaluated, to obtain final compound, using a route of only 5 steps (figure 38). This makes EVT-701 synthesis scalable for pre-clinical and clinical development. EVT-701 was selected to inhibit EPO release, decrease HIF-1 stabilization under hypoxia and mitochondrial ATP production (Meneyrol, 2016). In addition, no Drug Metabolism and Pharmacokinetics (DMPK) issues were identified for EVT-701 (Meneyrol, 2016).

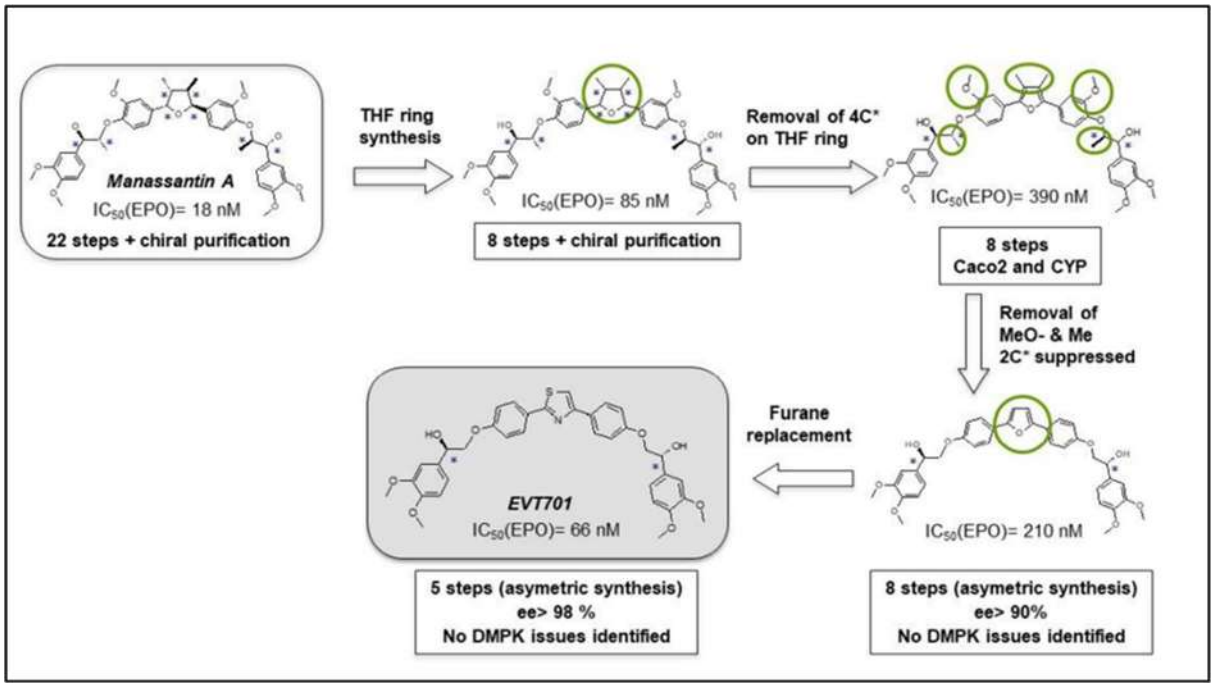

Figure 38. Overview of Medicinal chemistry optimization of Manassantin A into EVT-701.

In brief

Cancer cells are dependent on ETC function for tumor initation, progression, as well as for anti-cancer therapy resistance and for cancer stem cell maintenance. Thus, targeting ETCI to block OxPhos seems a relevant therapeutic approach. At the beginning of this thesis, the available ETCI inhibitors were either pleiotropic, such as metformin, or exerted unacceptable toxicity through the induction of lactic acidosis resulting unsafe in humans, such as phenformin or BAY87-2243. This indicated the need for new, safe and selective ETCI inhibitors that would exert anti-cancer activity with limited toxicity.

Summary

It is undeniable that mitochondria play an essential role in cancer initiation and progression. Functional mitochondria sustain numerous cellular processes, both energetic and anabolic that are tightly interconnected, and key for cell proliferation and survival. For example, functional ETC is essential to support cell proliferation by enabling nucleotide production. In fact, many cancer cells show increased mitochondrial function compared to their normal counterparts in non-transformed tissue. Remarkably, OxPhos is also involved in the maintenance of cancer stem cells. Resistance to both chemotherapy and targeted therapies have been reported to be accompanied by OxPhos phenotype in several cancer types, including AML. Thus, targeting mitochondrial functions and structures (including the inhibition of the first component of the ETC) could potentially be an interesting approach to improve anti-cancer therapy. Historically, metformin has been the main drug used to target ETC in pre-clinical studies, due to its use in the treatment of type 2 diabetes (T2D), what makes it easily accessful. Nevertheless, metformin is a pleiotropic drug with a wide variety of cellular targets which makes hard to definitively and exclusively link the effects observed upon metformin treatment to ETCI inhibition. Targeting OxPhos can lead to an adaptive mechanism by which the cells upregulate lactic fermentation to try to compensate ETC blockade and maintain energy balance. This is known as Pasteur effect. Phenformin is another biguanide, more potent at inhibiting ETCI but it had to be withdraw from the market precisely due to the induction of lactic acidosis. ETCI inhibition with BAY87-2243 is reputed selective and strong, but clinicals trials had to be stopped due to unacceptable toxicity. That is why the development of novel, safe and selective ETCI inhibitors is strongly needed. EVT-701 is an optimized lead compound derivate of Manassantin A, resulting from extensive medicinal chemistry campaign executed in collaboration with Sanofi and Evotec to facilitate synthesis path and scalability, improve ADME profile while maintaining potency of the natural compound. Metformin beneficial effect in the antitumor immune response has been suggested by several studies, despite the mechanisms involved remain elusive. Importantly, many of the studies showing anti-tumor activity upon ETCI inhibition are performed in NSG mice, especially in AML. Immunodeficient mice allow the study of the disease with human cells and even with patient molecular features, at the expense of a functional immune system. Thus, the study of ETCI inhibition in AML syngeneic models is required to better characterize the effect these compounds can have in the immune compartment when administered to "immunocompetent" humans. In this field, several questions were still unadressed at the beginning of this thesis: **Can the specific inhibition of ETCI be safe and show anti-cancer activity? How would ETCI inhibition impact the immune compartment in cancer? What other cell and non-cell autonomous functions might be controlled by ETCI activity? Does the inhibition of ETCI impact other aspects of mitochondrial, metabolic or cellular biology?**

2. Hypothesis and objectives

Aiming to answer these questions, my hypothesis was that blocking oxidative phosphorylation dependency of cancer and leukemic cells by a selective electron transport chain I inhibitor may be safe and impact and re-activate immune compartment to enhance treatment efficacy in cancer.

My PhD work aimed to address the two following objectives:

- To determine whether EVT-701 is a safe selective compound with anti-cancer activity in solid tumors.
- To assess the effects of EVT-701 in a murine syngeneic model of chemoresistant leukemia.

To assess the before mentioned questions, we used three following cancer models:

Non-Hodgkin lymphoma/ Diffuse large B cell lymphoma. Lymphomas are a heterogeneous group of lymphoid malignancies characterized by the presence of tumors predominantly in lymphoid structures, but also in extranodal tissues. (Scott and Gascoyne, 2014; Jiang et al., 2017). Among the different subtypes of lymphoma, non-Hodgkin lymphoma (NHL) accounts for 90% of the cases (Shankland et al., 2012). NHL are derived from B cells in 85-90% of the cases (Armitage et al., 2017) and among them, diffuse large B cell lymphoma (DLBCL) accounts for the 30-35% of NHL cases (Scott and Gascoyne, 2014; Siegel et al., 2019). DLBCL metabolic heterogeneity was first reported in 2005, when Monti and colleagues identified by whole genome array and multiple clustering using samples from newly diagnosed DLBCL patients a subtype of DLBCL whose signature was enriched in genes involved in OxPhos, mitochondrial function and ETC subunits (Monti et al., 2005, Caro et al., 2012). This entity is referred now as OxPhos-DLBCL and accounts for 30% of primary DLBCL (Caro et al., 2012). To study this disease, we used Eμ-myc lymphomas isolated from transgenic mice in which normal myc gene had become coupled to the Ig heavy chain enhancer, being thus constitutively expressed and leading to the development of NHL (Adams et al., 1985). This model recapitulates metabolic heterogenetity found in DLBCL, what is of great relevance when evaluating ETC as target for the disease.

Non-small cell lung carcinoma. Non-small cell lung carcinoma (NSCLC), that constitutes 85% of lung cancer cases. Approximately 30% of lung adenocarcinomas harbor activating mutations in Kirsten rat sarcoma viral oncogene homologue (KRAS) (Yang et al., 2019), what results in its constitutive activation. Metabolically, NSCLCs tumors have been reported to have increased glycolysis and TCA cycle activity than non-cancerous lungs (Hensley et al., 2016), as denoted by the enrichment in labelled lactate, alanine, succinate, glutamate, aspartate and citrate (Fan et al., 2009). Mitochondria is essential for KRAS-driven

lung cancer, since the deletion of TFAM, involved in mtDNA copy number maintenance, resulted in diminished tumorigenicity (Weinber et al., 2010). LKB1 (also known as STK11) inactivating mutations are detected in 20% of NSCLC cases (Gill et al., 2011). As mentioned in the point "1.3.2. Mitochondrial dependency in cancer and ETCI inhibition", KRAS driven-NSCLC tumors bearing LKB1 mutations are very sensitive to phenformin treatment, due to energetic imbalance (Shackelford et al., 2013). Given NSCLC dependency on mitochondria, as well as the interesting sensibility of LKB1 mutations to ETCI inhibition, we considered it to be a suitable context to evaluate EVT-701.

Acute myeloid leukemia. Acute myeloid leukemia (AML) is a clonal hematologic malignancy characterized by a block in terminal myeloid cell differentiation and uncontrolled proliferation (Sánchez-Mendoza et al., 2017). Despite significant increase in 5 year survival rates in the recent years, reaching up to 40-45% in young individuals and almost 30% in elderly patients, there is a persistent need of suitable and more effective therapies to eradicate the disease (Dombret et al., 2016; Kantarjian et al., 2021). After intensive chemotherapy, the majority of *de novo* AML patients achieve complete remission (CR). However, approximately two-thirds of patients end up with relapse, generally within the first 18 months (Yilmaz et al., 2019). The role of mitochondria as an essential organelle for AML, as well as in involvement in chemo-resistance, has been highlighted by many works and focusing on different aspects, as previously described in the chapter "Mitochondria in resistance to cancer therapy". Remarkably, increased OxPhos is the common denominator of resistance to classical AraC chemotherapy and to new therapeutic agents making it an attractive metabolic vulnerability to exploit therapeutically.

3. Results

3.1. EVT-701 is a novel selective and safe mitochondrial ETC 1 inhibitor with potent anti-tumor activity

Major results and conclusions

EVT-701 showed good bioavailability and a safe profile, not inducing the emesis observed with other competitors. EVT-701 demonstrated to decrease mitochondrial ATP production, oxygen consumption rate and proliferation in several cancer cell lines, at concentrations below micromolar range. Furthermore, EVT-701 proved therapeutic efficacy at reducing tumor weights and volume in several cancer models, including NSCLC and DLBCL with OxPhos clones. Finally, we found LKB1 expression to be a good stratification marker to identify higher sensitivity to EVT-701 in lung cancer.

Article

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EVT-701 is a novel selective and safe mitochondrial complex 1 inhibitor with potent anti-tumor activity in models of solid cancers

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Abbreviations:

AUC, Area Under Curve; DAPI, 4′,6-diamidino-2-phenylindole; DLBCL, Diffuse Large B Cell Lymphoma; ETC, Electron Transport Chain; FCCP, Carbonyl cyanide-p-trifluoromethoxy-phenylhydrazone; IC_{50}, half maximal inhibitory concentration; MC1, Mitochondrial Complex 1; NH, Non Hodgkin's; OCR, Oxygen Consumption Rate; OD, Optic Density; OxPhos : Oxydative Phosphorylation; PEG, PolyEthylene Glycol; TCA, TriCarboxylic Acid.

Section:

Drug Discovery and Translational Medicine

Abstract

Targeting the first protein complex of the mitochondrial electron transport chain (MC1) in cancer has become an attractive therapeutic approach in the recent years, given the metabolic vulnerabilities of cancer cells. The anticancer effect exerted by the pleiotropic drug metformin and the associated reduction in hypoxia-inducible factor 1α (HIF-1α) levels putatively mediated by MC1 inhibition led to development of HIF-1α inhibitors, such as BAY87-2243, with a more specific MC1 targeting. However, development of BAY87-2243 was stopped early in phase 1 due to dose-independent emesis and thus there is still no clinical proof of concept for the approach. Given the importance of mitochondrial metabolism during cancer progression, there is still a strong therapeutic need to develop specific and safe MC1 inhibitors. We recently reported the synthesis of compounds with a novel chemotype and potent action on HIF-1α degradation and MC1 inhibition. We describe here the selectivity, safety profile and anti-cancer activity in solid tumors of lead compound EVT-701. In addition, using murine models of lung cancer and of Non-Hodgkin's B cell lymphoma we demonstrated that EVT-701 reduced tumor growth and lymph node invasion when used as a single agent therapy. Altogether these results support further evaluation of EVT-701 alone or in combination in preclinical models and eventually in patients.

Significance statement

The present study elucidates the mechanism of action and supports further preclinical and clinical investigation of EVT-701, either as a single agent or in combination for the treatment of solid tumors. LKB1 deficiency in lung cancer was identified as potential indicator of accrued sensitivity to EVT-701, allowing stratification and selection of patients in clinical trials. In addition, emesis associated with BAY87-2243 was not observed for EVT-701 at therapeutic doses, providing a key difference and a strong justification for further development.

INTRODUCTION

Since 2000, a growing body of evidence has rehabilitated the importance of mitochondrial metabolism in tumor growth, muzzled for long by Warburg effect supremacy. The very aggressive type of Non-Hodgkin's (NH) B-cell lymphoma, i.e. Diffuse Large B Cell Lymphoma (DLBCL) was the first tumor entity in which an increase in the expression of mitochondrial-related genes was identified in the so-called OxPhos-DLBCL subset (Monti *et al.*, 2005). Two other independent studies unraveled the key role of mitochondrial metabolism in supporting growth of KRAS-driven lung tumors in mouse models (Weinberg *et al.*, 2010; Guo *et al.*, 2011). Since then, many studies demonstrated increased mitochondrial metabolism in melanoma, lung and brain tumors compared to normal tissues (Maher *et al.*, 2012; Momcilovic and Shackelford, 2015; Hensley *et al.*, 2016; Ždralević *et al.*, 2018).

Furthermore, tumors seem to develop from clones with amplified and functional mitochondria (Ju *et al.*, 2014; Reznik *et al.*, 2016) while clones with mitochondrial dysfunction cannot undergo tumorigenesis (Joshi *et al.*, 2015). Recent work suggests that such metabolic reprogramming is a continuous process, allowing dynamic adaptation as tumor requirements change from initiation to metastasis (Faubert *et al.*, 2020), and in response to anti-cancer therapies (Farge *et al.*, 2017).

Mitochondrial Complex 1 (MC1) is the first and major component of the electron transport chain (ETC), oxidizing NADH from the tricarboxylic acid (TCA) cycle (and fatty acid β-oxidation pathway) to initiate the electron and proton transfer necessary for ATP synthesis (Brand and Nicholls, 2011; Zickermann *et al.*, 2015). It comprises 44 subunits of which 14 are necessary and sufficient for catalytic activity (Fiedorczuk *et al.*, 2016; Zhu *et al.*, 2016). Given the number of accessory subunits, its roles in metabolism and cell fitness are multiple and difficult to disentangle. Nevertheless, recent studies shed light to important roles of MC1 in tumorigenesis and tumor adaptation in nutrient- and oxygen-poor surrounding.

Firstly, the electron transport chain is operational in hypoxic environments, even at oxygen levels below 0.5% (Rumsey *et al.*, 1990) and it can still contribute to ATP production and tumor cell survival (Jain *et al.*, 2002; Le *et al.*, 2014).

Secondly, another important role of ETC is to support aspartate biosynthesis. Aspartate is a proteogenic amino acid involved in nucleotide (purines and pyrimidines) biosynthesis, and therefore in proliferation. Active ETC also favors aspartate biosynthesis in order to maintain the level of TCA cycle intermediate levels compatible with cell energetic needs. Furthermore, increased expression and activity of MC1 is correlated with resistance to chemotherapy in pancreatic cancer (Masoud *et al.*, 2020) and with metastatic potential in colon cancers (Rai *et al.*, 2020). Finally, MC1 is fully functional in moderate hypoxic conditions and so sustainably maintains low levels of oxygen in tumor. This in turn restricts damaging ROS levels and stabilizes the transcription factor HIF-1α.

Consistent with the important role played by MC1 in tumor survival and growth, a handful of approved drugs inhibiting MC1 (among other targets) have shown anticancer effects in clinical studies. Metformin, for instance, improves survival of patients with breast, colorectal, ovarian and liver tumors (Morales and Morris, 2015; Bost *et al.*, 2016). Mechanistically, metformin prevents stabilization of HIF-1 under hypoxic conditions in hepatocellular carcinoma (Zhou *et al.*, 2016). This reprogramming of HIF1-driven metabolism eventually prevents mitochondrial-dependent biosynthesis (Griss *et al.*, 2015).

Among more specific compounds developed over the last decades, BAY87-2243 is of special interest as it was identified from a screen for inhibitors of HIF-1α-mediated gene transcription under hypoxic conditions. This very potent compound was shown to inhibit MC1 and to induce mitochondrial depolarization and ATP depletion (Ellinghaus *et al.*, 2013). It also increased ROS production, leading to activation of AMP-activated protein kinase (AMPK) signaling and apoptosis (Schöckel *et al.*, 2015). Unfortunately, clinical development of BAY87-2243 was terminated at phase 1 due to massive and dose-independent induction of emesis in patients. IACS-010759 is a potent and selective MC1 inhibitor identified through a medicinal chemistry program building on the structures of HIF-1α modulators and known to inhibit oxidative phosphorylation. The agent showed some promise in the treatment of brain tumor and leukemia (Molina *et al.*, 2018), and a recent phase 1 study in 18 patients with advanced solid tumors showed that the compound was well-tolerated, with initial indications of anti-tumor activity (Yap *et al.*, 2019).

Nevertheless, the clinical potential of MC1 inhibition in cancer remains largely unexplored. Given the complex nature of the target and the limited number of specific agents available, the development of new potent and specific MC1 inhibitors based on different chemotypes is highly desirable.

MATERIAL & METHODS

Cell lines and reagents

Lewis lung carcinoma tumor cells, LL/2 (CRL-1642™), Hep3b.1-7 (HB-8064™), MDA-MB231 (HTB-26™), H460 (HTB-177™) and H1299 (CRL-5803™) cells were purchased from ATCC ® (Manassas, VA, USA), KARPAS 422 were from ECACC, purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). The NCI-H460-luc2 human lung cancer cell line was kindly provided by Dr Alain Le Pape (Research Director, Scientific Director of Centre For Small Animal Imaging, CIPA of CNRS Orleans Campus, Orléans, France). LL/2 and MDA-MB-231 were grown in DMEM, H460 and H1299 in RPMI-1640 and Hep3b in MEM, each supplemented with 10% Fetal Bovine Serum, 2 mM Glutamax, 1 mM sodium pyruvate and 1mM MEM NEAA (non-essential amino acids). ATCC-formulated DMEM, MEM-Earle's medium, RPMI1460, PBS, Hepes, sodium pyruvate, MEM NEAA trypsin, Glutamax and L-glutamine were from Gibco, fetal bovine serum (FBS), BSA, rotenone, antimycin A, iodoacetate, Carbonyl cyanide-p-trifluoromethoxy-phenylhydrazone (FCCP), sodium bicarbonate, D-glucose were from Sigma-Aldrich, Penicillin and streptomycin from Life Technologies, PBS-buffered formalin from Fisher Scientific, mouse standard diet from SAFE, Rompun®2% from Bayer Health Care, Imalgene®1000 from Merial, sodium pentobarbital from Ceva, Isoflurane® from Axience, NaCl 0.9% from Aguettant, Hexomedine® from Sanofi Aventis, Matrigel® from BD Biosciences, D-Luciferine salt from Molecular Probes, PEG200 from Alfa Aesar and Solutol®HS15 from BASF.

Mouse primary Eμ-*Myc* cells (murine malignant B-cells) obtained from B-cell lymphomas of independent C57BL/6 Eμ-*Myc* transgenic mice (line hosted at C3M), were isolated as described previously (Chiche *et al.*, 2015), maintained in DMEM supplemented with 10% FBS, 2-mercaptoethanol (50 μM), L-asparagin (0.37 mM) and HEPES (pH 7.4, 10 mM) and previously characterized as OxPhos or Glycolytic (Chiche *et al.*, 2019).

Anti-human Ki-67 (Rabbit IgG) was obtained from Abcam (all used 1/200X), anti-human carbonic anhydrase IX (Goat IgG) from R&D System (used 1/100X), anti-human Caspase-3 (Rabbit IgG) from Cell Signaling Technology (used 1/500X), anti-mouse CD31 (Rat IgG) from Biolegend (all used 1/500X); Anti-rabbit, anti-rat HQ, anti-goat HQ and anti-HQ-HRP, Ultra Map DAB kit, ChromoMap

DAB Kit, Omnimap anti-Rb HRP and haematoxylin were obtained from DISCOVERY, Roche Diagnostics (all used 1/500X).

In vitro inhibition of Complex 1

MitoTox™ Complex I OxPhos Activity Assay Kit (ab109903), designed for testing the direct inhibitory effect of compounds on Complex I, was used according to the supplier's instruction. The assay uses Complex I purified from bovine heart, immunocaptured by specific antibodies on the plate. Complex I activity is observed as decrease in absorbance at OD 340 nm, which denotes the oxidation of NADH by mitochondrial complex I.

Mitochondria respiration profiling

20,000 cells/well were seeded in VP3-PS cell culture microplate adapted to a XF96 analyzer (Seahorse Bioscience, Billerica, MA, USA), and allow to adhere overnight. Growth medium was exchanged with pre-warmed assay medium (XF base medium supplemented with 25 mM glucose, 2 mM glutamine and 1 mM sodium pyruvate; pH 7.4) and incubated at 37 °C for 1 h without CO_2 to allow to pre-equilibration in assay medium. Pre-warmed oligomycin, FCCP and rotenone plus antimycin A were loaded into injection ports A, B and C of the sensor cartridge, respectively, to give final concentrations of 1 µg/ml oligomycin, 1 µM FCCP, 1 µM rotenone & 1 µM antimycin A. For assessment, compound was loaded into the injector port A and oligomycin, FCCP and rotenone plus antimycin A into the ports B, C and D respectively. The cartridge was calibrated by the XF96 analyzer, and the assay performed with following parameters: 4 min mix, 3 min measure. Oxygen consumption rate (OCR) was detected under basal conditions followed by the sequential addition of 10X solutions containing the different doses of the compound to evaluate, followed by 3 sequential additions of FCCP at a final concentration of 0.3 µM, 1 µM and 3 µM to uncouple mitochondria. Control injections were with medium. Data are presented as the fold of the OCR of the given dose of compound to vehicle. Supplemental figure 4 shows a representative OCR curve of a Seahorse experiment with H460 cell line.

Quantification of total and mitochondrial ATP content

Total and mitochondrial ATP content was measured by using Cell Titer Glo assay (Promega) according to the manufacturer's protocol. Iodoacetate was used as specific glycolysis inhibitor to define origin of ATP and to isolate the effect of compounds on mitochondrial function, since it inhibits glycolysis by the alkylation of the essential cysteine residue in the active center of GAPDH, which results in impaired enzyme activity (Sabri and Ochs, 1971; Schmidt, 2009). Thus, compound effect is measured on the total ATP content and on the mitochondrial ATP content (measured in the presence of 100 µM iodoacetate). Cells were seeded at 8,000 cells/well in 80 µl of complete medium (96 well plates) and incubated at 37 °C (95% air; 5% CO_2) for 18 h. Compounds were added (10 µl of a 10X solution in culture media to obtain half-log dose response with final concentrations in 10 nM to 10 µM range) to pairs of plates and the cells were incubated for 1h in presence or not of iodoacetate (addition of 10µl of culture media or of 10 µl of a 10X iodoacetate solution). At the end of the incubation time, the ATP content was measured by using the Cell Titer Glo according to the manufacturer's protocol. The luminescence signal was recorded with a microplate reader (2103 EnVision™ Multilabel Plate Readers – PerkinElmer). Raw data (Relative Luminescence Units) were analyzed using Microsoft Excel 2016 software; values represent mean ± standard deviation. Mitochondrial ATP is determined with iodoacetate, global ATP without.

Proliferation & apoptosis measurement assays

Cells were seeded at 5,000 cells/well in 80 µl/well in culture media using 96-well plates. After an overnight incubation at 37 °C (95% air, 5% CO_2), 10 µL of 10X solutions of the compound were added to the appropriate wells (in culture media to obtain dose response with final concentrations in 10 nM to 10 µM range). For apoptosis determination, an additional 10 µL of Caspase-3/7 green reagent (Essen Bioscience, Catalog # 4440) diluted at 1/200 in corresponding media was added to the wells. Caspase-3/7 green reagent only emits fluorescence when it is cleaved by activated caspase-3/7, resulting in the release of the DNA dye and green fluorescent staining of nuclear DNA, which allows the monitoring of the kinetic activation of caspase-3/7. Plates were immediately placed in Incucyte live-cell, time-lapse imaging system allowing the visualization and quantification of cell proliferation and apoptosis

over time (Incucyte Zoom, Essen Bioscience Sartorius, Gottingen, Germany) for image acquisition. Two images were acquired under phase and green fluorescence (400 ms acquisition time by default) every 3 h, at 10X magnification for 96 h for the first experiment and then for 72 h for the three subsequent experiments. Assessment of cell confluence was based on phase images and apoptosis assessment was based on green fluorescence images. The Incucyte software (Incucyte ZOOM Version 2018A) was used to generate the kinetic graphs from the proliferation and apoptosis data. Changes in % of cell confluence for proliferation and of fluorescent counts for apoptosis were represented as % or fold to vehicle both for apoptosis and proliferation. ***Proliferation:*** Although there were changes in cell morphology as cells proliferate and confluence increases, we decided to use confluence as the measure of cell proliferation to assess the effect of the small molecules on cell proliferation. Cell proliferation was monitored by analyzing the occupied area (% confluence) of cell images over time. ***Apoptosis:*** Apoptosis was monitored using the caspase-3/7 green apoptosis assay reagent. When added to tissue culture medium, this inert, non-fluorescent reagent crosses the cell membrane where it is cleaved by activated caspase-3/7 that releases the DNA dye and that results green fluorescent staining of nuclear DNA. Apoptosis was determined by counting the number of fluorescence objects in each well and was expressed as cells/mm^2. The fluorescence threshold was set to 1 GCU, meaning that each object above this threshold were detected in the image.

***In vitro* cell death assay in the Eμ-*Myc* model**

Eμ-*Myc* cells (2x10^5) were seeded in 96 well-plates in the presence or absence of EVT-701 (at indicated doses) for 24 hours. Cells were then labeled with DAPI (Molecular Probes; 0.5 μg/mL) and analyzed immediately by flow cytometry using a MACSQuant Analyzer (Miltenyi Biotec). Cell death induction represents the percentage of DAPI positive cells in EVT-701-treated conditions divided by the percentage of DAPI positive cells in control (DMSO) conditions.

Animals

Female C57BL/6, male Balb/CJ-Nude and female CB17 SCID mice were purchased from Charles River Laboratories, France. The nycthemeral cycle in the housing room is 12-12-hour light/dark, room temperature was at 22±2 °C with 55±10% of relative humidity. The mice were fed *ad libitum* with standard diet and filtered tap water. The laboratory animal care program and the animal facility have been fully accredited by AAALAC organization. All the experimental procedures were approved by the local ethics committee of the company and were registered at the French Ministry of Higher Education and Research.

Orthotopic LL/2 lung tumor model

Cell implantation procedure*:** LL/2 cells were grown in a 5% CO_2 humidified atmosphere (90%) at 37°C, in DMEM medium supplemented with 3% FBS and L-glutamine 2 mM. The cells were trypsinized, washed with PBS and adjusted to a concentration of 50,000 cells/ml in Matrigel and PBS/BSA 0.1% (1:4; v:v). The cell suspension was kept at 4°C until inoculation into mice. 7-week-old female C57/Bl6 mice were anesthetized with a mixture of Rompun®2% (20 mg/kg) and Imalgene®1000 (100 mg/kg) in NaCl 0.9% at 10 mL/kg by intraperitoneal injection. A small skin incision to the left chest wall (<1 cm) was made at about 5mm tail side from the scapula. Subcutaneous fat and muscles were separated from costal bones. LL/2 cells (1,000 cells suspended in 20 µL) were directly injected through the intercostal space into the left lung. The area of injection was cleaned with Hexomedine®. The incision was closed with a surgical skin clip. At study termination, the mice were euthanized by barbiturate overdose (sodium Pentobarbital). The ribcage was opened and the left lung and the mediastinal lymph nodes were removed. Lung tumors were measured using an electronic caliper in order to determine the tumor volume (V) according to the formula: $V= l^2xLx0.52$ (l=width and L=length) and the mediastinal lymph nodes were weighed. Statistical analysis was performed using t-test for tumor volumes and lymph node weights. ***Treatment schedules: Mice were allocated to experimental groups based on body weight at the initiation of the treatment. At the end of the studies, mice without tumors were excluded from the statistical analysis. LL/2 tumor-bearing mice were dosed orally with either placebo (vehicle) or with EVT-701, 14 mice were allocated to each group. Mice received 14 (from D10

to D23 post cell inoculation) or 4 (from D19 to D22 post cell inoculation) consecutive single oral daily administrations with 10 and 30 mg/kg of compound according to the study design. EVT-701 was prepared daily as 1 and 3 mg/mL solutions in PEG200/Solutol®HS15/water (30%/5%/65%) and administered to mice at 10 mL/kg by oral gavage.

Orthotopic H460 lung tumor model

Cell implantation procedure*:** H460-Luc2 cells were grown in a 5% CO_2 humidified atmosphere (90%) at 37°C, in RPMI1640 medium supplemented with 10% FBS, D-Glucose (4.5 g/L), Glutamax 2mM, Sodium pyruvate 1X, sodium bicarbonate (2 mM) and Hepes buffer (10mM). The cells were trypsinized, washed with PBS and adjusted to a concentration of 20 million cells per ml in PBS. Cell suspension was kept at 4°C until inoculation into mice. 7-week-old male Balb/c Nude mice were anesthetized with a mixture of Rompun®2% (20 mg/kg) and Imalgene®1000 (100 mg/kg) in NaCl 0.9% at 10 mL/kg by intraperitoneal injection, and subsequently placed on an angular board in order to visualize the glottis using a light source, an otoscope and a modified spatula. The mice were inoculated with 25 µL of cell suspension using a S/L First PICC 26G (1.9F) blunt-end silicon catheter inserted into the bronchus through the mouth. Tumor progression was followed by bioluminescence imaging (BLI) at D7, D14 and D21 post cell inoculation. Animals were anesthetized with isoflurane® vaporized to 2.5% in O_2/Air = 1L/min. D-luciferin was applied by intraperitoneal injection (2 mg/mouse in 200 µL). Bioluminescence was measured 12 minutes after using an IVIS spectrum imager (PerkinElmer). At termination the ribcage was opened, the lungs were removed and tumors were measured as described for the LL/2 model. Statistical analysis was performed using t-test for tumor volumes and weights, and two-way repeated measures ANOVA for bioluminescence. ***Treatment schedule: Mice were allocated to experimental groups based on bioluminescent signal of the tumors at the initiation of the treatment (D7 post cell inoculation). H460-Luc2 tumor-bearing mice received 15 consecutive single daily oral administrations with 30 mg/kg of compound according to the study design. EVT-701 was prepared daily as 3 mg/mL solutions in PEG200/Solutol®HS15/water (30%/ 5%/ 65%) and administered to mice at 10 mL/kg by oral gavage (n=9 mice/group). Control mice received PEG200/Solutol HS15/water (30%/5%/65%) as vehicle (n=9 mice/group)

EVT-701 level assessment in plasma and tumor of LL/2 tumor-bearing mice

Blood samples were collected from the abdominal aorta and transferred into glass tubes containing lithium heparin. Immediately after sampling, the individual blood samples were centrifuged at 8000g for 10 min at 4°C. Plasma samples were frozen at - 20°C until analysis. LL/2 tumors were collected, pooled (3 mice per sampling time), weighed and frozen at -20°C until analysis. One gram of tissue being mixed with 2 mL of water for homogenization. After addition of the precipitant solution (CH_3CN), EVT-701 was quantified by LC-MS/MS. ***LC-MS/MS conditions:*** Analysis performed on Thermo Accela LC with HTC CTC-Pal autosampler. Column: Luna C18 Phenomenex (50 x 2.1 mm) 3µm; Elution conditions: Eluent A= H_2O + ammonium acetate 0.015% (w/v) + formic acid 0.2% (v/v); Eluent B= MeOH 20% (v/v) + ammonium acetate 0.015% (w/v) + formic acid 0.2% (v/v), CH_3CN to 100% volume. T= 25 °C. Gradient : 10% B for 0.5 min, to 90% in 1.5 min, 1 min 90% B, back to 10% in 0.5 min and 1.5 min 10% B; Flow rate= 0.7 ml/min; injection 5 µl, retention time 2.3 min. Analysis conditions: Thermo TSQ Quantum Ultra 4 mass spectrometer, ES+, cone tension 46 V, capillary T=350 °C, vaporizer T= 350 °C. *m/z* transition 630.03 => 270.05; Software : Excalibur version 2.1.0. ***Preparation of quality control and calibration samples:*** Quality Control samples and Calibration Samples were prepared daily by spiking mouse plasma with working solutions prepared from independent EVT-701 weightings. The calibration curve was calculated from calibration levels at 1 (n=3), 2.5, 5, 10, 25, 50, 100, 200 (n=3), 500, 1000 and 2000 (n=3) ng/mL. The concentrations for Quality Controls were 5, 50, 150, 450 and 1500 ng/mL. Analysis used quadratic regressions (not forced through the origin) weighted by 1/x. Limit of quantification (LOQ) was estimated to 2.5 ng/mL for the plasma and 10 ng/mL for the LL/2 tumors. The pharmacokinetic parameters were calculated from the arithmetic mean of the plasma concentrations or the LL/2 tumor concentrations using the program WinNonLin 5.2, non-compartmental model 200.

B-cell lymphoma *in vivo* studies

Lymphoma transfer of isolated Eμ-*Myc* clones was realized into syngeneic, non-transgenic, 6-week-old C57BL/6J Ola Hsd females (Envigo) by tail vein injection of 1×10^5 viable Eμ-*Myc* cells per recipient mouse (in 150 μL of sterile PBS). As the inguinal lymph nodes became "palpable" (six days after cell injection), the animals were treated daily with 30 mg/kg of EVT-701 (oral; 10 ml/kg) or vehicle. EVT-701 powder was dissolved daily in PEG200/Solutol HS15/water (30%/5%/65%). Control mice received PEG200/Solutol HS15/water (30%/5%/65%) as vehicle (n=10 mice/group). Food and water were given ad libitum. Mice weight and palpation of the inguinal lymph nodes were determined every two or three days. Eμ-*Myc* lymphoma-bearing animals sacrificed as soon as they presented several signs of illness. Upon sacrifice all lymph node tumors and spleens were collected and weighted. 2 hours after the last EVT-701 administration, mice were anesthetized with pentobarbital (75 mg/kg i.p). 500 μl of blood was collected for plasma isolation (lithium heparinate-coated tubes (BDMicrotainer®) were centrifuged at 6000 g for 1.5 min at room temperature, according to the manufacturer´s instructions) before mice sacrifice. Upon sacrifice all lymph node tumors and spleens are immediately collected and weighted.

Mice were maintained in specific pathogen-free conditions and experimental procedures were approved by the Institutional Animal Care and Use Committee and by the regional ethics committee (PEA232 from Comité Institutionnel d'Ethique Pour l'Animal de Laboratoire – AZUR).

Gene set enrichment analysis

The publicly accessible transcriptomic data from The Cancer Genome Atlas Lung Adenocarcinoma (TCGA-LUAD) and The Cancer Genome Atlas Lung Squamous Cell Carcinoma (TCGA-LUSC) cohorts were used in this study. GSEA was performed using DESEQ2 and fGSEA R packages. Gene sets were downloaded from GSEA website (https://www.gsea-msigdb.org/gsea/msigdb/). Volcano plots and enrichment maps were used for visualization of the GSEA results. For each gene signature, its Normalized Enrichment Score (NES) and False discovery rate (FDR) considering the p-adjusted values (padj) were evaluated. NES with FDR < 0.05 were considered significant.

Statistical analysis

Data are expressed as mean (± SD). Otherwise notified, these are means of three independent experiments, with 3 replicates per experiment. Differences in calculated means between groups were assessed by two-sided Student t tests. For time-to-event variables, the survival functions were estimated with Kaplan–Meier method and compared with log-rank tests. All statistical analyses were done with Prism software. A p-value of less than 0.05 was considered to indicate statistical significance (*p<0.05, **p<0.01 and ***p<0.001).

RESULTS

EVT-701 is a highly potent, selective and safe Mitochondrial Complex 1 inhibitor

EVT-701, 1-(3,4-dimethoxyphenyl)-2-[4-[2-[4-[2-(3,4-dimethoxyphenyl)-2-hydroxy-ethoxy]phenyl]-thiazol-4-yl]-phenoxy]ethanol (Fig. 1A) results from our efforts to identify novel, potent and selective compound degrading HIF-1α (Méneyrol *et al.*, 2016). We first investigated time- and dose-dependent effect of EVT-701 on isolated MC1 complex. EVT-701 reduces NADH abundance with an IC_{50} of 300 nM (Fig 1B). To assess the effect of EVT-701 on respiration of isolated mitochondria, we performed polarographic studies. EVT-701 dose-dependently inhibits NADH oxidation and phosphorylation of ADP into ATP by 80% at 7.5 µM (Fig. 1C). Next, we selected three different cancer cell lines in which metformin was reported to dose-dependently inhibit mitochondrial function and/or ATP production, namely MD-MB-231 (Gao *et al.*, 2016), Hep3B (Gao *et al.*, 2020) and U87 (Sesen *et al.*, 2015) cells. While MDA-MB-231 and U87 cells mainly produce ATP through glycolysis, Hep3B cells produce only 40% of their total ATP through glycolysis (Fig. 1C). Nevertheless, EVT-701 inhibited mitochondrial ATP production at nanomolar concentrations in all cell lines (IC_{50} of 52±18, 81±12 and 74±38 nM for MD-MB-231, Hep3B, and U87 respectively), regardless of their basal metabolism (Fig. 1E). Selectivity of EVT-701 was assessed on live Jurkat cells through a kinase selectivity panel from DiscoverX®, based on the KiNativ® In situ kinase profiling assay (Patricelli *et al.*, 2011). Among the 198 kinases evaluated in the panel, no kinase activity was inhibited by more than 30% and only MLK2 activity was increased by more than 30% by 10 µM EVT-701 (Fig. 1F), as determined by assessing the phosphorylation state of specific target peptides for each kinase. To evaluate developability of EVT-701, we performed further safety and ADME assays. The first of these was the i*n vitro* safety screen from Cerep®, based on the

ability of ET701 at 10 µM to compete for the binding of reference ligands to 300 receptors. Only one receptor (A2AR) showed ligand binding decreased by more than 50%, while increase greater than 50% was only seen with CCK1R and CCK2R (Supplemental Fig. 1A). While evaluating EVT-701 in mouse acute myeloid leukemia, we verified that its inhibitory effect on respiration was rescued by succinate (Luna Yolba, *in revision*). Pharmacokinetic parameters of EVT-701 were determined using C57BL/6J mice following intravenous administration of EVT-701 (3 mg/kg) and escalating oral dosing (10, 30 and 75 mg/kg). Blood was sampled over 48 h to obtain key PK variables (C_{max}, t_{max}, AUC, $t_{1/2}$, t_{last}, clearance, volume of distribution, and bioavailability). The plasma concentrations of EVT-701 over the 48 h time-course are shown in Supplemental Fig. S1B and PK parameters summarized in Supplemental Fig. 1C. Key features of the data were the extended $t_{1/2}$ (4.1 h), good bioavailability (64%) and the observation that dose-proportionality was conserved from 10 to 75 mg/kg. These measures are all supportive of further *in vivo* evaluation using oral route and a QD regimen. Tissue distribution was measured in the same strain of mice after single oral administration of 30 mg/kg, this showed accumulation of EVT-701 (compared to plasma) in all tissues except the brain (Supplemental Fig. 1D), making EVT-701 potentially suitable for treatment of several cancer types to the exception of CNS tumors. Specifically, tissue vs plasma AUC ratio was 5.9 for liver, 4.5 for kidney and pancreas, 4.2 for heart, 2.3 for lung and 0.25 for brain. In order to investigate the mechanism underlying this widespread distribution and address potential efflux issues, we evaluated interaction of EVT-701 with key transporters from ATP–binding cassette (ABC) and solute carrier (SLC) families, expressed either in CHO, HEK or MDCKII cells. The compound inhibited OATP1B1, OATP1B3 and BCRP-mediated transport with respective IC_{50}s of 1.1±0.1, 2.4±0.2 and 4.1±2.7 µM, whereas IC_{50}s for OAT1, OCT1 and MRP2 were higher than 30 µM and P-gp transport was not affected (Supplemental Fig. 1E). Collectively, these results are consistent with a massive uptake of EVT-701 in the liver, kidney, and intestine but could also provide a potent mechanism of elimination via kidney excretion. Finally, to examine to what extend safety issues reported for BAY87-2243 could be relevant for EVT-701, we tested the compounds in a ferret model of emesis at Porsolt. Ferrets were exposed to increasing doses of both compounds. For BAY87-2243, we chose as target concentration C_{max} reported in first patients treated with recommended starting dose in clinical phase 1; for EVT-701, we chose as target dose the estimated C_{max} obtained at steady state in patients to

be treated with predicted Dose-to-Man. This was assessed by allometric methods, using rat & mouse PK parameters and considering *in vitro* proliferation IC_{50} as minimum concentration at steady state. Dose escalation was eventually limited by toxicity for BAY87-2243 and by solubility for EVT-701. In conclusion, BAY87-2243 induced retches and vomits from half of the first patient C_{max}, whereas in similar conditions EVT-701 didn't, showing its safer profile.

EVT-701 reduces mitochondrial ATP production and the development of OxPhos but not of glycolytic NH B-cell lymphomas

Targeting mitochondrial metabolism of OxPhos-dependent NH B-cell lymphomas demonstrated a high potential as anti-cancer strategy (Ricci and Chiche, 2018). Accordingly, EVT-701 treatment dose-dependently inhibited mitochondrial ATP production with IC_{50} of 393±83 nM (Fig. 2A) and proliferation with IC_{50} of 188±50 nM (Fig. 2B) in the human OxPhos-DLBCL cell line KARPAS 422 (Caro *et al.*, 2012). This was accompanied by an increased glucose consumption and lactate release (Fig. 2C).

We previously established that murine primary *Myc*-driven B-cell lymphomas arising from the Eμ-*Myc* mice model (Adams *et al.*, 1985) display distinct metabolic profiles and energetic status (Chiche *et al.*, 2019). Moreover, we demonstrated that Eμ-*Myc* lymphomas relying on OxPhos for energy production are sensitive to specific inhibitors of mitochondrial metabolism such as phenformin, in *in vitro* and *in vivo* settings while glycolytic counterparts were resistant (Chiche *et al.*, 2019). *In vitro*, using five independent OxPhos-Eμ-*Myc* and five independent glycolytic-Eμ-*Myc* lymphoma cells, we show that OxPhos-Eμ-*Myc* cells are more sensitive to 10 μM EVT-701 than glycolytic-Eμ-*Myc* cells (Fig. 2D-E). To further test the specific anti-tumor efficacy of EVT-701 on B-cell lymphoma development, one OxPhos-Eμ-*Myc* (clone 506) and one glycolytic-Eμ-*Myc* (clone 504) lymphoma were transferred to syngeneic wild-type immunocompetent C57BL/6 mice. Six days after cell injection, mice were daily dosed with 30 mg/kg of EVT-701 (or Vehicle) given orally. EVT-701 treatment had no effect on the overall survival of glycolytic-Eμ-*Myc*-bearing mice (Fig. 2F) nor on the progression-free survival (data not shown). Accordingly, the weight of lymphomas from Vehicle or EVT-701-treated glycolytic-Eμ-*Myc*-bearing mice is similar (Fig. 2G). However, EVT-701 treatment significantly reduced the

progression of OxPhos-Eμ-*Myc*-lymphomas as shown by a significant increase in the overall survival (Fig. 2H) and in the progression-free survival (not shown) of those mice. Lymphomas harvested from EVT-701-treated OxPhos-Eμ-*Myc*-bearing mice were significantly smaller than those of Vehicle-treated OxPhos-Eμ-*Myc*-bearing mice (Fig. 2I). Altogether, our results demonstrated that EVT-701 holds potential as anti-cancer treatment in the context of highly aggressive type of NH B-cell lymphomas dependent on mitochondrial metabolism.

EVT-701 inhibits OxPhos metabolism and proliferation of non-small cell lung cancer (NSCLC) lines *in vitro* and *in vivo*

A previously published cohort study of over 7,000 patients with NSCLC identified that those receiving concomitant metformin treatment had improved outcomes, suggesting that the anti-metabolic drug could influence cancer progression (Chuang *et al.*, 2018). We therefore considered that NSCLC was a good candidate for further evaluation of the anti-tumor potential of EVT-701. We used a syngeneic mouse model of NSCLC involving orthotropic injection of LL/2 cells. This cell line was selected as the parent line - Lewis lung carcinoma (LLC) - is reportedly relevant for assessment of both antitumor and anti-metastatic effects (Doki *et al.*, 1999). LL/2 cells poorly rely on glycolysis (18%) to produce ATP (not shown) which suggests an important role of mitochondrial metabolism. *In vitro*, EVT-701 dose-dependently inhibited mitochondrial energetics in LL/2 cells, as shown by a reduction in ATP production with an IC_{50} of 512±109 nM (Fig. 3A) and proliferation with an apparent IC_{50} of 295±52 nM (Fig. 3B). *In vivo*, when used as a single agent, EVT-701 reduced tumor growth of LL/2 cells injected orthotopically into C57BL/6 mice left lung. Both doses of 10 and 30 mg/kg of EVT-701 significantly inhibited tumor growth (-57% and -69% volume respectively). PK performed during first day of treatment showed accumulation of EVT-701 in tumor compared to plasma, tumor/plasma AUC ratios being 2.9 and 2.5 for 10 and 30 mg/kg respectively (Fig. 3C-D). We then evaluated influence of time to treatment onset on metastasis in addition to tumor growth. LL/2 orthotopic tumors were treated with EVT-701 (30 mg/kg) either for 14 days from D10 (early onset) or for 4 days from D19 (late onset). The effect on primary tumor growth was similar (66% and 65% decrease of tumor volume respectively)

whereas early onset gave an enhanced benefit in terms of lymph node invasion (64 vs 34% decrease of lymph node weight – Fig. 3E-F).

Among NSCLC tumors, those carrying LBK1 mutations or with LKB1 downregulation (~20% of all NSCLC) have been reported as highly sensitive to metformin and phenformin (Shackelford *et al.*, 2013). We therefore used the NCI-H460 (hereafter H460) cell line as a model of LKB1-deficient NSCLC. *In vitro*, EVT-701 not only showed dose-dependent inhibition of mitochondrial ATP production (with an IC_{50} of 203±53 nM; Fig. 3G); OCR (Fig. 3H); and proliferation of H460 cells (with apparent IC_{50} of 68±27 nM; Fig. 3I), but also induced apoptosis with an IC_{50} of 96±28 nM (Fig. 3J) whereas no significant cell death was induced in LL/2 even at 10 µM.

We next assessed activity of BAY87-2243 in same model under the same conditions and identified a clear discrepancy between the effects of EVT-701 and BAY87-2243. While mitochondrial ATP production, proliferation and apoptosis were significantly inhibited by BAY87-2243 at doses less than or equal to 1 nM, we only detected an effect on OCR at doses above 100 nM (Supplemental Fig. 2A-C). These findings support the hypothesis that the undesirable emetic side-effects of BAY-2243 are due to off-target effect(s) rather than inhibition of oxidative metabolism.

To evaluate *in vivo* effect of EVT-701 in LKB1-deficient NSCLC, luciferase-expressing NCI-H460 cells, hereafter H460-luc2, (Raes *et al.*, 2016)) were orthotopically injected into Balb/CJ-Nude mice and treated daily for 14 days with EVT-701 30 mg/kg starting from D7 (Supplemental Fig. 3A). Mice were randomized based on bioluminescence imaging (BLI – Supplemental Fig. 3B). Unfortunately, BLI couldn't be used to monitor tumor growth until the end of the experiment as signal was partially lost after D14 (data not shown), presumably due to large the hypoxic and apoptotic zones appearing in vehicle-treated group tumors (Supplemental Fig. 4C shows CAIX, Ki67 and Caspase 3 staining in those tumors). Nevertheless, tumors could be harvested at D21, allowing us to determine that EVT-701 significantly decreased tumor volume by 60% and weight by 57% (Supplemental Fig. 3D-E).

LKB1 status is an important factor driving sensitivity of NSCLC cells to EVT-701

Metformin and Phenformin are reportedly highly active on LKB1 mutant NSCLC models. This is potentially linked to the inability of LKB1 mutated cells to modulate AMPK signaling and adapt to

metabolic stress. According to this view, LKB1 tumor cells continue uncontrolled growth despite the inhibition of OxPhos, leading to rapid depletion of energy resources and eventually triggering apoptosis (Shackelford *et al.*, 2013; Momcilovic and Shackelford, 2015). To evaluate LKB1 proficiency/deficiency effect on EVT-701 activity, we established LKB1-overexpressing H460 cell line (H460-LKB1) as compared to wild type H460. Both cell lines were relying more on glycolysis to produce ATP (59% and 75% respectively - not shown). In H460-LKB1, similar to the effect observed with wild type H460, EVT-701 dose-dependently inhibited mitochondrial ATP production with IC_{50} of 232±50 nM (Fig. 4A); however, we saw no induction of apoptosis even at 10 µM in the LKB1 proficient cells oppositely to LKB1 deficient cells (Fig. 4B). In addition, we could monitor that the phosphorylation cascade downstream of AMPK is activated in EVT-701-treated H460-LKB1 cells, as shown by an increased phosphorylation state of 4E-BP1 on Serine 65, Raptor and Acetyl CoA carboxylase (ACC) within minutes after exposure to EVT-701, whereas parental H460 couldn't (Fig. 4C). A growing body of evidence is accumulating of increased TCA cycle activity, mitochondrial membrane potential and mitochondrial respiration (Faubert *et al.*, 2014; Kaufman *et al.*, 2014; Whang *et al.*, 2016) in LKB1-deficient cancer cells to support their proliferation and survival (Zhang *et al.*, 2021). Borzi and colleagues proposed that even NSCLC showing low expression of wild-type LKB1 could undergo this metabolic evolution and may accordingly be more sensitive to antimetabolic drugs (Borzi *et al.*, 2020). We therefore interrogated the TCGA database to see if there was evidence to support this metabolic evolution model in larger NSCLC patient cohorts. We addressed lung adenocarcinoma and squamous cell carcinoma cohorts (LUAD and LUSC respectively) separately. Within each cohort, patients were divided into *LKB1* high and *LKB1* low sub-cohorts based on median *LKB1* mRNA expression levels. We performed gene set enrichment analysis (GSEA) in *LKB1* low versus *LKB1* high sub-cohorts and observed that mitochondrial metabolism related pathways are enriched in *LKB1* low group (Fig. 4D-E), in alignment with the conclusions reached by Kaufman and Borzi. Altogether, those results support use of EVT-701 in LKB1 deficient and LKB1-low NSCLC.

DISCUSSION

We present here the preclinical characterization of EVT-701, a structurally novel mitochondrial complex 1 inhibitor. The results demonstrate that EVT-701 is a highly potent and selective MC1 inhibitor in a range of biochemical and cell-based assays. Significantly the compound is free of the side effects that interrupted the clinical development of BAY87-2243. While first-in-human trial results with IACS-010759 were reassuring with regard to toxicity, it shares the BAY87-2243 backbone and so uncertainty about its safety remains. EVT-701 is the product of a discovery program aimed at finding HIF-1α-destabilizers with new scaffold to circumvent the aforementioned side effects.

Pointing to the therapeutic potential of EVT-701 for solid tumor treatment, proliferation of non-small cell lung cancer (NSCLC) and diffuse large B-cell lymphoma (DLBCL) cell lines *in vitro* and tumor growth in syngeneic and xenograft NSCLC and NH B-cell lymphoma models were potently inhibited by EVT-701. Mice bearing NH B-cell lymphoma tumors originating from high OxPhos clones showed prolonged survival after EVT-701 treatment. Functional studies showed that reduction of tumor cell growth was correlated with inhibition of NADH oxidation, oxygen consumption rate and mitochondrial ATP formation, paralleled with increase in glucose consumption and lactate production, altogether concurring with MC1 inhibition. Using and growth of syngeneic and xenograft NSCLC and NH B-cell lymphoma models we demonstrated that EVT-701 hold potential as anti-cancer agent dedicated to OxPhos-dependent tumors since it prolonged survival of OxPhos-Eμ-*Myc* lymphoma-bearing mice, but not for glycolytic-Eμ-*Myc*-lymphoma-bearing mice.

It became clear over the last decades that despite their glucose addiction, tumors still largely use oxidative phosphorylation to support survival and growth.

Liver kinase B1 (LKB1), also known as serine/threonine kinase (STK11) has been characterized as a tumor suppressor gene. In cancer setting, LKB1 suppressed malignant cell transformation and drastically decreased tumor progression (Korsse *et al.*, 2013). Mutation in LKB1 has been identified as a possible causative factor for Peutz-Jeghers syndrome (Jenne *et al.*, 1998), a genetic disorder where patients show an increased risk of cancer (Giardiello *et al.*, 1987).This has been evidenced for pancreatic and non-small cell lung cancers, where heterozygous LKB1 cooperates with KRAS mutation to the

development and progression of tumors (Morton *et al.*, 2010; Sanchez-Vega *et al.*, 2018; Galan-Cobo *et al.*, 2019; Hollstein *et al.*, 2019). LKB1 activates members of AMP kinase family (AMPK) which in turn, phosphorylate effectors to repress anabolic and stimulate catabolic processes, thereby re-establishing energy homeostasis to overcome energetic stress. AMPK represses lipogenesis by inhibiting sterol regulatory element binding transcription factor 1c (SREBP1c) and acetyl-coenzyme A (CoA) carboxylase (ACC) and impairs cholesterol synthesis by inactivating hydroxymethylglutaryl-CoA reductase (Munday *et al.*, 1988; Li *et al.*, 2011; Vara-Ciruelos *et al.*, 2019), while preventing oxidative stress secondary to defective mitochondria accumulation via ULK1-mediated mitophagy (Egan *et al.*, 2010). The idea of inducing a "metabolic catastrophe" to attack LKB1-deficient cancers has been tested with metformin (Moro *et al.*, 2018), phenformin (Shackelford *et al.*, 2013) and BAY87-2243 (Ellinghaus *et al.*, 2013). However, the poly-pharmacology of these compounds makes it hard to draw firm conclusions about how beneficial different aspects of metabolic catastrophe can be exploited to fight cancer. For example, LKB1-independent AMPK activation has been reported for metformin (Guo *et al.*, 2016), which could offer more flexibility to cancer cells to circumvent MC1 inhibition-mediated metabolic catastrophe. In this study, we show that EVT-701 did not modulate AMPK signaling in LKB1-deficient H460 cells and could inhibit tumor growth as single agent *in vivo*. Our findings therefore point to EVT-701 as a valuable tool to explore the metabolic catastrophe approach, particularly in combination with other therapeutic approaches. We reported partial activation of MLK2 in kinase screen. This kinase is part of the first subgroup of Mixed Lineage Kinase family of serine/threonine protein kinases (MLK1-MLK4, sharing high sequence identity) that regulate signaling via JNK and MAPKs (Kashuba *et al.*, 2011). MLKs role in disease has essentially been described in neurodegeneration, and potential role of MLK2 in promotion of proliferation and decrease of sensitivity to gemcitabine of pancreatic cancer was also reported (An *et al.*, 2013). Nevertheless, we do not expect this to be relevant for EVT-701 mechanism of action, since the circulating EVT-701 doses would need to be sustainably higher than 10 µM to potentially modulate MLK2, which was not the case in *in vivo* studies presented here. EVT-701-mediated HIF-1α degradation could be both MC1-dependent and independent. Indeed, it's been reported that Manassantin A, the natural compound from which EVT-701 was derived from (Méneyrol *et al.*, 2016), directly interacts with filamin A, preventing its hypoxia-

induced cleavage by calpain, and stabilizing its interaction with HIF-1α, eventually preventing its nuclear translocation and favoring its degradation (Geer Wallace *et al.*, 2016).

In summary, EVT-701 is a novel, highly potent and selective MC1 inhibitor with original chemical scaffold. Preclinical validation has shown *in vitro* and *in vivo* efficacy on models of NSCLC and NH B-cell lymphomas. EVT-701 is well distributed in mouse and accumulated in several tissues compared to blood, which supports its use in a range of indications. Importantly, EVT-701 presents a safer profile than previous compounds specifically designed to inhibit MC1 and to induce HIF-1α degradation. However, EVT-701 shows dose-dependent interaction with efflux pumps, which impairs its accumulation overtime when dosed chronically in QD regimen. Short-term treatments of tumors presenting *de novo* or acquired sensitivity to inhibition of respiration, and therefore vulnerable to induced metabolic catastrophe, would be preferred options. LKB1 deficiency, through mutation or low expression levels, increases sensitivity of NSCLC to EVT-701, probably due to the inability of such tumor cells to manage metabolic stress. OxPhos Non-Hodgkin's B-cell lymphomas, expressing low levels of GAPDH, are also highly susceptible to EVT-701. Patients with tumors of the aforementioned types may benefit from EVT-701 treatment. Another approach would be to combine with therapies that induce metabolic switch towards oxidative phosphorylation and so susceptibility to MC1 inhibition. EGFR inhibitor-resistant NSCLC could be a potential indication (De Rosa *et al.*, 2015) as well as acute myeloid leukemia treated with cytarabine-containing regimens (Farge *et al.*, 2017). Altogether, these results support further investigations of EVT-701 in additional preclinical models and, eventually, in patients.

AUTHORSHIP CONTRIBUTION

Participated in research design: Visentin, Hervé, Chiche, Ricci, Paillasse and Alet.

Conducted experiments: Luna Yolba, Visentin, Hervé, Chiche and Alet.

Contributed new reagents and analytic tools: Méneyrol.

Performed data analysis: Luna Yolba, Visentin, Hervé, Chiche, Ricci, Paillasse and Alet.

Wrote or contributed to the writing of the manuscript: Luna Yolba and Paillasse.

DISCLOSURE OF CONFLICTS OF INTEREST

Luna Yolba, Visentin, Hervé, Méneyrol, Paillasse and Alet and full time employees of Evotec.

Chiche and Ricci declare no financial conflict of interest.

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Footnotes

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FIGURE LEGENDS

Figure 1. EVT-701 is a MC1 inhibitor.

(A) Chemical structure of EVT-701. **(B)** Dose- and time-dependent inhibition of ETC I by EVT-701, using the MitoToxTM complex 1 kit. MC1 activity is monitored by following the decrease in absorbance at OD 340 nm due to the oxidation of NADH, which correlates with reduction of ubiquinone (NADH + H^+ + ubiquinone → NAD^+ + ubiquinol). **(C)** Plot of dose-dependent modulation of oxidation and phosphorylation rates by EVT-701, measured by polarographic experiment using isolated mitochondria isolated from liver of OF1 – female from Charles Rivers. **(D)** Glycolytic ATP percentage in Hep3B (OxPhos high), MDA-MB231 and U87MG (OxPhos low) cell lines. **(E)** Dose-dependent inhibition of mitochondrial ATP in Hep3b, MDA-MB231 and U87 cell lines by EVT-701. **(F)** Selectivity kinase DiscoverX panel performed on Jurkat live cells using 10 µM EVT-701, by assessing the phosphorylation state of specific substrate peptides for each kinase. DiscoverX panel has been run once, in duplicate and compared to vehicle treatment tested in duplicate or quadruplicate.

Figure 2. EVT-701 is active *in vitro* and *in vivo* in NH B-cell lymphoma models.

(A) Dose dependent inhibition of mitochondrial ATP by EVT-701 in KARPAS 422 cell line, with an IC_{50} of 394 nM. **(B)** Dose-dependent inhibition of KARPAS 422 proliferation by EVT-701, with an IC_{50} of 188 nM. **(C)** Dose-dependent modulation of glucose and lactate levels in KARPAS 422 by EVT-701. The compound induces a significant increase in glucose consumption and lactate secretion. **(D)** OxPhos-Eµ-*Myc* (lymphomas 249, 506, 549, 585, 508) and Glycolytic-Eµ-*Myc* (lymphomas 504, 251, 115, 136, 337) cells were seeded in the presence (EVT-701) or absence (DMSO) of indicated concentration of EVT-701 for 24 hours. Cell death was determined by DAPI staining and analyzed by FACS. Cell death induction represents the percentage of DAPI positive cells in EVT-701-treated conditions divided by the percentage of DAPI positive cells in control (DMSO) condition. **(E)** Summary as the mean of 4 independent experiments (n=5 independent clones per group) is presented. **(F)** Kaplan–Meier curve of overall survival of syngeneic C57BL/6 mice intravenously injected with glycolytic-Eµ-*Myc* lymphoma cells and treated with 30 mg/kg (p.o, QD) or vehicle until ethical endpoint was achieved. 10 mice per

group were used. **(G)** Inguinal, submaxillary, axillary lymphomas and spleen weights upon sacrifice of glycolytic-Eμ-*Myc*-bearing mice. **(H)** Kaplan–Meier curves of overall survival of syngeneic C57BL/6 mice intravenously injected with OxPhos-Eμ-*Myc* lymphoma cells and treated with 30 mg/kg (p.o, QD) or vehicle until ethical endpoint was achieved. 10 mice per group were used. **(I)** Inguinal, submaxillary, axillary lymphomas and spleen weights upon sacrifice of OxPhos-Eμ-*Myc*-bearing mice. *In vivo* data represent an average of two independent experiments.

Figure 3. *In vitro* and *in vivo* EVT-701 activity in lung cancer.

EVT-701 decreases OxPhos and proliferation *in vitro* in several lung cancer cell lines, and shows efficacy *in vivo* in LL/2 orthotopic model. **(A)** Dose dependent inhibition of mitochondrial ATP in LL/2 by EVT-701, with IC_{50} of 512 nM while in same conditions global ATP remains unchanged whatever the dose of EVT-701. **(B)** Dose-dependent inhibition of LL/2 proliferation by EVT-701. **(C)** EVT-701 efficacy in LL/2 *in vivo* orthotopic model. Comparison of efficacy of 10 and 30 mg/kg EVT-701QD dosing for 14 days. Lower dose inhibited tumor growth by 57% while 30 mg/kg inhibited by 69% and the difference between the effects of the two doses was not statistically significant. **(D)** Evaluation of EVT-701 blood exposure after single administration of 10 or 30 mk/kg. Exposures and exposure ratio (AUC) were consistent with pharmacodynamics studies shown in supplementary figure 1. **(E)** EVT-701 4-day treatment reduced tumor growth by -65% (right) and lymph node weight by -34% (left). **(F)** EVT-701 14-day treatment reduced tumor growth by -66% (right) and lymph node weight by -76% (left). **(G)** Dose dependent inhibition of mitochondrial ATP in H460 cell line by EVT-701, with IC_{50} of 203 nM. Under the same conditions, global ATP remains unchanged at all doses of EVT-701. **(H)** Dose-dependent reduction of oxygen consumption in H460 cells by EVT-701 measured by Seahorse. **(I)** EVT-701 dose-dependent inhibition of proliferation in H460. **(J)** EVT-701 dose-dependent apoptosis induction in H460 cells. *In vivo* data represent an average of two independent experiments.

Figure 4. LKB1 expression as stratification marker.

Exploring possible target patient populations responsive to EVT-701 treatment. The importance of LKB1 expression status on sensitivity to EVT-701. **(A)** Dose dependent inhibition of mitochondrial ATP in LKB1-proficient H460 cells, with IC_{50} of 230 nM. Under the same conditions, total ATP remains unchanged whatever the dose of EVT-701. **(B)** Comparison of the dose-dependent induction of apoptosis by EVT-701 between LKB1-proficient H460 and WT H460 cells. Absence of LKB1 expression sensitizes H460 cells to EVT-701 induced death. **(C)** Modulation of mTOR/AMPK pathway signaling by EVT-701 in LKB1-proficient H460 (left) or H460 WT (right) cells over 60 minutes. In LKB1-proficient H460 cells (left), 4E-BP1 and Raptor phosphorylation is increased by EVT-701 treatment whereas ACC phosphorylation is inhibited. However, in WT H460 cells (right) 4E-BP1, Raptor and ACC phosphorylation are unaffected by EVT-701 at any time point. **(D)** Left: volcano plot showing the signatures enriched in the low vs high LKB1 expression group in The Cancer Genome Atlas Lung Adenocarcinoma (TCGA-LUAD) cohort. The enriched OxPhos and mitochondrial gene sets are highlighted in black. Right: a plot showing differentially expressed gene sets in TCGA-LUAD low LKB1 expression group (FDR< 0.05) highlighted in the volcano plot with their normalized enrichment score (NES). **(E)** Left: volcano plot showing the signatures enriched in the low vs high LKB1 expression group in The Cancer Genome Atlas Lung Squamous Cell Carcinoma (TCGA-LUSC) cohort. The enriched OxPhos and mitochondrial gene sets are highlighted in black. Right: a plot showing differentially expressed gene sets in TCGA-LUSC low LKB1 expression group (FDR< 0.05) highlighted in the volcano plot with their normalized enrichment score (NES).

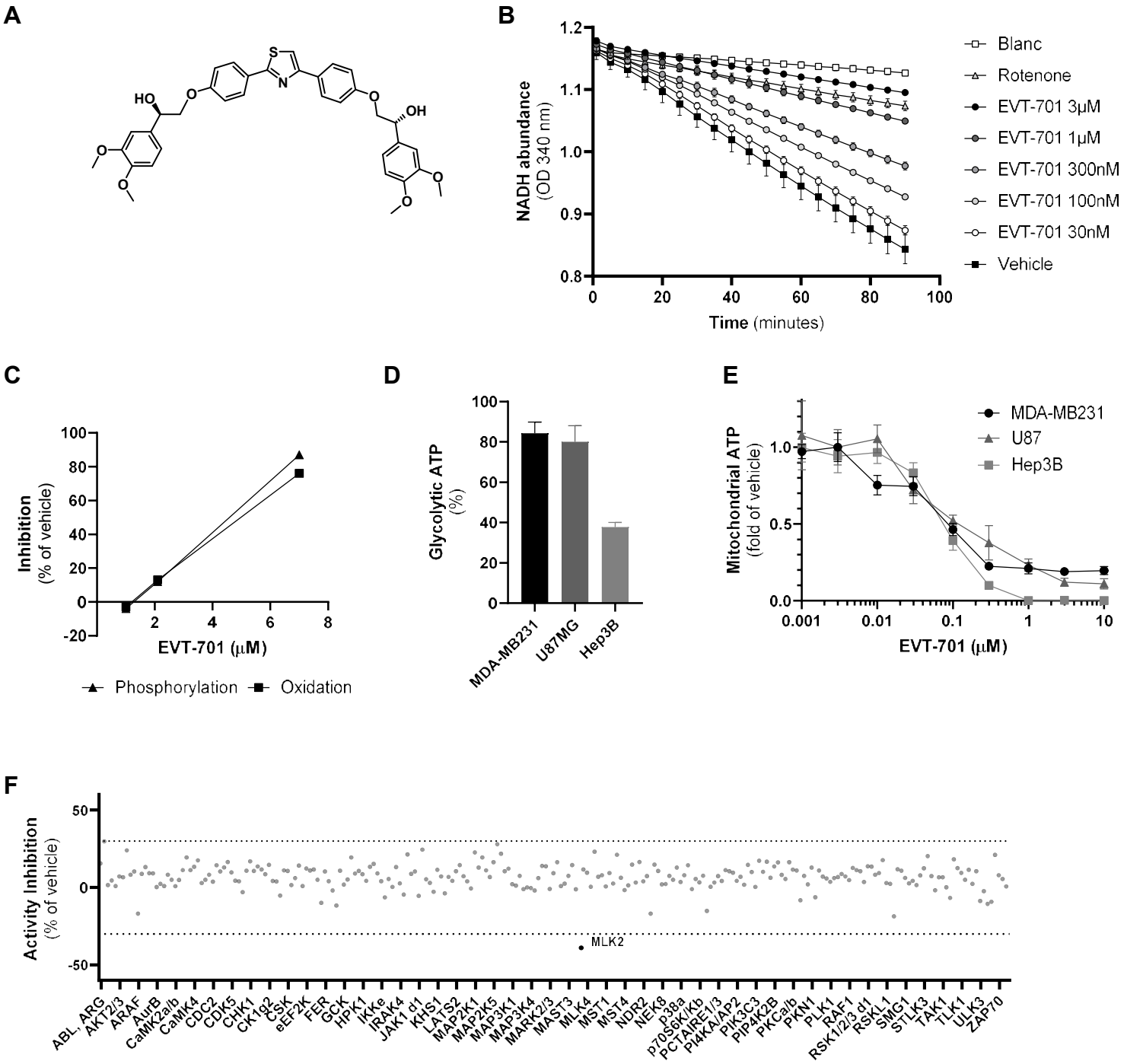

A
B
NADH abundance (OD 340 nm)
Time (minutes)
Blanc
Rotenone
EVT-701 3µM
EVT-701 1µM
EVT-701 300nM
EVT-701 100nM
EVT-701 30nM
Vehicle
C
Inhibition (% of vehicle)
EVT-701 (µM)
Phosphorylation
Oxidation
D
Glycolytic ATP (%)
MDA-MB231
U87MG
Hep3B
E
Mitochondrial ATP (fold of vehicle)
EVT-701 (µM)
MDA-MB231
U87
Hep3B
F
Activity Inhibition (% of vehicle)
MLK2

Figure 1

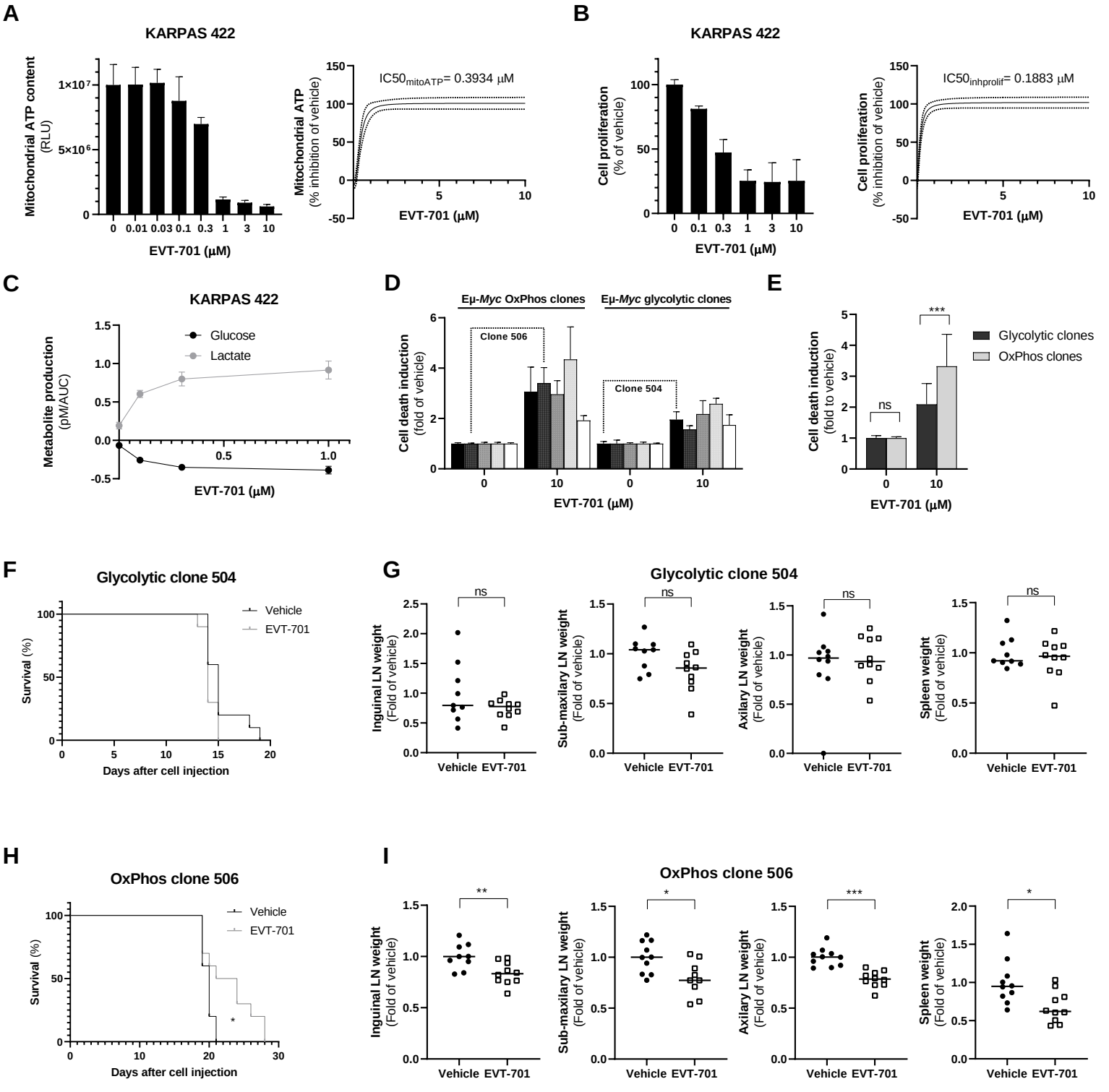

A
KARPAS 422
Mitochondrial ATP content (RLU)
EVT-701 (μM)
Mitochondrial ATP (% inhibition of vehicle)
IC50mitoATP= 0.3934 μM
B
KARPAS 422
Cell proliferation (% of vehicle)
IC50inhprolif= 0.1883 μM
C
KARPAS 422
Glucose
Lactate
Metabolite production (pM/AUC)
D
Eμ-Myc OxPhos clones
Eμ-Myc glycolytic clones
Clone 506
Clone 504
Cell death induction (fold of vehicle)
E
Glycolytic clones
OxPhos clones
Cell death induction (fold to vehicle)
ns
F
Glycolytic clone 504
Vehicle
EVT-701
Survival (%)
Days after cell injection
G
Glycolytic clone 504
Inguinal LN weight (Fold of vehicle)
Sub-maxillary LN weight (Fold of vehicle)
Axillary LN weight (Fold of vehicle)
Spleen weight (Fold of vehicle)
H
OxPhos clone 506
I
OxPhos clone 506

Figure 2

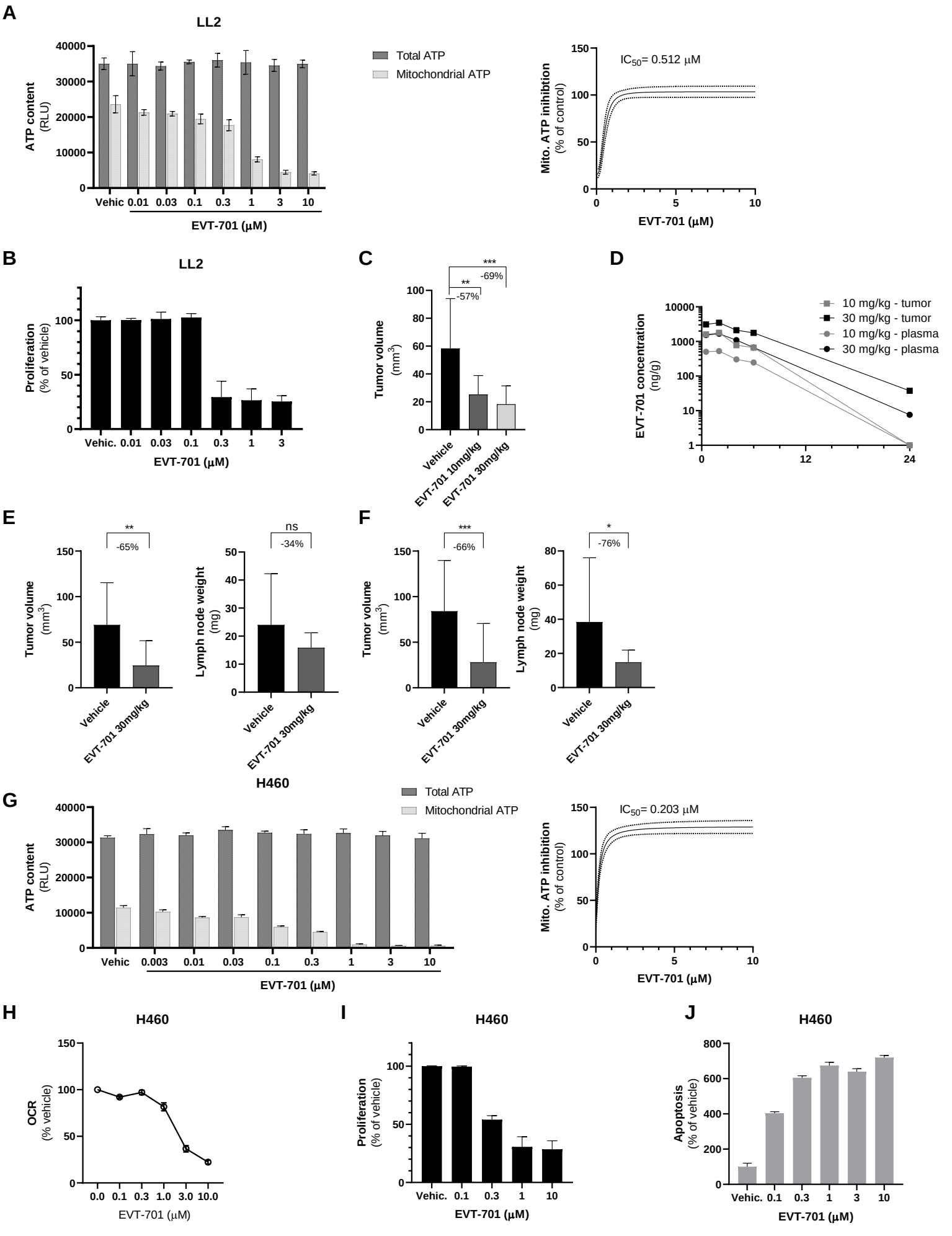

A
LL2
ATP content (RLU)
Total ATP
Mitochondrial ATP
Vehic 0.01 0.03 0.1 0.3 1 3 10
EVT-701 (μM)
Mito. ATP inhibition (% of control)
IC50= 0.512 μM
EVT-701 (μM)
B
LL2
Proliferation (% of vehicle)
Vehic. 0.01 0.03 0.1 0.3 1 3
EVT-701 (μM)
C
Tumor volume (mm3)
-57%
-69%
Vehicle
EVT-701 10mg/kg
EVT-701 30mg/kg
D
EVT-701 concentration (ng/g)
10 mg/kg - tumor
30 mg/kg - tumor
10 mg/kg - plasma
30 mg/kg - plasma
E
Tumor volume (mm3)
-65%
Lymph node weight (mg)
ns
-34%
Vehicle
EVT-701 30mg/kg
F
Tumor volume (mm3)
-66%
Lymph node weight (mg)
-76%
Vehicle
EVT-701 30mg/kg
G
H460
ATP content (RLU)
Total ATP
Mitochondrial ATP
Vehic 0.003 0.01 0.03 0.1 0.3 1 3 10
EVT-701 (μM)
Mito. ATP inhibition (% of control)
IC50= 0.203 μM
EVT-701 (μM)
H
H460
OCR (% vehicle)
0.0 0.1 0.3 1.0 3.0 10.0
EVT-701 (μM)
I
H460
Proliferation (% of vehicle)
Vehic. 0.1 0.3 1 10
EVT-701 (μM)
J
H460
Apoptosis (% of vehicle)
Vehic. 0.1 0.3 1 3 10
EVT-701 (μM)

Figure 3

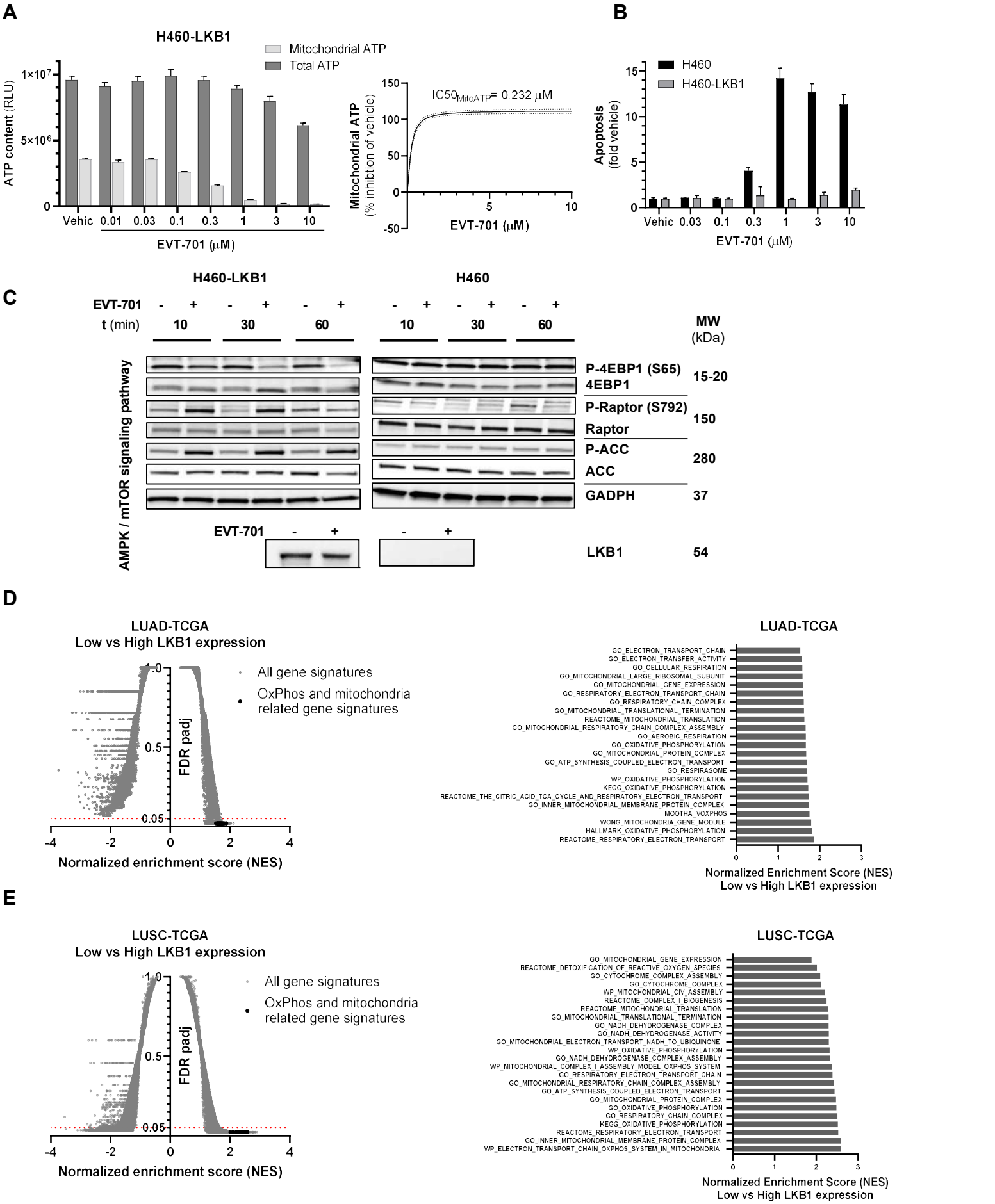

A
H460-LKB1
Mitochondrial ATP
Total ATP
ATP content (RLU)
1×10^7
5×10^6
0
Vehic 0.01 0.03 0.1 0.3 1 3 10
EVT-701 (μM)
Mitochondrial ATP (% inhibition of vehicle)
IC50MitoATP= 0.232 μM
150
100
50
0
-50
5
10
EVT-701 (μM)
B
H460
H460-LKB1
Apoptosis (fold vehicle)
15
10
5
0
Vehic 0.03 0.1 0.3 1 3 10
EVT-701 (μM)
C
H460-LKB1
H460
EVT-701 - + - + - + - + - + - +
t (min) 10 30 60 10 30 60
MW (kDa)
AMPK / mTOR signaling pathway
P-4EBP1 (S65)
4EBP1
15-20
P-Raptor (S792)
Raptor
150
P-ACC
ACC
280
GADPH
37
EVT-701 - + - +
LKB1
54
D
LUAD-TCGA
Low vs High LKB1 expression
All gene signatures
OxPhos and mitochondria related gene signatures
FDR padj
1.0
0.5
0.05
-4 -2 0 2 4
Normalized enrichment score (NES)
LUAD-TCGA
GO_ELECTRON_TRANSPORT_CHAIN
GO_ELECTRON_TRANSFER_ACTIVITY
GO_CELLULAR_RESPIRATION
GO_MITOCHONDRIAL_LARGE_RIBOSOMAL_SUBUNIT
GO_MITOCHONDRIAL_GENE_EXPRESSION
GO_RESPIRATORY_ELECTRON_TRANSPORT_CHAIN
GO_RESPIRATORY_CHAIN_COMPLEX
GO_MITOCHONDRIAL_TRANSLATIONAL_TERMINATION
REACTOME_MITOCHONDRIAL_TRANSLATION
GO_MITOCHONDRIAL_RESPIRATORY_CHAIN_COMPLEX_ASSEMBLY
GO_AEROBIC_RESPIRATION
GO_OXIDATIVE_PHOSPHORYLATION
GO_MITOCHONDRIAL_PROTEIN_COMPLEX
GO_ATP_SYNTHESIS_COUPLED_ELECTRON_TRANSPORT
GO_RESPIRASOME
WP_OXIDATIVE_PHOSPHORYLATION
KEGG_OXIDATIVE_PHOSPHORYLATION
REACTOME_THE_CITRIC_ACID_TCA_CYCLE_AND_RESPIRATORY_ELECTRON_TRANSPORT
GO_INNER_MITOCHONDRIAL_MEMBRANE_PROTEIN_COMPLEX
MOOTHA_VOXPHOS
WONG_MITOCHONDRIA_GENE_MODULE
HALLMARK_OXIDATIVE_PHOSPHORYLATION
REACTOME_RESPIRATORY_ELECTRON_TRANSPORT
0 1 2 3
Normalized Enrichment Score (NES)
Low vs High LKB1 expression
E
LUSC-TCGA
Low vs High LKB1 expression
All gene signatures
OxPhos and mitochondria related gene signatures
FDR padj
1.0
0.5
0.05
-4 -2 0 2 4
Normalized enrichment score (NES)
LUSC-TCGA
GO_MITOCHONDRIAL_GENE_EXPRESSION
REACTOME_DETOXIFICATION_OF_REACTIVE_OXYGEN_SPECIES
GO_CYTOCHROME_COMPLEX_ASSEMBLY
GO_CYTOCHROME_COMPLEX
WP_MITOCHONDRIAL_CIV_ASSEMBLY
REACTOME_COMPLEX_I_BIOGENESIS
REACTOME_MITOCHONDRIAL_TRANSLATION
GO_MITOCHONDRIAL_TRANSLATIONAL_TERMINATION
GO_NADH_DEHYDROGENASE_COMPLEX
GO_NADH_DEHYDROGENASE_ACTIVITY
GO_MITOCHONDRIAL_ELECTRON_TRANSPORT_NADH_TO_UBIQUINONE
WP_OXIDATIVE_PHOSPHORYLATION
GO_NADH_DEHYDROGENASE_COMPLEX_ASSEMBLY
WP_MITOCHONDRIAL_COMPLEX_I_ASSEMBLY_MODEL_OXPHOS_SYSTEM
GO_RESPIRATORY_ELECTRON_TRANSPORT_CHAIN
GO_MITOCHONDRIAL_RESPIRATORY_CHAIN_COMPLEX_ASSEMBLY
GO_ATP_SYNTHESIS_COUPLED_ELECTRON_TRANSPORT
GO_MITOCHONDRIAL_PROTEIN_COMPLEX
GO_OXIDATIVE_PHOSPHORYLATION
GO_RESPIRATORY_CHAIN_COMPLEX
KEGG_OXIDATIVE_PHOSPHORYLATION
REACTOME_RESPIRATORY_ELECTRON_TRANSPORT
GO_INNER_MITOCHONDRIAL_MEMBRANE_PROTEIN_COMPLEX
WP_ELECTRON_TRANSPORT_CHAIN_OXPHOS_SYSTEM_IN_MITOCHONDRIA
0 1 2 3
Normalized Enrichment Score (NES)
Low vs High LKB1 expression

Figure 4

Supplementary data: EVT-701 is a novel selective and safe mitochondrial complex 1 inhibitor with potent anti-tumor activity in models of solid cancers

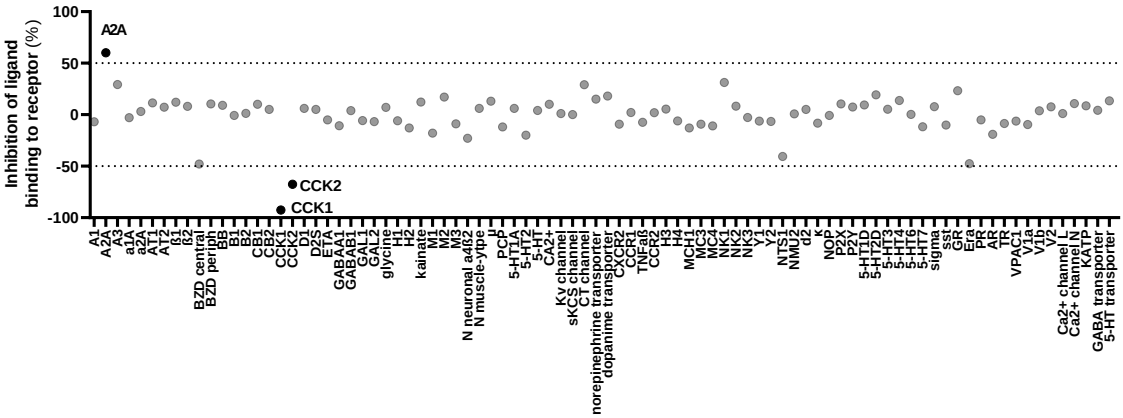
Raquel Luna Yolba[1], Virgile Visentin[1], Caroline Hervé, Johanna Chiche, Jean-Ehrland Ricci, Jérôme Méneyrol, Michaël R. Paillasse & Nathalie Alet

[1] Authors contributed equally to the work as first authors

Supplemental Figures & legends

Supplemental Figure 1. Pharmacology, pharmacokinetics and pharmacodynamics of EVT-701. **(A)** *In vitro* safety screen from Cerep based on competitive binding of 10 µM EVT-701 with reference ligands. Only A_2AR ligand binding was inhibited by more than 50% while only binding to CCK1R & CCK2R were increased by more than 50%. **(B)** Pharmacokinetics study of EVT-701 plasma concentration in C57BL/6J mice. Concentrations of compound, after oral administration of 10, 30 or 75 mg/kg is plotted over time. **(C)** Table summarizing C_{max}, t_{max}, AUC, $t_{1/2}$ and t_{last} calculated from the study shown in B. Bioavailability (F(%)) of EVT-701 was determined by comparing AUC of the 10 mg/kg oral dose vs AUC of the 1 mg/kg intravenous dose. Dose proportionality was maintained up to 75 mg/kg dose. **(D)** Tissue distribution of EVT-701was evaluated in C57BL/6J male mice over 24 h after oral administration of 30 mg/kg solution. The compound showed marked accumulation in liver while brain penetration was limited. **(E)** Evaluation of dose-dependent *in vitro* transporter inhibition. MRP2 and P-gp were expressed in HEK and MDCKII respectively. All the other transporters were expressed in CHO cells. EVT-701 inhibited OATP1B1, OATP1B3 and BCRP with respective IC_{50}s of 1.1, 2.4 and 4.1 µM. **(G)** Evaluation of induction of emesis in ferret by EVT-701 and BAY87-2243. *In vivo* data represent an average of two independent experiments. Safety screen has been run once, in duplicate, and compared to activity of reference ligands tested in duplicate.

A



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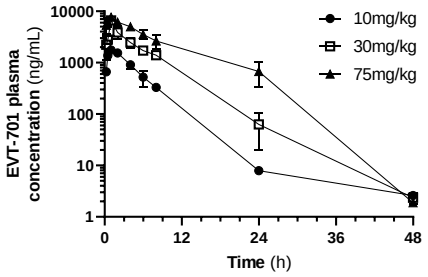


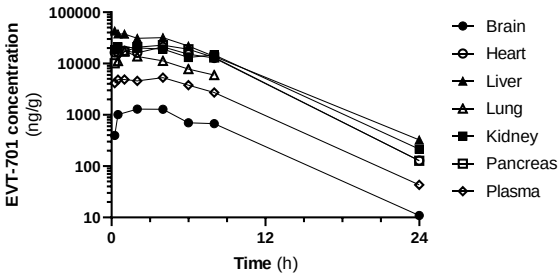
C

PK parameters of EVT-701 intravenous and oral administrations

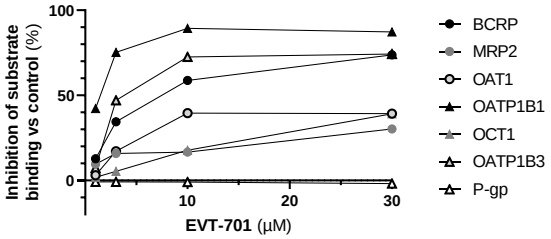
Matrix	C_0 (ng/mL)	$AUC_{(0\text{-last})}$ (ng.h/mL)	t_{last} (h)	$AUC_{(0\text{-24h})}$ (ng.h/mL)	$t_{1/2}$ (h)	Cl (L/h/kg)	Vd_{ss} (L/kg)
Plasma	5280	4700	24	4700	3.0	0.63	1.5
rating	-	-	-	-	Moderate	Moderate	Large

Dose (mg/kg)	C_{max} (ng/mL)	t_{max} (h)	$AUC_{(0\text{-last})}$ (ng.h/mL)	t_{last} (h)	$AUC_{(0\text{-48h})}$ (ng.h/mL)	$t_{1/2}$ (h)	F (%)	"X/10 mg/kg" (AUC(0-inf) ratio)
10	1760	1	10000	48	10000	4.1	63.8	-
rating	-	-	-	-	-	Long	Good	-
30	5980	1	34000	48	34000	4.3	-	3.4
rating	-	-	-	-	-	Long	-	-
75	7680	1	73000	48	73000	4.1	-	7.3
rating	-	-	-	-	-	Long	-	-

D



E



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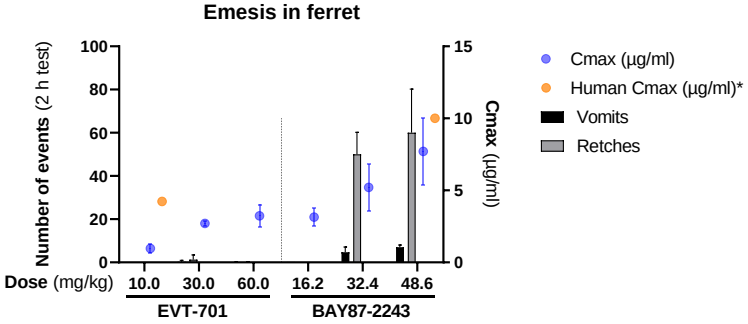

Figure S1

Supplemental Figure 2. *In vitro* evaluation of BAY87-2243 on H460 cancer cell line.

BAY87-2243 strongly decreases mitochondrial ATP and induces apoptosis in H460 lung cancer cell even at concentrations at which respiration is unaffected. **(A)** Dose dependent inhibition of mitochondrial ATP by BAY87-2243. Mitochondrial ATP is inhibited by more than 50% compared to control even at 10 pM. **(B)** Dose-dependent modulation of oxygen consumption in H460 cell line by BAY87-2243. **(C)** Dose-independent inhibition of proliferation and induction of apoptosis by BAY87-2243. Proliferation and apoptosis are inhibited by more than 50% compared to control even at dose of 1 nM. AUC: area under the curve.

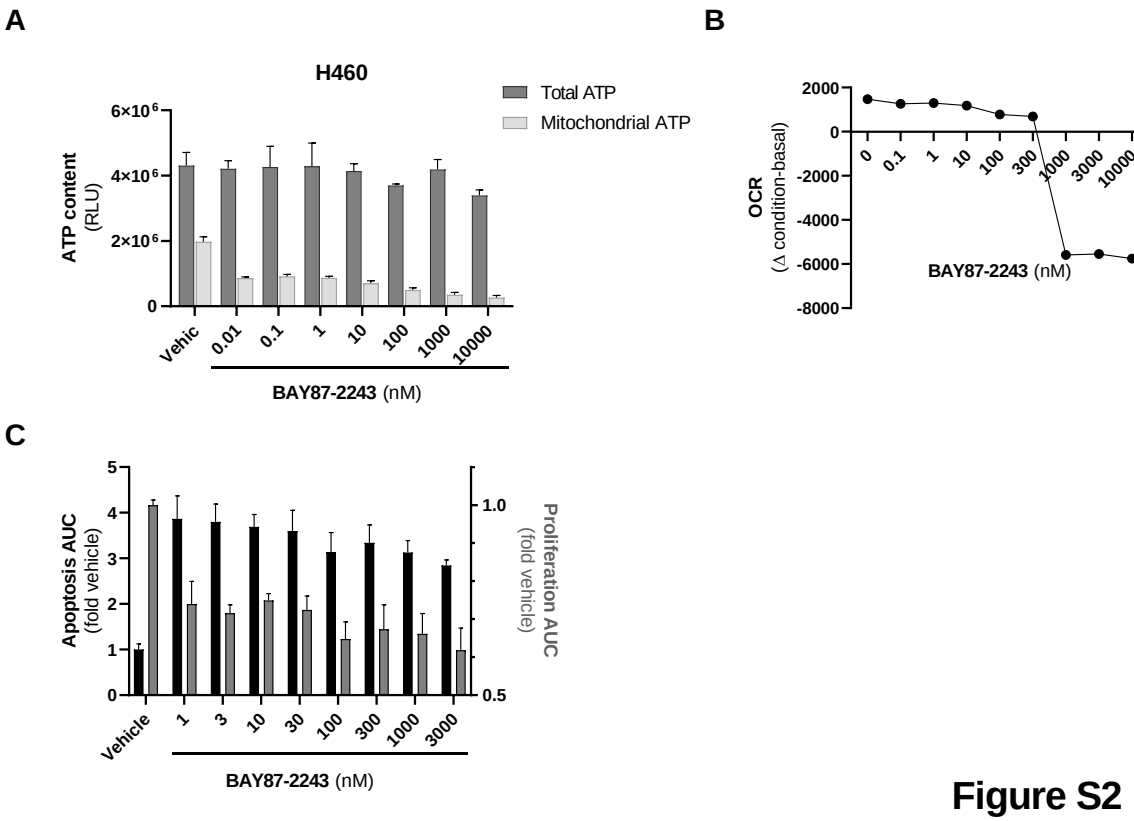

Figure S2

Supplemental figure 3. EVT701 *in vivo* activity in H460 orthotopic model.

(A) Schematic view of study design. H460-Luc2 cell suspension in PBS was injected orthotopically and treated daily by oral route from D7 for 15 days. **(B)** Characterization of the model by immuno-histochemistry. Representative images are presented, showing hypoxic, proliferating and necrotic zones, at D7 (top) and D21 (bottom), characterized by carbonic anhydrase IX, Ki67 and caspase-3 staining respectively, from left to right. **(C)** Representative bioluminescence imaging (BLI) used to randomize mice at D7 and monitor tumor progression, following intraperitoneal injection of luciferin. **(D)** Tumor growth inhibition induced by 30 mg/kg EVT-701 QD dosing for 15 days using tumor volume as readout. **(E)** Tumor growth inhibition induced by 30 mg/kg EVT-701 QD dosing for 15 days using tumor weight as readout. Tumor growth was inhibited by 60% and tumor weight by 55%.

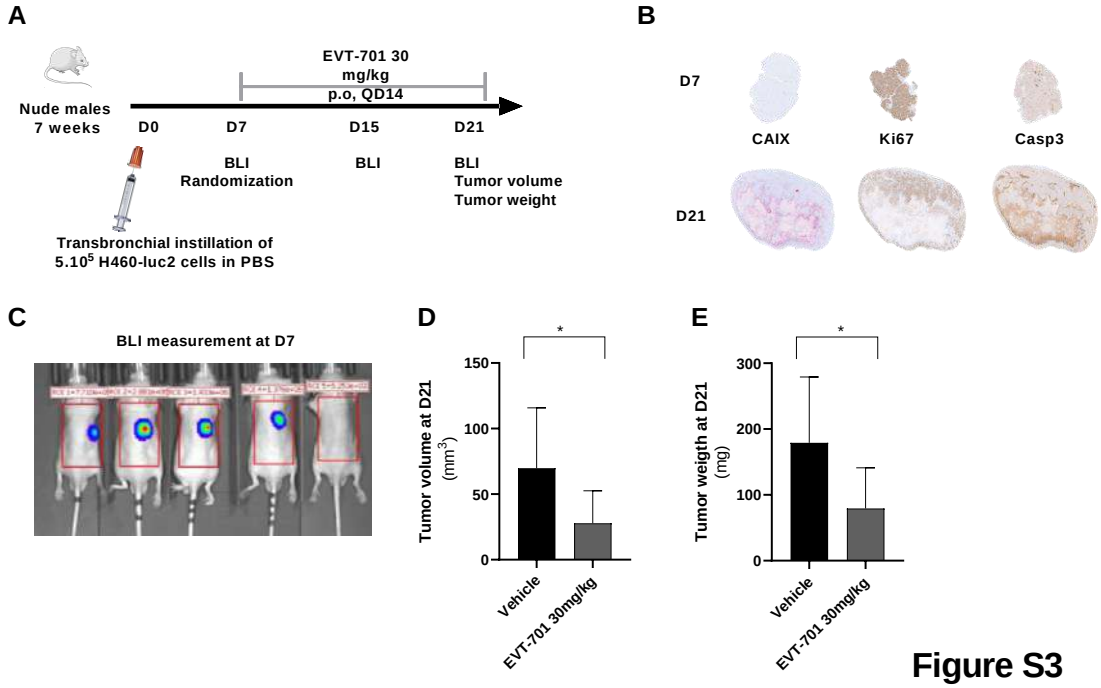

Figure S3

3.2. OxPhos is a common mechanism of response to chemotherapy in human and murine AML. Mitochondrial Electron Transfer Chain Complex I disruption decreases Immune Checkpoints in Murine and Human Acute Myeloid Leukemic Cells

Major results and conclusions

AML patients with a shorter overall survival displayed gene signatures enriched in OxPhos and mitochondrial metabolism. Furthermore, we identified that AraC-resistant murine leukemic exhibited an OxPhos phenotype. We also characterized the metabolic changes and compensatory reprogramming induced by selective ETC1 inhibitor EVT-701 on human and leukemic cells. Interestingly, we found that ETC I inhibition led to decreased expression of the immune checkpoint molecules PD-L1 and CD39 in murine and human leukemic cells.

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Disrupting Mitochondrial Electron Transfer Chain Complex I Decreases Immune Checkpoints in Murine and Human Acute Myeloid Leukemic Cells

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Simple Summary: Despite all of the advancements made in recent years in the treatment of acute myeloid leukemia (AML), long-term survival is achieved by only 30–40% of AML patients. Thus, new therapeutic strategies are strongly needed. This work confirms the increase in oxidative phosphorylation upon cytarabine resistance in AML murine cells, reinforcing the interest of targeting it. In addition, it identifies a new role of the first complex of the electron transport chain (ETCI) in the regulation of the immune checkpoints PD-L1 and CD39 in murine and human leukemic cells. Thus, this work opens the door to the evaluation of ETCI inhibitors in combination with immunotherapy.

Abstract: Oxidative metabolism is crucial for leukemic stem cell (LSC) function and drug resistance in acute myeloid leukemia (AML). Mitochondrial metabolism also affects the immune system and therefore the anti-tumor response. The modulation of oxidative phosphorylation (OxPHOS) has emerged as a promising approach to improve the therapy outcome for AML patients. However, the effect of mitochondrial inhibitors on the immune compartment in the context of AML is yet to be explored. Immune checkpoints such as ectonucleotidase CD39 and programmed dead ligand 1 (PD-L1) have been reported to be expressed in AML and linked to chemo-resistance and a poor prognosis. In the present study, we first demonstrated that a novel selective electron transfer chain complex (ETC) I inhibitor, EVT-701, decreased the OxPHOS metabolism of murine and human cytarabine (AraC)-resistant leukemic cell lines. Furthermore, we showed that while AraC induced an immune response regulation by increasing CD39 expression and by reinforcing the interferon-γ/PD-L1 axis, EVT-701 reduced CD39 and PD-L1 expression in vitro in a panel of both murine and human AML cell lines, especially upon AraC treatment. Altogether, this work uncovers a non-canonical function of ETCI in controlling CD39 and PD-L1 immune checkpoints, thereby improving the anti-tumor response in AML.

Keywords: OxPHOS; Immune checkpoints; AML

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Citation: Luna-Yolba, R.; Marmoiton, J.; Gigo, V.; Marechal, X.; Boet, E.; Sahal, A.; Alet, N.; Abramovich, I.; Gottlieb, E.; Visentin, V.; et al. Disrupting Mitochondrial Electron Transfer Chain Complex I Decreases Immune Checkpoints in Murine and Human Acute Myeloid Leukemic Cells. *Cancers* **2021**, *13*, 3499. https://doi.org/10.3390/cancers13143499

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1. Introduction

The overall survival in acute myeloid leukemia (AML) patients is poor due to relapses after a usually successful induction therapy [1]. This is due to residual leukemic cells

after chemotherapy that regrow, resulting in a relapse [2]. Energy metabolism and redox homeostasis have emerged as hallmarks of carcinogenesis and their role in the response to chemotherapy in cancer cells is now fully acknowledged [3,4]. A large body of evidence links both cancer cell stemness and chemotherapy resistance to OxPHOS metabolism [5–9], rendering OxPHOS inhibitors important therapeutic agents to disrupt tumorigenesis and improve the chemotherapy outcome. Mitochondrial metabolism has been shown to be a promising target to overcome relapses in leukemia. Recent research has shown that mitochondria in AML cells surviving after chemotherapy have a higher transmembrane potential [10,11]. In B-progenitor acute lymphoblastic leukemia (B-ALL), diagnosis relapse initiating clones (dRI) transcriptionally enriched for mitochondrial metabolism are present from the diagnosis [12]. In addition, the direct or indirect inhibition of the electron transport chain (ETC) [13–15] or the oxidation of substrates such as fatty acids [16] have proven to be of therapeutic benefit in leukemia, synergizing with AraC or with BCL-2 inhibition [17,18]. One much less studied aspect is the effect of OxPHOS inhibitors in an immunocompetent context, especially in AML, as the vast majority of the studies use NSG mice to allow the study of human cell lines or samples. In other cancer types, metformin has been demonstrated to decrease the expression of immune checkpoint components such as PD-L1 [19], CD39 and CD73 [20] both in cancer and immunosuppressive cell populations, enabling a CD8 T cell cytotoxic response [19,21]. However, due to the pleiotropic effects of metformin, it is not clear if these effects are predominately mediated by ETCI inhibition. EVT-701 is a novel ETCI inhibitor that has shown efficacy in different solid tumor models but its effect on AML has not previously been evaluated [22,23].

In this study, we report that murine leukemic AML cell lines exposed to AraC behave similar to chemo-resistant human AML cells by upregulating OxPHOS to escape chemotherapy, making our murine model translational to the human pathology. We also show that AraC increased the PD-L1 and CD39 immune checkpoint expression at the cell surface membrane. Interestingly, the inhibition of the ETCI complex by EVT-701 decreased the levels of both immune checkpoints in vitro. Altogether, our findings show that disrupting mitochondrial metabolism through the inhibition of ETCI modulates the immune checkpoints PD-L1 and CD39 and may improve the anti-tumor response in AML.

2. Materials and Methods

2.1. Cell Culture

Leukemic cell lines were maintained in a DMEM medium containing 1 g/L of glucose, 2 mM glutamax (#35050-038), 1X NEAA (#11140-035, Gibco) and 1 mM sodium pyruvate (#11360-039), all acquired from ThermoFisher Scientific (Illkirch Graffenstaden, France) and 10% fetal bovine serum (#F7524, Sigma-Aldrich, Saint Quentin Fallavier, France). The cells were incubated at 37 °C with 5% CO_2 and split every 3 days to maintain an exponential growth phase. The cells were purchased from the American Type Culture Collection (Rockville, MD, USA). Table 1 summarizes the clinical and mutational features of the leukemic cell lines used in this study.

Table 1. Clinical and mutational information about the AML cell lines used in this study.

	FAB	Karyotype	Sex	FLT3		NPM1	IDH1	Kit	N/K
				ITD	TKD		IDH2		Ras
Human									
MOLM13	M5-AML	ins(11;9)(q23;p22p23)	M	ITD	WT	WT	WT	WT	WT
MV4-11	M5-AML	Complex	M	ITD	WT	WT	WT	WT	WT
THP-1	M5-AML	Complex	M	WT	WT	WT	WT	WT	NRAS p.G12D
U937	M5-AML	t(10;11)(p13;q14)	M	WT	WT	WT	WT	WT	WT

2.2. IncuCyte Assay

The cells were seeded in triplicate in 384-well ViewPlates (#6007480, Perking Elmer, Villebon sur Yvette, France) at a density of 1000 cells/well. After an overnight, the cells were stimulated with the compounds and placed into the IncuCyte Zoom (Ozyme, Saint-Cyr-l'Ecole, France) reader until the end of the experiment (5 days after). Images were taken every 3 h. The confluence for each condition was analyzed using the masks of the IncuCyte Zoom software.

2.3. TMRE and MTDR Assay

The cells were seeded in fresh media 24 h prior to FACs experiments. To prepare the samples, the cells were washed in PBS and stained to detect the live cells with violet live dead staining (#L34955, Thermo Fisher). Tetramethylrhodamine, ethyl ester (TMRE) (#T669, Thermo Fisher Scientific) was then added for the characterization of the mitochondrial membrane potential and MitoTracker Deep Red FM (#M22426, Thermo Fisher Scientific) was used for the assessment of the mitochondrial mass. After incubation at 37 °C for 20 min, the cells were washed, resuspended in a FACs buffer and analyzed in a FACs Canto (BD Biosciences, Le Pont De Claix, France). FCCP (Sigma-Aldrich) was added as a control for specific mitochondrial membrane potential staining.

2.4. ATP Content Assay

The ATP cell content was measured using Cell Titer Glo (#G7571, Promega, Charbonnières Les Bains, France). In summary, the cells were seeded at 2000 cells/well in three replicates in 384-well ViewPlates (Perking Elmer). After an overnight, oligomycin A (Sigma-Aldrich) and sodium iodoacetate (Sigma-Aldrich) were added, either both or in combination. Following a 1 h incubation, the cell confluence was assessed with IncuCyte for further normalization and 40 µL of Cell Titer Glo reaction mix was added to each well, reaching a final volume of 80 µL. The plates were then analyzed for luminescence with an EnVision 2105 Multimode Plate Reader (Perkin Elmer). By comparing the different conditions, the global ATP and the percentages of both glycolytic and mitochondrial ATP were determined.

2.5. Cell Proliferation

The cell proliferation was calculated by measuring the number of viable cells with trypan blue dye and Nexcelom Bioscience Cellometer™ Auto T4 (Ozyme) or staining the cells with live fixable staining (#L34955, Thermo Fisher Scientific) and adding counting beads (#C36950, Thermo Fisher Scientific) for a further analysis with a BD LSR Fortessa™ cell analyzer and FlowJo™ software (Version 10, Becton, Dickinson and Company, Ashland, OR, USA).

2.6. Seahorse

The cells were incubated overnight with the vehicle or with EVT-701. Following the incubation time, the cells were suspended in pre-warmed Seahorse XF media (Agilent, Les Ulis, France) containing 10 mM glucose, 2 mM glutamine and 1 mM pyruvate. A total of 10^5 cells were seeded per well of Seahorse 96-well plates, previously coated with Cell-tak (#10317081, Thermo Fisher Scientific). The Mito Stress Test was performed with 1–3 µM oligomycin, 1–10 µM FCCP (both optimized for each cell line), 50 µM antimycin/rotenone and 50 mM 2-DG. The OCR and ECAR values were normalized to a cell confluence percentage with IncuCyte.

To assess the restoration of oxygen consumption by succinate in EVT-treated cells, 10^5 cells were seeded per well in replicates of five. After plate centrifugation, the Seahorse medium was replaced by a pre-warmed mitochondria assay solution (MAS) medium containing 10 mM pyruvate, 4 mM ADP and 2 mM malate, prepared as described by Agilent (https://www.agilent.com/cs/library/usermanuals/public/insert-xf-pmp-reagent-web.pdf, 23 January 2020). A total of 10 mM of succinate was added to the wells where

the bypass of the ETCI blockade was assessed. Plasma membrane permeabilizer (PMP, Agilent) was added just before the introduction of the plates into the Seahorse reader. Unless otherwise mentioned, all reagents were bought from Sigma-Aldrich.

2.7. Western Blot

The cells were lysed with a RIPAS buffer (#98065, Cell Signaling Technology, Ozyme) containing a protease inhibitor cocktail (#5872S, Cell Signaling Technology, Ozyme). The proteins were quantified by the Pierce BCA protein assay kit (#23227, Thermo Fisher Scientific) and the protein concentration for each sample was adjusted to 500 µg/mL by adding a loading buffer containing DTT or β-mercaptoethanol. Once reduced, the samples were heated to 55 °C to complete the protein denaturalization without affecting the thermosensitive band of the SDHB (ETCII) for the assessment of the ETC components or to 95 °C for 5 min for the assessment of STAT and p-Stat1. The samples were resolved in 4–20% Criterion™ TGX™ Precast Midi Protein Gels from Biorad (#5671093, Marnes-la-Coquette, France) by electrophoresis, transferred to nitrocellulose membranes and incubated with a rodent OxPHOS antibody cocktail (#ab110413) from Abcam (Amsterdam, The Netherlands) used at a dilution of 1:250, a STAT1 antibody (#9172S, Cell Signaling) used at a dilution of 1:1000, Phospho-Stat1 (Tyr701) (#9167S, Cell Signaling) used at 1:1000 or actin (#A2228, Sigma-Aldrich) diluted 1:20,000. An incubation with the secondary antibody goat anti-mouse IgG-HRP (#7076, Cell Signaling Technology, Ozyme) or anti-rabbit IgG HRP-linked antibody (#7074, Cell Signaling) and the further addition of a SuperSignal West Pico Chemiluminescent Substrate (#34580, Thermo Fisher Scientific) then revealed the protein bands in the membranes. Images were captured with Syngene PXi and quantified with GeneTools from Syngene (Thermo Fisher Scientific).

2.8. Data Sets and the Gene Set Enrichment Analysis (GSEA)

All publicly accessible transcriptomic data sets used in this study are listed below:

- TCGA: [24]
- GSE12417: [25]
- GSE97631: [10]
- MOLM14 treated with metformin: GSE97346.

GSEA was performed using DESEQ2 and fGSEA R packages. Gene sets were downloaded from the GSEA website (https://www.gsea-msigdb.org/gsea/msigdb/, 21 November. 2020) and the Farge_High OxPHOS signature from [10]. Volcano plots and enrichment maps were used for the visualization of the GSEA results. For each gene signature, its normalized enrichment score (NES) and false discovery rate (FDR) considering the p-adjusted values (padj) were evaluated.

2.9. Metabolomics

A total of four replicates of 7×10^5 cells/mL were seeded in 6-well plates per experiment. The cells were stimulated with EVT-701 or the vehicle. After a 24 h incubation with the compounds, 10 µL of media were sampled for metabolic extraction while the remaining media were discarded and the cells were washed with PBS. Metabolite extractions were performed by adding 1 mL of a cold extraction mix consisting of methanol (#1060351000, Sigma-Aldrich), acetonitrile (#1000291000, Sigma-Aldrich) and water (#1153331000, Sigma-Aldrich) in a proportion of 5:3:2. All solvents were LC-MS grade. The samples were vortexed for 10 min at 4 °C and then immediately centrifuged at the maximum speed for 10 min at 4 °C. Clear supernatants were transferred to Eppendorf tubes and kept at −80 °C until shipping to the Technion—Israel Institute of Technology for an LC-MS analysis. The LC-MS metabolomics analysis was performed as described previously [26]. Briefly, a Thermo Ultimate 3000 high-performance liquid chromatography (HPLC) system coupled to a Q-Exactive Orbitrap Mass Spectrometer (Thermo Fisher Scientific) was used with a resolution of 35,000 at 200 mass/charge ratio (m/z), electrospray ionization and polarity switching mode to enable both positive and negative ions across a mass range of 67 to 1000 m/z.

The HPLC setup consisted of a ZIC-pHILIC column (SeQuant; 150 mm × 2.1 mm, 5 µm; Merck) with a ZIC-pHILIC guard column (SeQuant; 20 mm × 2.1 mm). A total of 5 µL of the metabolite extracts were injected and the compounds were separated with a mobile phase gradient of 15 min, starting at 20% aqueous (20 mM ammonium carbonate adjusted to pH 0.2 with 0.1% of 25% ammonium hydroxide) and 80% organic (acetonitrile) and terminated with 20% acetonitrile. The flow rate and column temperature were maintained at 0.2 mL/min and 45 °C, respectively, for a total run time of 27 min. All metabolites were detected using mass accuracy below 5 ppm. Thermo Xcalibur was used for the data acquisition. The analyses were performed with TraceFinder 4.1 (Thermo Fisher Scientific). The peak areas of the metabolites were determined by using the exact mass of the singly charged ions. The retention time of the metabolites was predetermined on the pHILIC column by analyzing an in-house mass spectrometry metabolite library that was built by running commercially available standards. The cell number was used for data normalization.

2.10. Transcriptomics

The cells were seeded in 20 µL at a density of 7500 cells/well in 384-well plates (#3542, Corning, Boulogne-Billancourt, France) and treated with different doses and combinations of AraC (#PHR1787, Sigma-Aldrich, St. Louis, MO, USA), EVT-701 and IACS or DMSO for 24 h. After a 24 h incubation, the plates were centrifuged and the media were replaced by a lysis buffer containing an RNasin® ribonuclease inhibitor (#2511, Promega). The plates with lysates were frozen to −80 °C and sent to EVOTEC Gottingen where RNAseq was performed using Evotec's proprietary high-throughput platform, ScreenSeq.

Briefly, the plates were thawed and the oligo(dT)-containing oligos were distributed among the wells using an Agilent Bravo automated liquid handling system. Each oligo had a unique barcode specific for the particular well. In addition, there was a stretch of random nucleotides (unique molecular identifiers, UMIs) next to the barcode to uniquely label the original mRNA molecules. After the mRNAs in the wells were barcoded, all of the material from the wells of one plate was pooled together followed by the library preparation. The quality of libraries was controlled using BioAnalyzer. There was one library generated per plate.

The sequencing reaction was performed using a NovaSeq 6000 Illumina platform and paired-end reads covering the gene and the barcoded UMI parts. The raw data were processed to identify the genes, to demultiplex the samples from different wells based on the well barcodes and to identify all original mRNA/cDNA molecules based on the UMIs.

Subsequently, the demultiplexed files were aligned to the reference genome (Mus_musculus.GRCm38.97) using a STAR aligner and a table with counts per gene was obtained (the raw count matrix in the Supplementary Material).

The data analysis was performed with PanHunter (EVOTEC platform), DESEQ2 and fGSEA R packages.

2.11. NAD+/NADH Ratio Assessment

The NAD+/NADH ratio was evaluated using NAD/NADH Glo (#G9072, Promega). In summary, 4×10^5 cells/mL were seeded in 96-well plates (Perking Elmer) and stimulated with different doses of EVT-701 in a final volume of 200 µL/well. After the 24 h incubation time, the NAD+/NADH was evaluated as described (https://france.promega.com/-/media/files/resources/protocols/technical-manuals/101/nad-nadh-glo-assay-protocol.pdf?la=en, 15 December 2020). Briefly, a twice-serial 2-fold dilution of the cells was performed before diluting another 2-fold with 1% DTAB to lyse the cells. From each well, 50 µL were transferred to a microtube for NAD+ detection in acidic media (25 µL of 0.4 N HCl were added) or to an empty tube for NADH detection in basic media. The samples were heated at 60 °C for 15 min, then left for 10 min at room temperature. A total of 25 µL of Trizma base were added to the acidic NAD+ sample to neutralize HCl. A total of 50 µL of Trizma-HCl were added to the basic samples containing NADH. From each tube, four replicates of 20 µL were seeded in 384-well plates (Perking Elmer). The NAD/NADH detection reagent was

prepared immediately before its addition to the well in a ratio of 1:1 (20 µL/well). After the incubation time (30–40 min) at room temperature, the luminescence was read by using an EnVision 2105 Multimode Plate Reader (Perkin Elmer).

2.12. PD-L1 and CD39 Expression Assessment

Murine AML cell lines were seeded in fresh media 72 h prior to the FACs experiment and stimulated with murine IFN-γ at 25 ng/mL (#575306, BioLegend, Ozyme), 1 µM EVT-701, 0.03 µM (L1210) or 0.1 µM (C1498) AraC or a combination of the compounds. For human AML cell lines, the cells were seeded in fresh media 24 h prior to the FACs experiment and stimulated with human 25 ng/mL IFN-γ (#285-IF/CF, R&D Systems, Bio-Techne SAS, Noyal Châtillon sur Seiche, France), 1 µM EVT-701 or 0.3 µM AraC or a combination of the compounds. The cells were then washed in PBS and stained with violet live dead staining (#L34955, Thermo Fisher). The samples were then incubated for 30 min at 4 °C with the corresponding CD39 and PD-L1 antibody mix or the control isotypes (further detailed in Table 2). The cells were then washed and resuspended in a FACs buffer followed by an analysis on BD LSRFortessa™ (murine AML cell lines) or BD FACSCanto™ (human AML cell lines) cytometers and FlowJo™ software. For the NAD supplementation experiments, 0.5 mg/mL NAD+ (#1.24542.0005, Merk) at pH 7 was used. The median fluorescence intensity for each experiment is represented.

Table 2. CD39 and PD-L1 antibodies used in this study.

Antibody	Dilution	Supplier	Catalogue Number
Anti-mPD-L1-APC	1:50	Biolegend, Ozyme	#124312
APC-anti-Rat IgG2b,κ	1:50	Biolegend, Ozyme	#400611
Anti-mCD39-PE-Cy7	1:50	Biolegend, Ozyme	#143806
PE/Cyanine7-anti-Rat IgG2a,κ	1:50	Biolegend, Ozyme	#400522
Anti-hPD-L1-APC	1:100	Biolegend, Ozyme	#329708
APC-anti-Mouse IgG2b,κ	1:100	Biolegend, Ozyme	#400320
Anti-hCD39-PE-Cy7	1:100	Biolegend, Ozyme	#328212
PE/Cyanine7-anti-Mouse IgG1,κ	1:100	Biolegend, Ozyme	#400126

2.13. Statistical Analyses

GraphPad Prism Software version 8 (La Jolla, CA, USA) was used for the statistical analysis. The results were expressed as a mean ± SD. T-tests were used for the comparison of the two groups whereas a one-way Anova with a Tukey post-test was carried out to compare one variant in > 2 groups or a two-way Anova was used to compare more than one variant in > 2 groups. The significance is represented by stars in which * is $p < 0.05$, ** is $p < 0.01$, *** is $p < 0.005$ and **** is $p < 0.001$.

2.14. Graphical Summaries

Graphical schemes were generated using scientific illustration toolkits from Motifolio (Motifolio Inc., PO Box 2040, Ellicott City, MD, USA).

3. Results

3.1. OxPHOS Phenotype and Mitochondrial Gene Signatures Are Enriched in AML Patients with a Shorter Survival

The publically available human AML data sets from TCGA-AML [24] and the Metzeler [25] cohort were analyzed to assess whether there was an association between the mitochondrial and OxPHOS gene expression with overall survival. Briefly, each cohort was divided into short or long overall survival (OS > or < to 1 year for both cohorts; Figure 1a,b) and a gene set enrichment analysis (GSEA) was performed. In both cohorts, OxPHOS- and mitochondrial metabolism-related gene signatures were enriched in the short overall survival groups of patients (Figure 1a,b). In addition, sets of genes involved in the unfolded protein response and mitochondrial stress were also enriched in short

survival patients from the TCGA cohort. Interestingly, a higher expression of OxPHOS and NADH-dehydrogenase ETCI complex-related gene signatures derived from AML patients [10] was also seen in shorter survival groups of both TCGA and Metzeler cohorts (Figure 1c), suggesting an involvement of ETCI and mitochondrial metabolism as adverse prognosticators for AML patients.

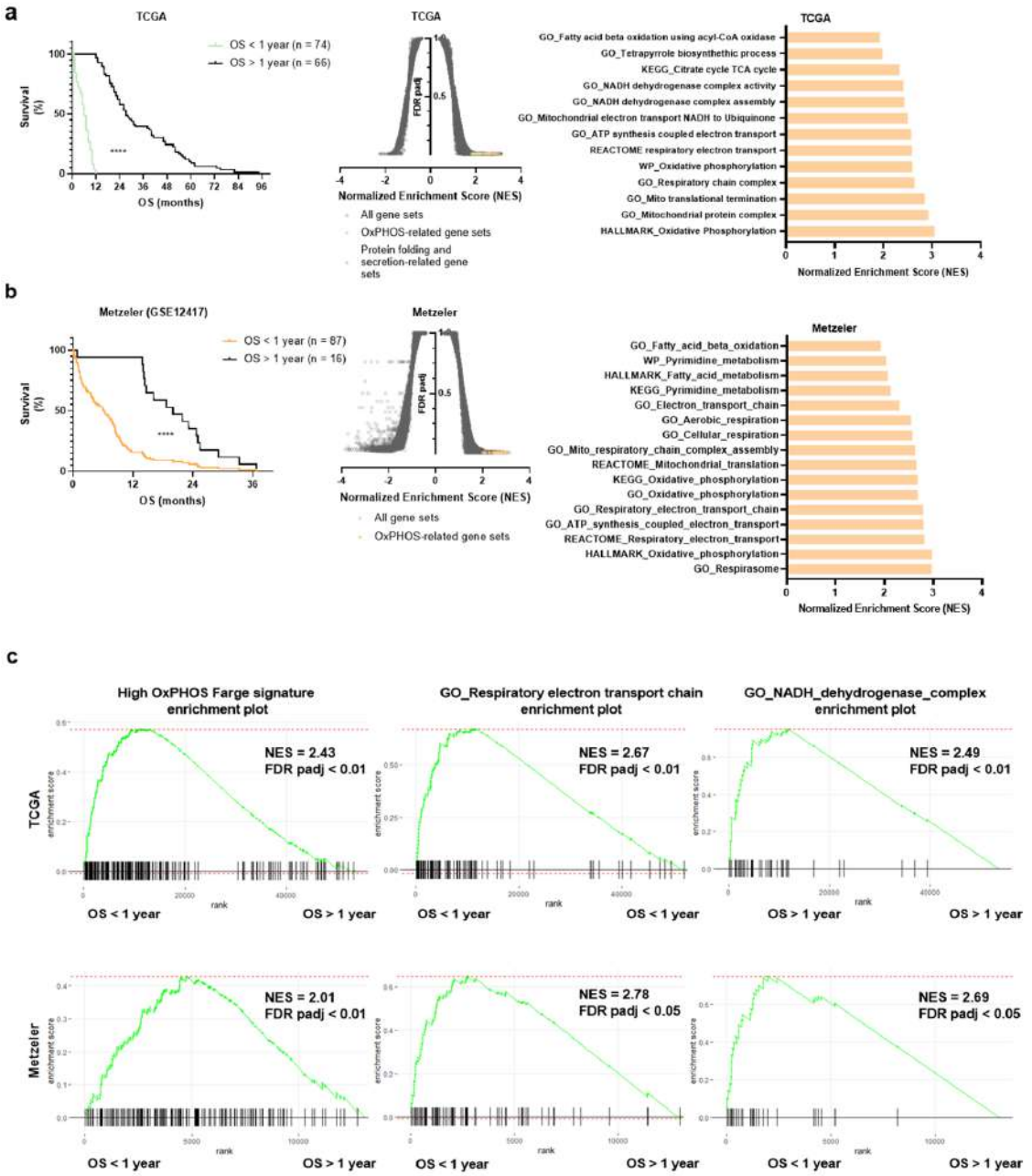

Figure 1. OxPHOS and mitochondrial gene expression is associated with a shorter overall survival in human AML. (**a**) Kaplan–Meier survival curves of the TCGA-AML cohort for patients with a short (OS > 1 year) or long (OS < 1 year) overall survival (**left**). Volcano plot showing GSEA results in the TCGA cohort comparing short vs. long survival (**middle**). OxPHOS-related gene sets enriched in the short survival group (FDR < 0.01) are highlighted in orange and protein folding and secretion-related gene sets are in green. On the (**right**), a plot showing TCGA short vs. long survival OxPHOS-related differentially expressed gene sets (FDR < 0.05) with their normalized enrichment scores (NES). (**b**) On the left, Kaplan–Meier survival curves of the Metzeler cohort for patients with a short (OS > 1 year) or long (OS < 1 year) overall survival. In the middle, the volcano plot showing GSEA results in the Metzeler cohort comparing short vs. long survival. OxPHOS and

mitochondria-related gene sets enriched in the short vs. long survival group (FDR < 0.05) are highlighted in orange. On the right, a plot showing short vs. long survival OxPHOS and mitochondria-related differentially expressed gene sets (FDR < 0.05) with their normalized enrichment scores (NES). (**c**) Gene set enrichment plots of the Farge_High OxPHOS gene signature, the GO_respiratory electron transport chain and NADH dehydrogenase complex gene signatures in the TCGA (**left**) and in Metzeler (**right**) cohorts.

3.2. AraC-Tolerant Murine Leukemic Cells Exhibit a High OxPHOS Phenotype also Seen in Drug-Resistant Human AML Cells

As we were interested in an immunocompetent context, we assessed whether the previously described high OxPHOS phenotype of AraC-resistant human cells was also observed in murine AML cells. To this end, C1498 and L1210 murine AML cell lines were grown in the presence of AraC, whose concentration was increased up to 10 µM for C1498 and up to 0.1 µM for L1210 (Figure 2a). AraC-tolerant cells (C1498-R, L1210-R) displayed higher basal and maximal respiratory rates as well as a higher mitochondrial membrane potential, mass and ATP levels compared with AraC-sensitive cells (C1498-S, L1210-S; Figure 2b–e). Western blots revealed a higher expression of ETC complex subunits, especially ETCI, in C1498-R-resistant cells compared with C1498-S cells (Figure 2f, Supplementary Materials Figures S3 and S4) whereas no consistent changes in ETC complex subunits were observed in L1210-R cells compared with L120-S (Figure S1a, Supplementary Materials Figures S3 and S4). In addition, AraC-resistant C1498 cells also showed an enrichment in OxPHOS-related gene sets including the high OxPHOS gene signature (Figure 2g,h). Therefore, murine leukemic cells able to grow in the presence of AraC exhibited an increased OxPHOS metabolism observed in chemo-resistant human leukemic cells.

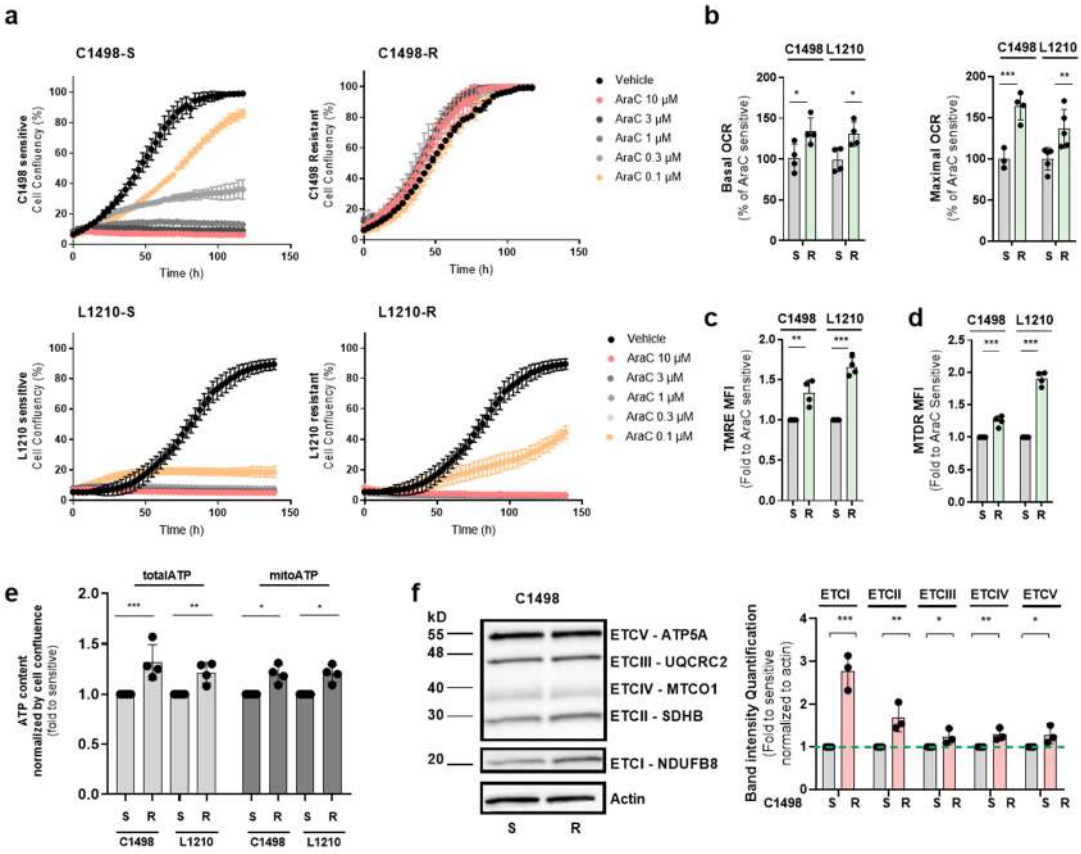

Figure 2. *Cont.*

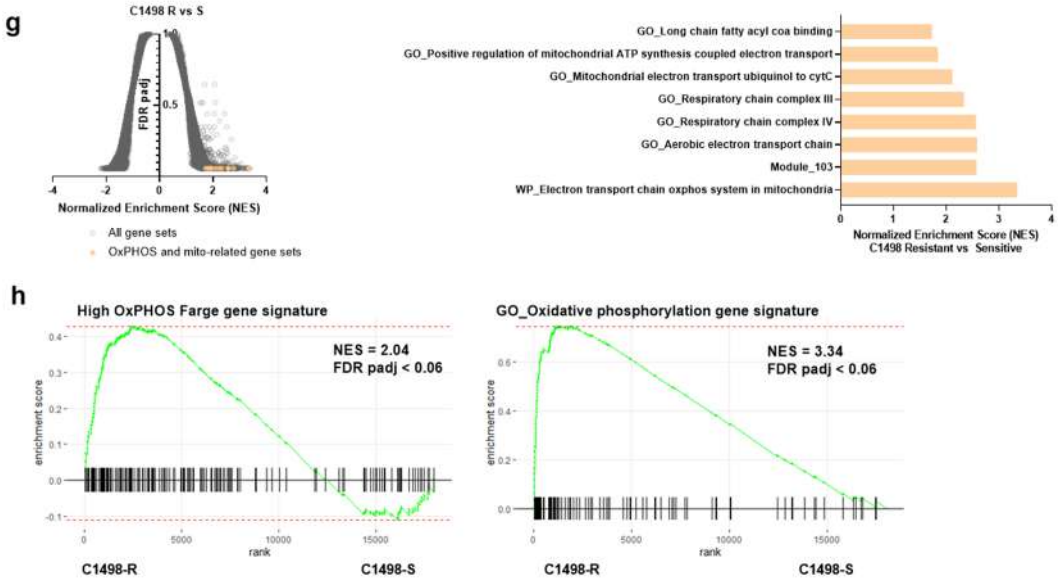

Figure 2. AraC-tolerant leukemic cells have more active mitochondria. (**a**) Proliferation IncuCyte assay of murine leukemic C1498 and L1210 cell lines sensitive (C1498S, L1210S) and resistant (C1498R, L1210R) to AraC. (**b**) Seahorse assessment of basal and maximal respiration in murine leukemic cells sensitive or tolerant to AraC (n = 4). (**c**) FACs assessment of mitochondrial membrane potential with the TMRE probe in AraC-sensitive vs. -resistant conditions for both cell lines (n = 4); (**d**) Mitochondrial mass assessment with MTDR (MitoTracker Deep Red) in both cell lines in the condition of sensitiveness and resistance to AraC (n = 4). (**e**) Total and mitochondrial ATP content measured by Cell Titer Glo in C1498 and L1210 cell lines sensitive and resistant (n = 4). (**f**) Protein expression levels assessed by Western blot of the ETC complexes in C1498 sensitive and resistant to AraC (n = 3). (**g**) Volcano plot showing the signatures enriched in AraC-resistant vs. -sensitive C1498 cells (FDR q-val < 0.05) on the left and a plot showing OxPHOS and mitochondrial-related gene sets differentially expressed in resistant vs. sensitive conditions with their normalized enrichment scores (NES). (**h**) Gene set enrichment plot of the GO oxidative phosphorylation and Farge_High OxPHOS gene signatures in resistant vs. sensitive C1498 cells. * is $p < 0.05$, ** is $p < 0.01$, *** is $p < 0.005$, ns is statistically non-significant.

3.3. EVT-701 Blocks OxPHOS by Inhibiting ETCI and Induces Metabolic Compensatory Reprogramming in Human and Murine AML Cells

As shown in solid tumors [23], EVT-701 was demonstrated here to inhibit oxygen consumption in both murine and human leukemic cell lines grown in the presence of glucose and pyruvate (Figure 3a,b). The blockade in mitochondrial respiration was bypassed in the presence of succinate, which donates electrons at the level of ETC complex II (Figure 3c), denoting that EVT-701 does not inhibit the electron transport chain beyond the ETCI complex. EVT-701 induced a shift toward a glycolytic phenotype as observed by an increase in glucose consumption and lactate production in the exometabolome of the murine leukemic cells (Figure 3d). EVT-701 did not affect the expression of ETC complexes assessed by Western blot on AML cells (Figure S1b, Supplementary Materials Figures S3 and S4). At the intracellular level, EVT-701 decreased the levels of glucose, UDP-GlcNAc, succinic acid, butyryl-carnitine, serine and aspartate and it increased the intracellular lactate in both the C1498 and L1210 cell lines (Figure 3e). EVT-701 decreased the cell proliferation as a single agent in murine leukemic cells (Figure 3f). Importantly, EVT-701 similarly affected the mitochondrial function of four human AML cell lines, decreasing OCR, the mitochondrial ATP level and the cellular NAD+/NADH ratio (Figure 3g–i) measured in the presence of glucose and pyruvate while it induced a decrease in cell viability (Figure 3j) and showed an additive effect to AraC to decrease the viability of human AML cells (Figure 3k).

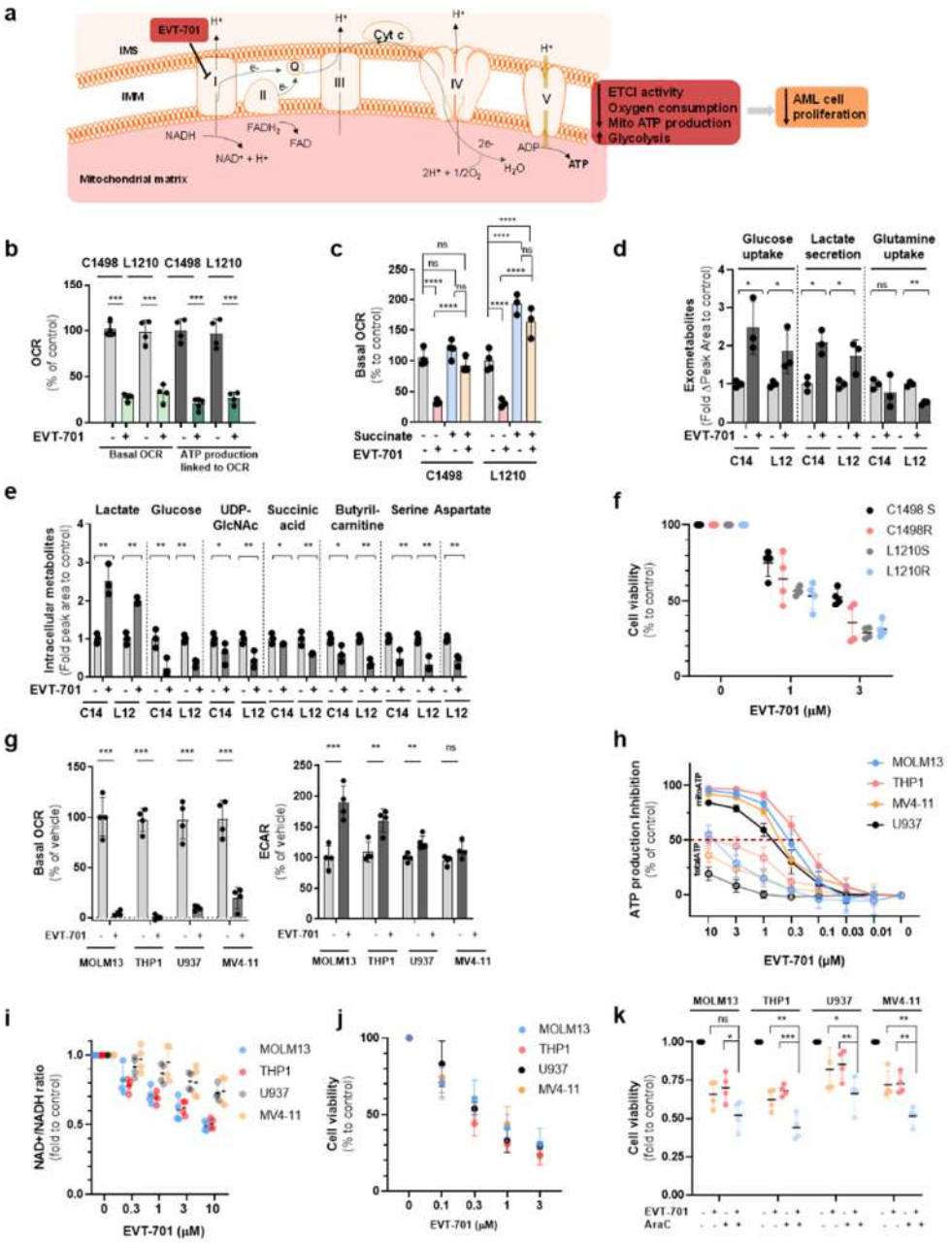

Figure 3. EVT-701 blocks OxPHOS by inhibiting ETCI and induces a profound metabolic compensatory reprogramming. (**a**) Schematic representation of the mode of action of EVT-701. (**b**) Effect of EVT-701 on the basal oxygen consumption rate and ATP production linked to OCR in murine AML cells, assessed by Seahorse (n = 4). (**c**) Effect of 10 mM succinate supplementation upon ETCI inhibition with EVT-701 in permeabilized leukemic cells (n = 4 C1498, n = 3 L1201). (**d**) Effect of EVT-701 on lactate and glucose in the exometabolome of C1498 and L1210 after 24 h incubation (n = 3). (**e**) Effect of EVT-701 on intracellular metabolites in C1498 and L1210 cells after 24 h incubation (n = 3). (**f**) Effect of EVT-701 on C1498 and L1210 proliferation (n = 4). (**g**) Effect of EVT-701 on oxygen consumption rate and extracellular acidification rate in human AML cell lines assessed by Seahorse (n = 4). (**h**) Dose-response effect of EVT-701 on total ATP and mitochondrial ATP production in human AML cell lines assessed by Cell Titer Glo (n = 4). (**i**) NAD+/NADH ratio change in response to EVT-701 in human AML cell lines (n = 4). (**j**) Dose-response effect of EVT-701 on human AML cell line proliferation (n = 4). (**k**) Effect of EVT-701, AraC or their combination on human AML cell line proliferation (n = 4). * is $p < 0.05$, ** is $p < 0.01$, *** is $p < 0.005$, **** is $p < 0.001$, ns is statistically non-significant.

3.4. EVT-701 Decreases the Expression of Immune Checkpoint Markers in Murine and Human Leukemic Cells

GSEA showed that gene sets associated with the interferon-γ response or signaling pathways that lead to an increased expression of PD-L1 were enriched in AraC-residual AML cells (Figure 4a) and in patients with a shorter overall survival (OS; Figure 4b,c), indicating a key role of immune checkpoints in a relapse to chemotherapy in AML. Interestingly, the transcriptome of murine-resistant cells was also enriched in the interferon-γ response gene signature (Figure 4 and Supplementary Material Figure S5). To confirm this observation, we assessed CD39 and inducible PD-L1 levels by flow cytometry in murine and human leukemic cell lines treated with EVT-701, AraC or their combination. AraC increased the cell surface expression of both inducible PD-L1 and CD39 in leukemic cells while EVT-701 as single agent as well as in combination with AraC decreased these proteins in the cellular membrane, suggesting potential as an immunomodulatory agent (Figure 4d–f). Interestingly, ETCI inhibition with IACS also resulted in decreased CD39 and inducible PD-L1 membrane levels (Supplementary Figure S2c,d). In addition, CD39 mRNA was also decreased by metformin (Supplementary Figure S2f). EVT-701 as single agent and in combination with AraC decreased the cellular NAD+/NADH ratio (Figure 4g), which has recently been proposed as the driving factor of inducible PD-L1 decreased expression [27]. Indeed, media supplementation with NAD+ restored PD-L1 levels in the presence of EVT-701 or IACS while CD39 levels were not altered by exogenous NAD+ in the presence of ETCI inhibitors (Figure 4h), showing a differential modulatory effect of ETCI inhibition on the two checkpoints. In addition, while PD-L1 expression was induced by IFN-γ, CD39 was unaffected (Supplementary Figure S2a,b,e), highlighting a different regulatory mechanism for both immune checkpoints. In sum, EVT-701 may be able to decrease tumor-mediated immunosuppression by decreasing inducible PD-L1 and CD39 expression on leukemic cells.

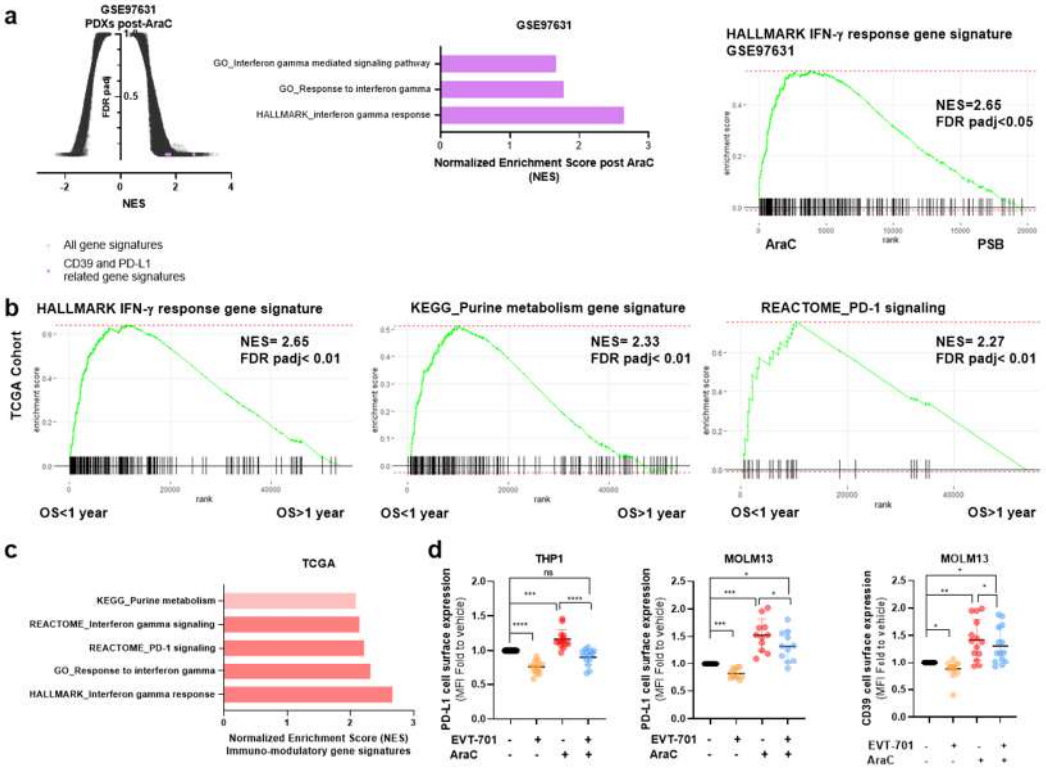

Figure 4. *Cont.*

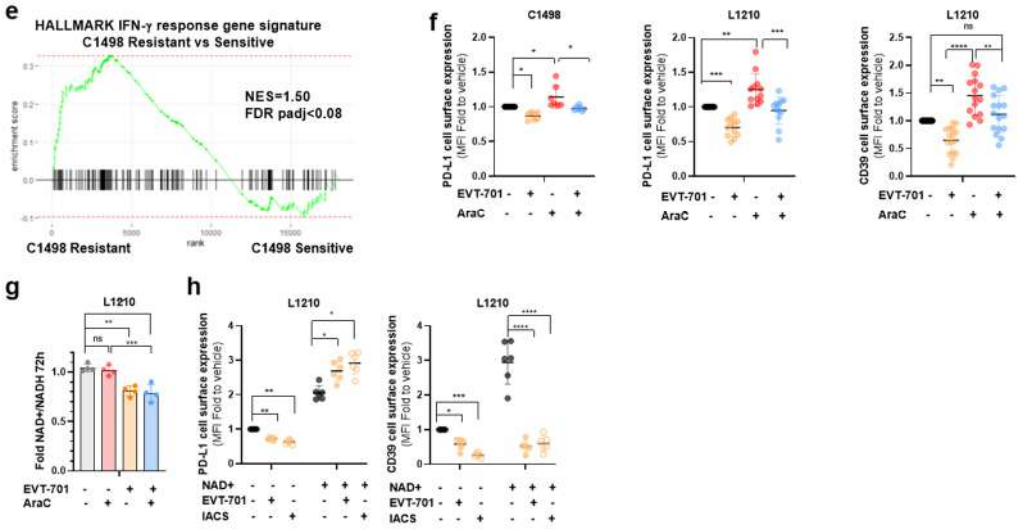

Figure 4. EVT-701 decreases the immune checkpoint expression to boost the immune response and increase the therapeutic benefit. (**a**) Volcano plot showing GSEA results in the GSE97631 data set containing the transcriptomic profiles of residual AML in PDXs AraC or vehicle-treated. Immune modulation-related gene signatures are in purple. In the middle, a plot showing AraC vs. PBS-treated PDXs differentially expressed gene sets with their normalized enrichment scores (NES). On the right, the enrichment plot of the hallmark Interferon-γ gene signature compatible with PD-L1 increases after AraC in vivo. (**b**) Enrichment plots of the hallmark Interferon-γ gene signature (**left**), KEGG purine metabolism-containing CD39 (**middle**) and Reactome PD-1 signaling (**right**) in the short group survival of the TCGA cohort. (**c**) Plot showing CD39- and PD-L1-related signatures in the short group of the TCGA cohort with their normalized enrichment scores (NES). (**d**) FACS assessment of the modulation of inducible PD-L1 and CD39 membrane expression on THP1 and MOLM13 cells by EVT-701, AraC or a combination of both ($n > 10$). (**e**) Enrichment plot of the hallmark Interferon-γ gene signature in C1498-resistant vs. -sensitive cell transcriptomes. (**f**) FACs assessment of the modulation of inducible and CD39 membrane expression on C1498 ($n = 7$) and L1210 ($n > 10$) by EVT-701, AraC or a combination of both at 72 h. (**g**) Effect of EVT-701, AraC or a combination of both on the NAD+/NADH ratio in L1210 at 72 h ($n = 4$). (**h**) Effect of exogenous NAD+ on the modulation of PD-L1 and CD39 membrane levels by EVT-701 or with IACS ($n = 6$). * is $p < 0.05$, ** is $p < 0.01$, *** is $p < 0.005$, **** is $p < 0.001$, ns is statistically non-significant.

4. Discussion

In summary, we have demonstrated that the OxPHOS phenotype induced by the metabolic adaptation to chemotherapy is a common mechanism in murine and human AML cells and many other forms of cancer [10,11]. In addition, we have shown that the genetic signatures of shorter survival groups within human AML cohorts are enriched in OxPHOS and mitochondrial metabolism gene sets, underscoring the involvement of mitochondria in the progression and relapse of the disease. We characterized the effect of EVT-701, a new selective ETCI inhibitor, on the metabolism in human and murine AML cell lines, characterizing the metabolic reprogramming it exerts on AML cells by inducing a shift toward glycolysis, increasing glucose consumption and lactate production while decreasing mitochondrial ATP production and the cellular NAD+/NADH ratio. The metabolic changes observed upon EVT-701 suggest an impairment of the OxPHOS function and all have been previously observed with other ETCI inhibitors. The glycolytic shift upon ETCI inhibition (Pasteur effect) has been already observed with other ETCI inhibitors such as metformin [28]. In addition, a fall in aspartate levels also occurred with IACS-01759 (hereafter IACS) [15]. This is explained by the drop in the NAD+/NADH ratio induced by ETCI inhibition, which impairs aspartate biosynthesis by interfering with the mitochondrial malate dehydrogenase (MDH2) activity [29,30]. It has been also reported in several works that de novo serine biosynthesis is impaired upon ETCI inhibition [31,32] while being still catabolized via the folate pathway [32], leading to the reduction in serine levels. The decrease in succinic acid could suggest decreased TCA activity though the

levels of other TCA cycle intermediates were not consistently reduced in this study. This could be compatible with TCA replenishment through the glutamine-derived reductive carboxylation of α-KG via NADPH-dependent isocitrate dehydrogenase 1 (IDH1) to generate citrate, also observed with other ETCI inhibitors [33,34] despite the fact that EVT-701 does not stimulate glutamine uptake (Figure 3d). In addition, EVT-701 decreased the proliferation of AML cells both as a single agent and in combination with AraC. Using in vitro models, we showed that AraC increased CD39 and inducible PD-L1 at the cell surface while EVT-701 as single agent decreased the expression levels of these proteins and, when used in combination with AraC, neutralized the increase induced by the chemotherapeutic agent. In addition, we showed that the decrease of immune checkpoints is not exclusive to EVT-701 because other ETCI inhibitors such as IACS and, as reported in the literature, metformin elicited similar responses [19,20].

As NAD+ supplementation abrogated the decrease of the inducible PD-L1 expression but not that of CD39 upon ETCI inhibition and considering also that only PD-L1 is induced by IFN-γ, we propose that two different regulatory mechanisms concur to achieve the modulation of these two immune checkpoint components. It is important to remark that despite the controversy around the NAD+ uptake by mammalian cells, it has been previously reported that exogenous NAD+ can rescue cells from death upon NAD depletion induced by the nicotinamide phosphoribosyl transferase (NAMPT) inhibitor FK866 [35]. In addition, NAD+ supplementation might also rescue cells from the block in DNA repair resulting from PARP1 inactivation upon FK866 NAD depletion [36], reinforcing the assumption that NAD+ can be uptaken and used by the cells. Remarkably, MCART1/SLC25A51 has been recently identified as the NAD importer in the mitochondria of mammal cells and necessary for ETCI activity [37,38]. Interestingly, previous studies showed that exogenous NAD+ can access mammalian mitochondria and increase respiration [39]. Therefore, supplementation with NAD+ could potentially affect the intracellular NAD+/NADH ratio changes induced by ETCI inhibition in our cellular context. The proposed regulatory mechanism of PD-L1 involves ETCI-driven NAD+ as a major coordinator. NAD+ is known to be a cofactor of sirtuin 1 (SIRT1), a class III histone deacetylase involved in epigenetic control [40]. Resveratrol is a SIRT1 agonist that has been reported to stimulate ETCI activity to increase the NAD+/NADH ratio and activate SIRT1 [41]. Interestingly, several studies described that resveratrol upregulated PD-L1 expression in several cancer types including colon, breast and lung cancer [42–44]. One of these studies supported that PD-L1 expression was upregulated upon SIRT1 activation with resveratrol via NF-κB [43]. Remarkably, PD-L1 inducible expression has been reported to be upregulated through IFN-γ activation of the NF-κB pathway [42]. Interestingly, a recent work identified that the ETCI function is essential for IFN-γ signaling because upon the genetic or pharmacological function abrogation of exclusively the first ETC complex, IFN-γ signaling is impaired and PD-L1 levels are decreased [45]. This study suggests that the NAD+ driven by the functional ETCI is essential for IFN-γ signaling as the impaired activity of exclusively ETCI is enough to decrease the IFN- γ signaling pathway and PD-L1 expression. Interestingly, it has been recently observed that OxPHOS cells have increased PD-L1 levels [46,47].

In addition, functional ETCI and NAD metabolisms have been shown to be involved in the epigenetic regulation of inducible PD-L1 expression [27]. In fact, this study proposed a mechanism by which NAD+ metabolism, via TCA intermediates such α-ketoglutarate (α-KG), activates TET1. TET1 then interacts with p-STAT1, previously phosphorylated and activated by JAK after IFN-γ IFNGR stimulation. They showed that the TET1-p-STAT1 interaction stabilized TET-1 and facilitated the TET1-mediated demethylation of the Irf1 promoter, which subsequently promotes the generation of IRF1 and induces PD-L1 expression [48–50]. The link between TET1 activity and PD-L1 expression has already been proposed in gliomas. Lower PD-L1 levels have been reported in patients bearing IDH mutations, increasing (R)-2-hydroxyglutarate (2-HG) oncometabolite production [51,52]. 2-HG is a competitive inhibitor of multiple α-KG-dependent dioxygenases, which includes the TET family of 5-methlycytosine hydroxylase [53,54] thereby explaining the loss of TET

activity concomitant with IDH mutations. It has recently been reported that in response to 2-HG inhibition, the PD-L1 levels in IDH1 mutant tumors were increased to the same levels observed in WT-IDH gliomas [55]. As IDH mutations are also present in several AML patients [56,57], the same profile regarding the IDH mutational state and PD-L1 levels might also be expected although this remains to be proven. In addition, TET1 repression has also been associated with increased tumor-infiltrating immune cells in several cancer types such as basal-like breast cancer (BLBC), melanoma, ovarian, lung and thyroid cancer [58]. Considering all of the above, we propose that ETCI-NADH dehydrogenase activity regulates the level and ratios of NAD+/NADH, which is essential for SIRT1- and/or TET1-mediated PD-L1 stimulation. Recent studies showed that CD39 expression was not induced by IFN-γ [59,60] whereas it can be induced under hypoxic conditions [61,62]. ETCI inhibitors have already been reported to interfere with HIF stabilization such as IACS [15,63] and metformin [64–66]. EVT-701 was also developed from a screening campaign to characterize HIF-1α inhibitors [22]; therefore, we propose an impairment of hypoxic signaling by interfering with HIF-1α stabilization as the mechanism by which EVT-701 decreases CD39 expression. Importantly, EVT-701 was shown to decrease the levels of the N-glycosylation unit UDP-GlcNAc, which can impair the CD39 function and localization in the cell membrane [67], highlighting another layer of the regulation of CD39 by EVT-701. Further studies will be needed to elucidate the exact mechanism involved in the ETCI regulation of PD-L1 and CD39 (Figure 5). Future perspective research would answer the question of whether ETCI inhibition decreases immune checkpoints in patient-derived samples and, eventually, in patient-derived xenografts (PDXs).

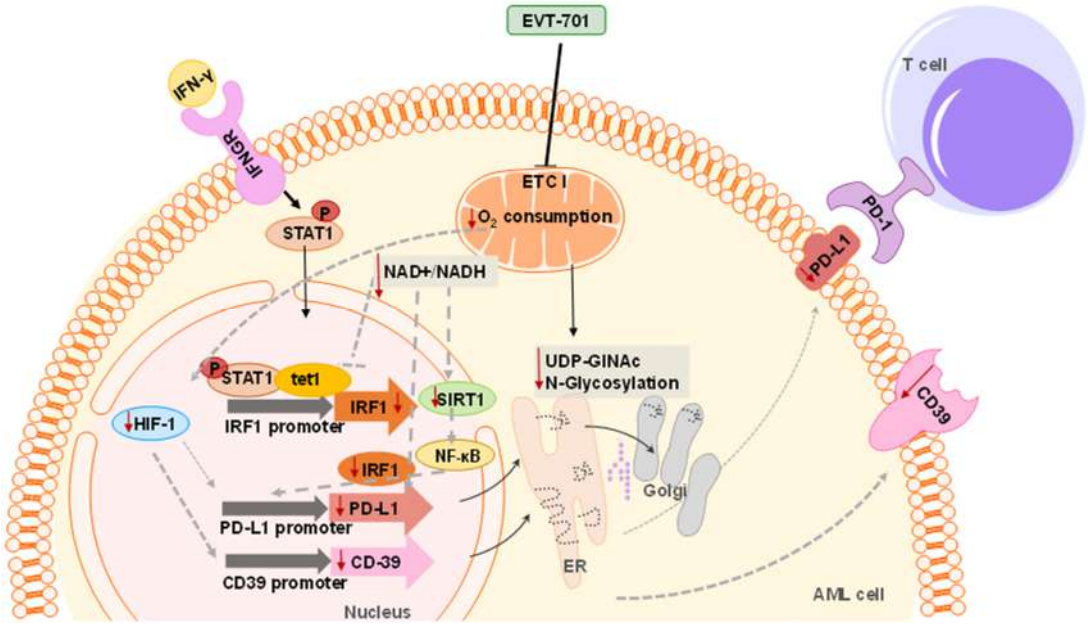

Figure 5. Schematic view of the proposed mechanism of action of EVT-701 as modulator of the immune checkpoints PD-L1 and CD39 membrane levels.

5. Conclusions

In conclusion, this work describes the opposed actions of chemotherapy and ETCI inhibitors on immune system modulation, providing a non-canonical function of ETCI in the regulation of CD39 and PD-L1 and a rationale to improve the anti-tumor response in AML including combinations with immune checkpoint therapies.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/cancers13143499/s1, Figure S1: ETC complexes expression in murine AML cells, Figure S2: Modulation of immune checkpoints on leukemic cells, Supplementary Material S3: Western blot raw

images, Supplementary Material S4: Densitometry readings of Western blot bands, Supplementary Material S5: Raw count and normalized count matrix of the transcriptomic data C1498 R vs. S.

Author Contributions: R.L.-Y., M.R.P., V.V., N.A. designed the studies and interpreted the data; R.L.-Y., J.-E.S. wrote the manuscript; M.R.P., V.V., J.-E.S. supervised the work; R.L.-Y. performed the experiments; X.M. contributed to the set-up, design and generation of transcriptomic samples; J.M., V.G. contributed to the Western blot experiments. E.B., A.S. contributed to the technical development and tools. E.G., I.A. supervised and helped analyze the metabolomics studies. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: GEO (Gene expression Omnibus, https://www.ncbi.nlm.nih.gov/geo/, 21 November 2020) accession codes of data sets used in the study are GSE12417; GSE97631; GSE97346. The transcriptomic data C1498 R vs. S are attached in Supplementary Material Figure S5_raw_and_normalized_count_matrix.

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Conflicts of Interest: R.L.-Y., J.M., V.G., N.A., V.V. and M.R.P. are full time employees of Evotec SE. The other authors declare no conflicting interests.

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3.3. *In vivo* study of EVT-701 in immunocompetent model of leukemia

3.3.1. Establishment of the leukemic syngeneic immunocompetent model

We then established a leukemic syngeneic model to evaluate the compound and several combinations *in vivo*. To that end, we injected 10^6 C1498-Fluc-eGFP cells in the lateral tail vein of C57/Bl6 mice (figure 39a). The model showed homogeneus AML progression, followed by the increase in bioluminescence signal (figure 39b). At endpoint, spleens were harvested and weighted, leading to the observation that the model was characterized by an increase in spleen weight compared to non-injected control mice (figure 39c), what had been previously reported as a characteristic of leukemia models (Jonas et al., 2016). We also observed a massive ovary size increase, resulting from the infiltration of the leukemic cells (figure 39c), making them the first homing niche for those cells in the model. This arouse the first questions regading the translationality of the model, as well as ethic concern since the increase in ovary size was tremendous. We further characterized the model, and observed a decreased lymphocyte count in blood in leukemic mice vs control mice (figure 39d).

Despite the ovary infiltration, we decided to pursue with the model and to evaluate its response to chemotherapy, to see its effect on disease clearance in combination with chemotherapy regime. With that aim, we injected C57/Bl6-Ly5.1 (to allow us to discriminate leukemic cells by FACs, since the host murine cells would express CD45.1 whereas the leukemic cells express CD45.2) females, and treated with AraC for 5 consecutive days, from day 10 to day 15 (figure 40a). We observed a response to chemotherapy that mimicked the human one: after AraC treatment, there was no detectable bioluminescence signal, therefore, there were not enough leukemic cells to produce BLI signal after luciferine mice injection (figure 40b-c). However, 9 days after the end of AraC administration, BLI signal arouse, denoting progression of the disease, what we considered to be a "relapse" (figure 40b-c).

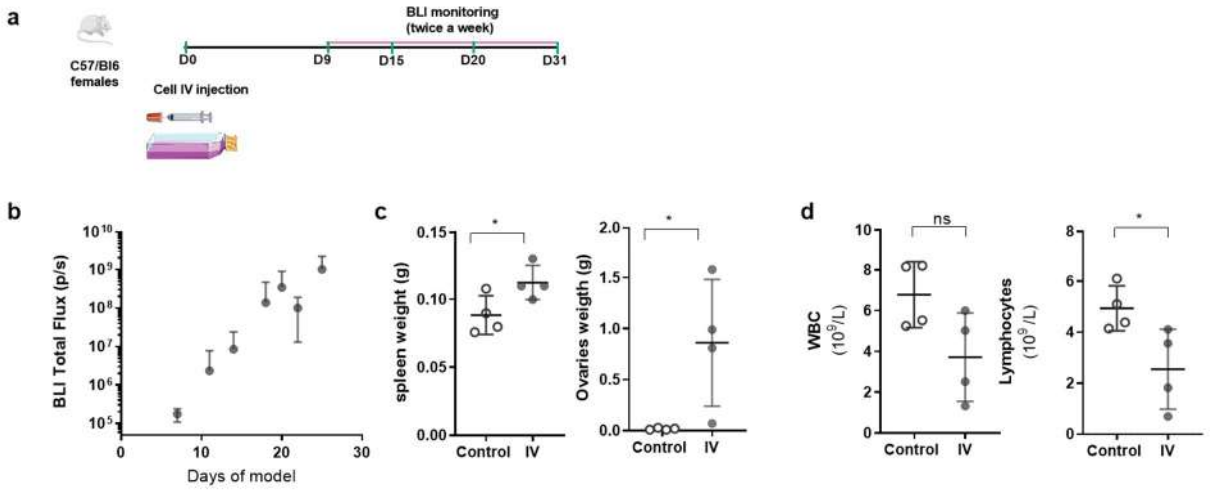


Figure 39. Set up 1 of the AML syngeneic model C1498-Fluc-eGFP in C57/Bl6 females. a) Schematic view of the study plan. b) Bioluminescence signal progression after cell injection. c) Spleen and ovary weights of C1498-injected mice vs control mice. d) HM5 measurement of White blood cells (WBC) and Lymphocyte counts in PB.

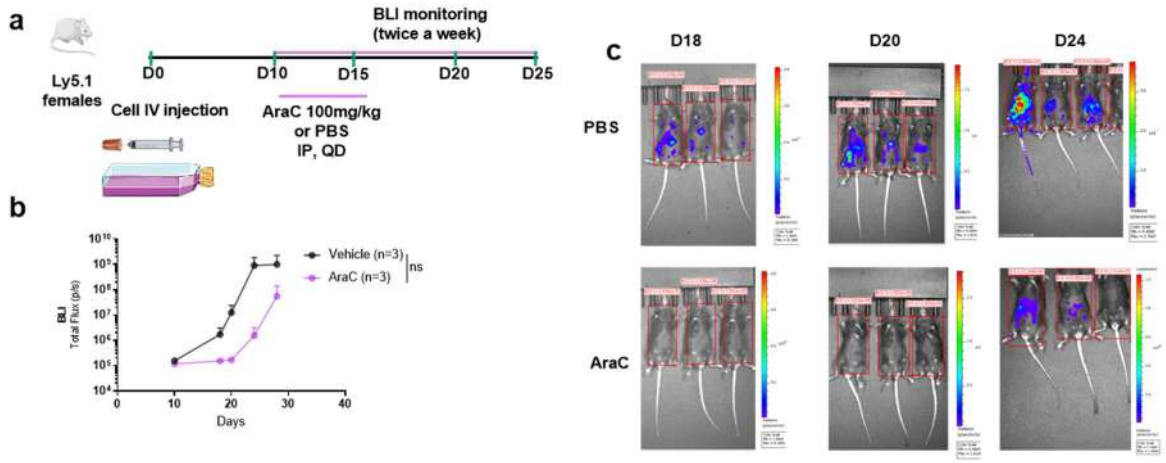


Figure 40. Assessment of AraC response in C1498 AML syngeneic model. a) Schematic view of the study plan. b) Bioluminescence signal progression upon AraC treatment vs PBS. c) BLI images at different time-points showing disease progression and AraC relapse.

We then assessed whether busulfan could impact leukemia engrafment and how did the gender of the mice affect the different organ colonization by leukemic cells (figure 41). Specifically, we wanted to assess if we could prevent ovaries to be the main homing organ for the leukemic cells. Busulfan is a myelosuppresive agent widely used to improve bone marrow engraftment and chimerism (Peake et al., 2015), so we tested it to enhance bone marrow engraftment of leukemic cells. We performed IP injections of 20 mg/kg busulfan during 3 consecutive days, reaching a final dose of 60 mg/kg. Four days after the last dose of busulfan administration, C1498 cells were injected in the lateral tail vein of female and male C57/Bl6 mice (figure 41a). Both females and males showed homogeneous AML progression (figure 41b-c). However, they main differences were observed when AML organ colonization was evaluated by FACs (as % of eGFP positive cells) (figure 41d). Bone marrow was more efficiently colonized by AML in males, and the leukemic cells were distributed among different organs (figure 41d). However, for females, ovaries were the primary niche of the leukemic cells (figure 41d). Accordingly, we focused on males for further experiments to be closer to the human disease.

We attempted to establish also the L1210 syngeneic model. For that, we infected L1210 cells with RediFect Red-Fluc-GFP lentivirus, and obtained cells that simultaneously expressed firely-luciferase and GFP (figure 42a, b) after selection of the clones that showed a similar growth pattern with the parental cell line. However, despite cells showing a linear BLI signal and the maintenance of both BLI and GFP signal along *in vitro* passages, the disease did not established *in vivo* and the BLI signal tended to decrease as time passed by (figure 42c), probably due to immune response. Since L1210 is a lymphocytic leukemia cell line, we decided to focus our *in vivo* work on the C1498 model, which is AML cell line and we had it already established and characterized.

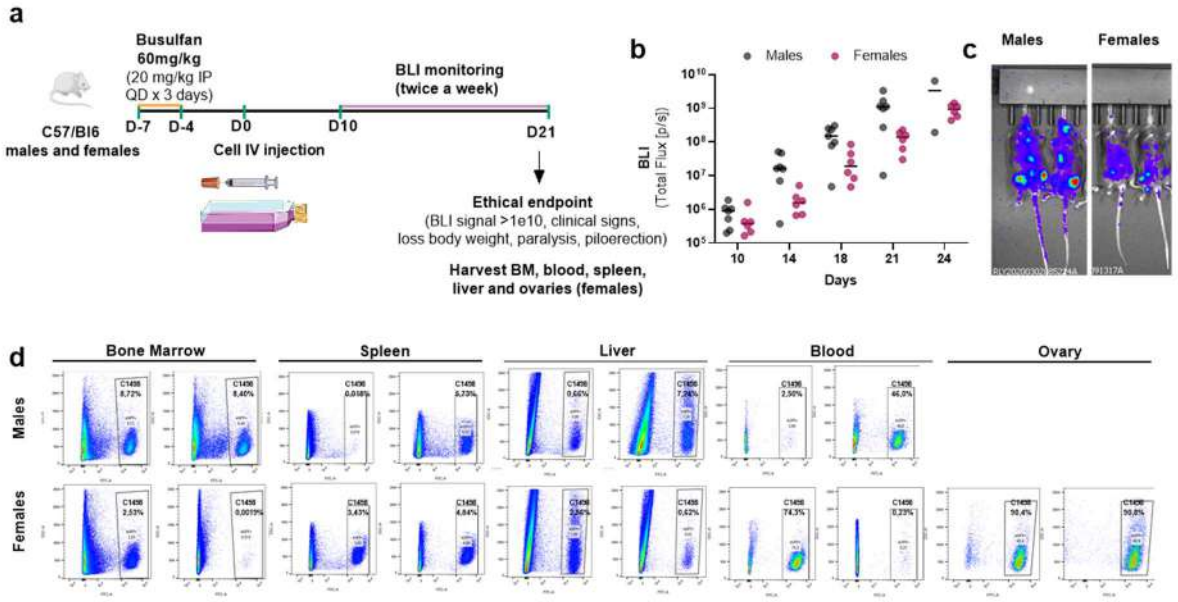


Figure 41. Evaluation of busulfan treatment and gender in C57/Bl6 mice. a) Schematic view of the study plan. b) Bioluminescence signal progression in females and males C57Bl6-C1498-Fluc-eGFP injected mice. c) Representative BLI images showing tumor cell distribution in females and males C57/Bl6. d) Assessment of leukemic cell infiltration in bone marrow, spleen, liver, blood and ovary in male and female leukemic mice.

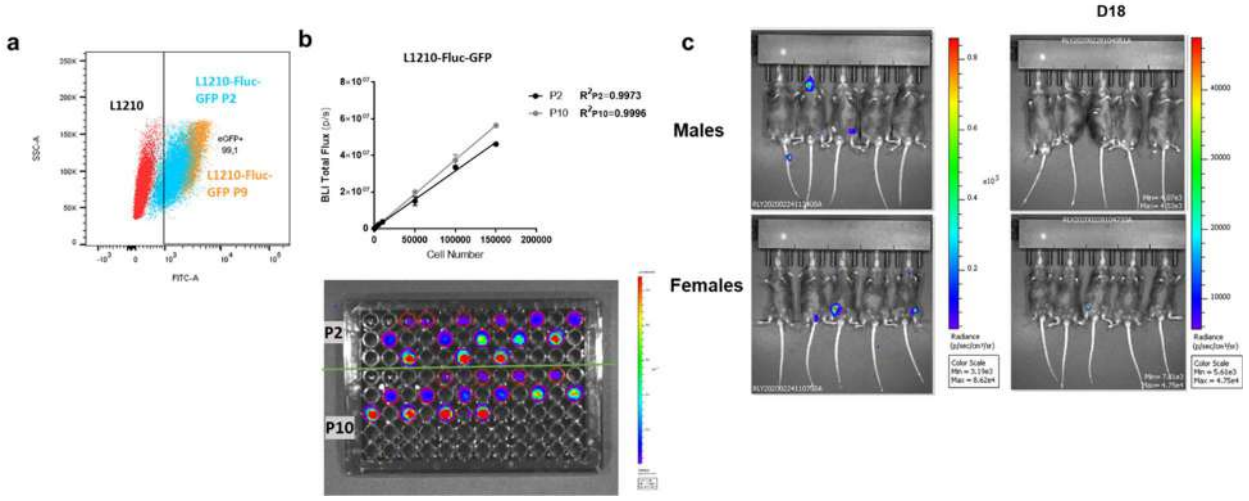


Figure 42. L1210-fLuc-GFP cell line generation and *in vivo* evaluation of the engraftment. a) GFP expression in L1210 infected with the retroviral particles. b) Luciferase *in vitro* expression in L1210 infected with the retroviral particles at cell passages 2 and 10. c) Bioluminescence images of C57/Bl6 mice intravenously injected with 1M L1210-fLuc-GFP cells at days 10 and 14 after cell injection.

3.3.2. *In vivo* efficacy assessment of EVT-701 in combination with chemotherapy

We then proceeded to the evaluation of EVT-701 in combination with chemotherapy.

The first attempt was performed in females (figure 43), and EVT-701 was administered from the first day of AraC treatment for 10 days, to abolish metabolic flexibility. However, we did not observe any benefit of the combination as measured by BLI (figure 43b). Mitochondrial membrane potential and

mitochondrial mass were also evaluated in the remaining leukemic cells in ovaries at endpoint, showing no significant difference among the different treatments (figure 43c).

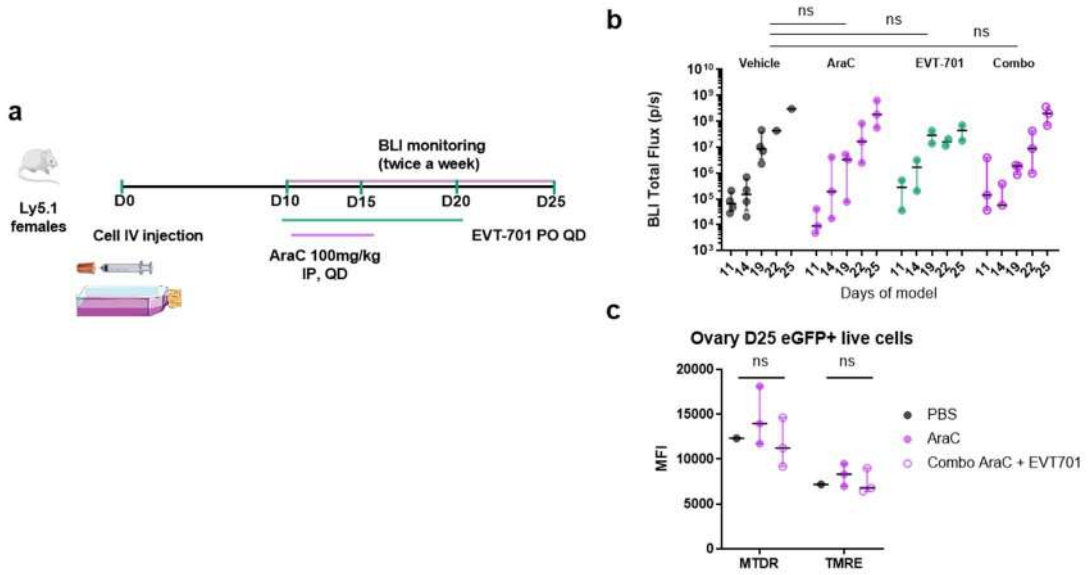


Figure 43. Evaluation of AraC, EVT-701 and their combination in C1498 syngeneic model. a) Schematic view of the study plan. b) Bioluminescence signal progression upon AraC, EVT-701 or their combination. c) FACs evaluation of the mitochondrial membrane potential and mitochondrial mass in leukemic cells in ovaries.

We then investigated the effect of busulfan in the response to AraC (figure 44). Interestingly, we could observe that mice treated with busulfan before cell injection did not relapse to disease after AraC treatment, whereas mice that did not received busulfan did (figure 44c-d). Considering that we were interested in the mitigation of AML relapse, we decided not to perform busulfan treatment for the following studies. Furthermore, busulfan also impacted white blood cell and lymphocyte counts which may result in the impairment of the immunocompetent context (figure 44b).

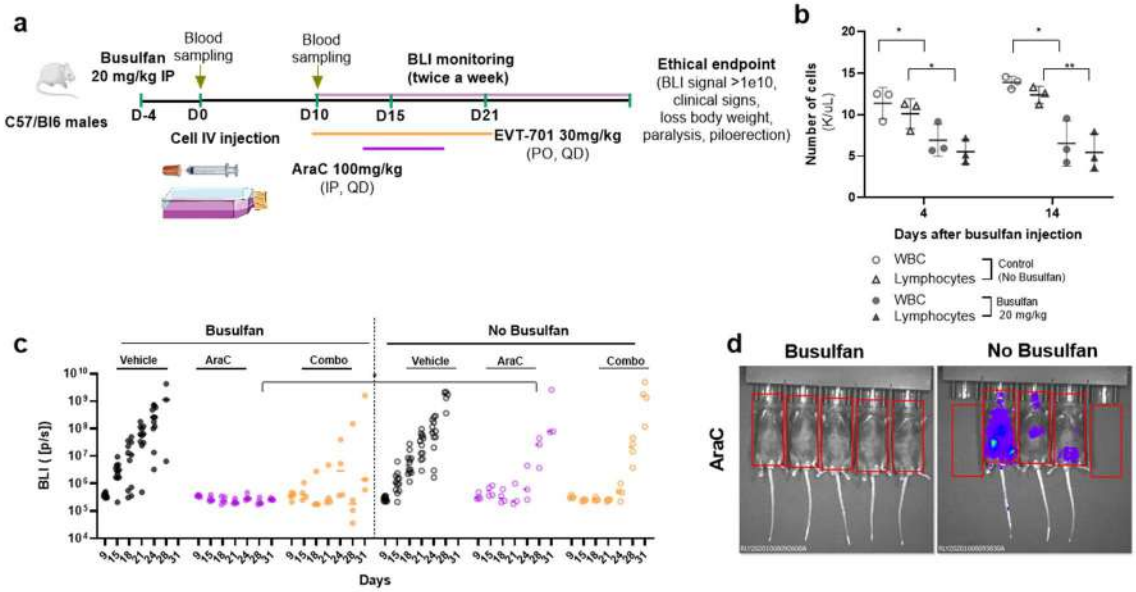


Figure 44. Evaluation of busulfan effect on the response to treatment in C1498 syngeneic model. a) Schematic view of the study plan. b) White blood cell count and lymphocyte count in mice pre-treated or not with busulfan c) Bioluminescence signal progression upon vehicle, AraC or AraC + EVT-701 combination with or without busulfan. d) BLI image of AraC treated mice pre-treated or not with busulfan.

Since in the previous experiment EVT-701 did not show benefit in combination with AraC, we decided to explore if the treatment schedule could vary the response. With that aim, we tried 2 different treatment sequences: the first one doing a priming with EVT-701 from d12, that is, 3 days before AraC administration, and maintaining EVT-701 daily treatment until last AraC administration; and the second one consisting of beginning EVT-701 treatment concomitantly with AraC, and then maintained for 3 days upon the end of chemotherapy (figure 45a). No significant difference between the different treatment schedules could be evidenced by bioluminescence imaging (figure 45b). Since bioluminescence is based on the reaction catalized by luciferase, that uses O_2 for the ATP-dependent oxidation of luciferin, we thougth that maybe bioluminescence signal could be impacted by the use of an ETCI inhibitor, that should increase the oxygen available for the luciferase. Therefore, we decided to go for an overall survival study to assess if any of the combinations increased survival. Nevertheless, none of the combinations improved mice survival when compared to AraC alone (figure 45c). Interestingly, there was one mice treated with EVT-701 that survived longer than any AraC treated mice (figure 45c). However, with such a small sample size, no conclusions could be drawn.

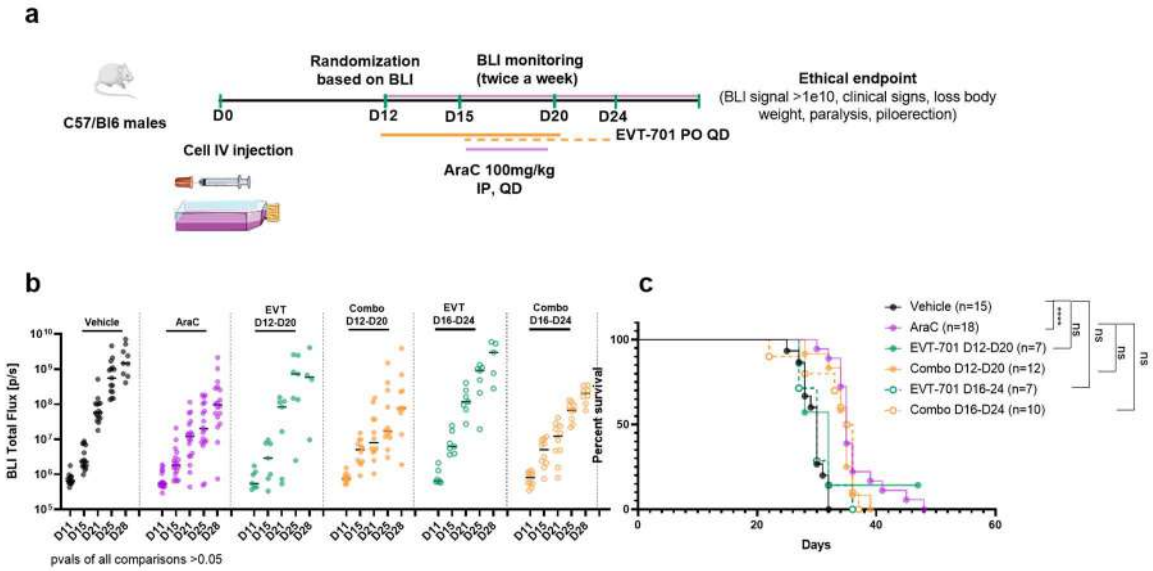


Figure 45. Evaluation of different EVT-701 administration schedules to find an effective combination regime in C1498 syngeneic model. a) Schematic view of the study plan. b) Bioluminescence signal progression upon vehicle, AraC (from d16 to d20), EVT-701 (beginning at d12 or at d16) or AraC + EVT-701 (before or after AraC) combination with different treatment schedules. c) Kaplan-Meier survival curves of the different treatment groups.

Then, we used lower doses of AraC, to discard the possibility that the AraC dose previously used were too high and it could hide any beneficial effect of its combination with EVT-701. In this study, we evaluated 25 mg/kg and 50 mg/kg, compared to the standard 100 mg/kg previously used. Since the pre-treatment with EVT-701 showed a slightly better outcome in the previous experiment, we decided to stick to that schedule and do a 3-day pre-treatment with EVT-701 before AraC administration, and then continue with EVT-701 until ethical endpoint (figure 46a). We were not able to observe any difference in bioluminescence signal, neither in survival among the different treatment groups (figure 46b-c). However,

AraC showed a dose response effect, since the higher dose of 50mg/kg slightly improved survival and decreased BLI signal when compared to 25mg/kg dose (figure 46b-c).

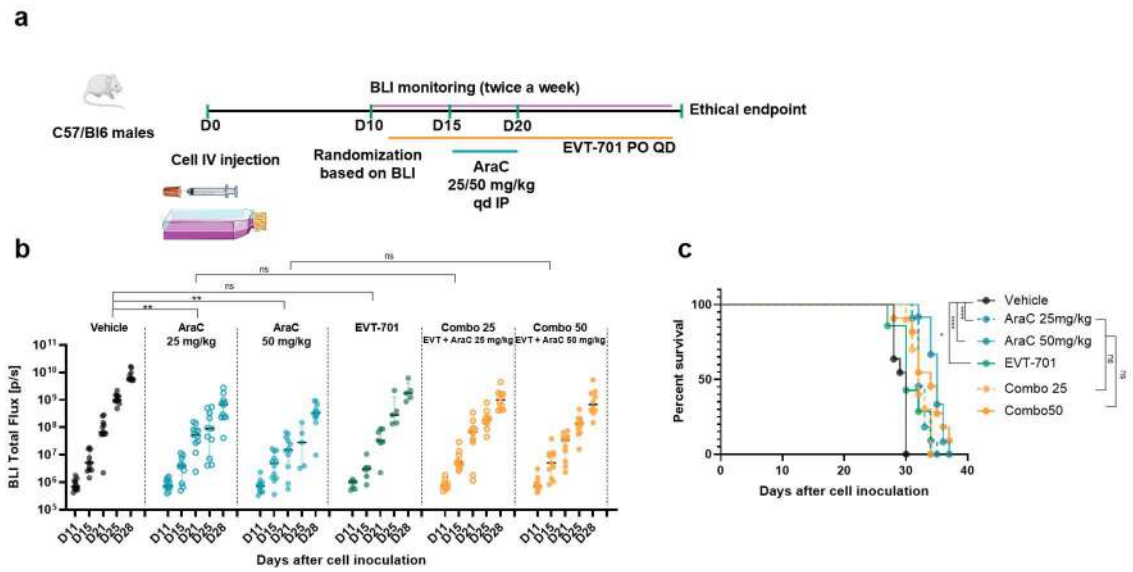
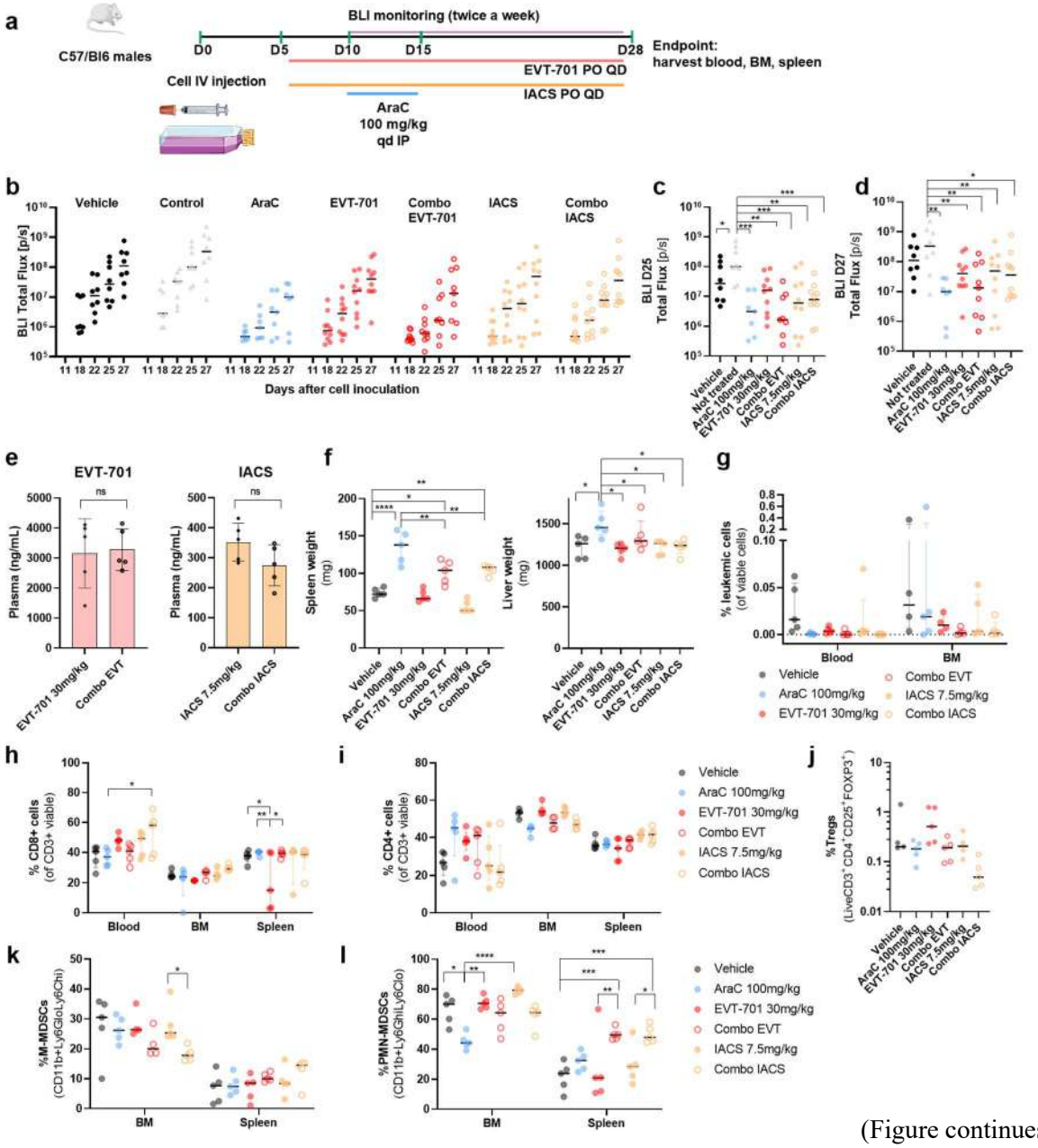


Figure 46. Low dose AraC (LDAC) regime evaluation in combination with EVT-701 in C1498 syngeneic model. a) Schematic view of the study plan. b) Bioluminescence signal progression upon vehicle, AraC 25 mg/kg or 50 mg/kg, EVT-701 or their combination. c) Kaplan-Meier survival curves of the different treatment groups.

We then decided to test IACS-010759 (hereafter, IACS) as reference compound for ETCI inhibition, and whose antileukemic activity was previously reported (Molina et al., 2018), to discriminate whether if EVT-701 lack of efficacy was model-dependent or compound-dependent. We began with an early EVT-701 or IACS pre-treatment, for 10 days before AraC administration (figure 47a) that was maintained until study termination, with the aim to maximize treatment window. We decided to use IACS at a dose of 7.5mg/kg, as previously reported for leukemia treatment in syngeneic models (Molina et al., 2018). Bioluminescence images at D25 suggested a beneficial effect of combination of EVT-701 with AraC (Figure 47 b-c), however, at D27 this effect was lost, being AraC the main driver of BLI signal decrease for all the combinations (Figure 47 b, d). Compound exposures in plasma were assessed, both for EVT-701, IACS and their combinations with AraC (figure 47e). There was no difference in the plasma concentration when comparing the chronic dosing of the compound as single agent or in combination with AraC (figure 47e). In terms of circulating concentrations, the C_{max} was 5.00µM for EVT-701 as single agent, and in combination 5.21 µM. For IACS, C_{max} in plasma were 0.69 µM when used as single agent and in combo it was 0.54 µM. Spleen weight was assessed, showing an increase in spleen weight upon AraC, observed in both IACS and EVT-701 combinations as well (figure 47f, left). Regarding liver, its weigth was only increased upon AraC treatment and in its combination with EVT-701, but not with IACS (figure 47f, right). Viable leukemic cells in peripheral blood as well as in bone marrow were evaluated, however, the percentage of eGFP-positive cells in these tissues were low (figure 47g). Nevertheless, mice treated with either of the combinations showed lower percentage of viable leukemic cells in bone marrow (BM) (figure 47g). Immune populations upon the different treatments were also evaluated (figure 47h-l).

IACs combination increased CD8 T cells (Live/CD3^{+}CD8^{+}) percentage in peripheral blood, whereas in spleen, CD8 were reduced upon EVT-701 treatment as single agent (figure 47h). Regarding CD4 T (Live/CD3^{+}CD4^{+}) cells and Tregs (Live/CD3^{+}CD4^{+}CD25^{+}FOXP3^{+}), no significant change was observed (figure 47i-j). MDSCs were also evaluated: M-MDSCs were decreased in BM upon the IACS combination with AraC vs IACS, whereas in spleen there were no detectable changes (figure 47k). PMN-MDSCs were decreased in BM upon AraC treatment, while both IACS and EVT-701 combinations with AraC increased them in spleen (figure 47l). Expression of the immune checkpoints PD-L1, CD39 and CD73 was assessed for both populations of MDSCs in spleen, finding that EVT-701 decreased PD-L1 expression when compared to AraC, EVT-701+AraC or IACS+AraC, whereas neither CD39 nor CD73 expression were altered (figure 47m). No change was identified for any of the ICs markers in spleen assessed on PMN-MDSCs (figure 47n).



(Figure continues)

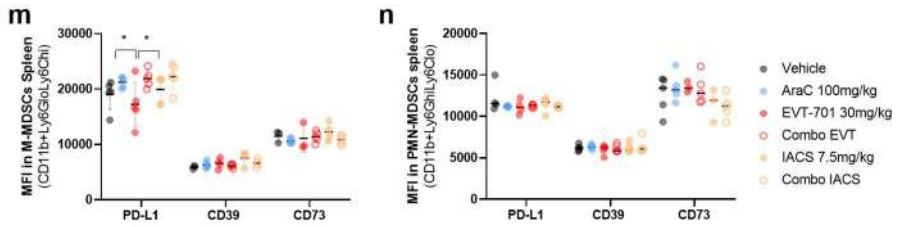


Figure 47. EVT-701 and IACS evaluation, as single agents or in combination with AraC in C1498 syngeneic model. a) Schematic view of the study plan. b) Bioluminescence signal progression upon AraC, EVT-701, IACS, or their combination with chemotherapy. c) Bioluminescence signal at D25. d) Bioluminescence signal at D27. e) EVT-701 and IACS plasma exposures, as single agents or in combination, after chronic dosing. f) Spleen (left) and liver (right) weights. g) Percentage of leukemic cells in blood and bone marrow upon the different treatments measured by FACs. h) Percentage of CD8 T cells in blood, bone marrow and spleen in the different treatment groups. i) Percentage of CD4 T cells in blood, bone marrow and spleen in the different treatment groups. j) Percentage of Tregs in spleen. k) Percentage of M-MDSCs cells in bone marrow and spleen in the different treatment groups. l) Percentage of PMN-MDSCs cells in bone marrow and spleen in the different treatment groups. m) PD-L1, CD39 and CD73 expression of M-MDSCs in spleen. n) PD-L1, CD39 and CD73 expression of PMN-MDSCs in spleen. Difference are ns unless otherwise specified.

3.3.3. *In vivo* efficacy assessment of EVT-701 in combination with immunotherapy

Since EVT-701 showed to decrease PD-L1 expression *in vitro,* we then proceeded to evaluate the combination of EVT-701 with immunotherapy *in vivo*, in our C1498-C57/Bl6 syngeneic model. We decided to combine EVT-701 with monoclonal anti-PD-1 therapy, to impair the PD-1/PD-L1 axis from "both sides" and eventually, improve immunotherapy outcome (figure 48). Mice were injected with the leukemic cells, and five days later, we began to treat with EVT-701 (figure 48a). From day 9 after cell injection, anti-PD-1 was administered each 3-4 days until day 24, while EVT-701 was dosed daily until D27 (figure 48a). BLI, monitored twice a week, showed that only a small proportion (2/15) of the mice responded to anti-PD-1 (figure 48b, d), what correlated with survival data (figure 48c). At D85 after cell injection, BLI images were taken from the remaining mice, showing that in mice treated with the combination EVT-701 + anti -PD-1 leukemia was not detectable, whereas in the remaining mouse treated with vehicle + anti-PD-1, leukemia was progressing (figure 48d-e). In addition, despite being a small sample size, the number of alive mice at D85 treated with the combination doubled that of mice treated just with immunotherapy (figure 48d-e). To further evaluate if there were any differences regarding immune response between the remaining mice, we performed ELISpot assay (Möbs and Schmidt, 2016). We observed IFN-γ production (as marker of T cell activation upon antigen recognition) upon re-estimulation with leukemic cells in both anti-PD1 and combo EVT-701+ anti-PD-1 groups (figure 48f,h). Nevertheless, there was no difference in IFN-γ production between the two groups (figure 48f,h). In parallel, no T cell response was detected upon C1498 stimulation of splenocytes coming from naïve mice, highlighting the specificity of the response (figure 48f,h). The positive control also worked, as anti-CD3 monoclonal antibody activated T cells in all the conditions tested (figure 48g,h).When evaluating the percentage of the different splenic T cell populations, we couldn't find either any difference between groups (figure 48i). We could conclude that in combo-treated surviving animals, immune system defeated tumors and returned to a steady state, with limited number of tumor-specific T effector memory cell

population.

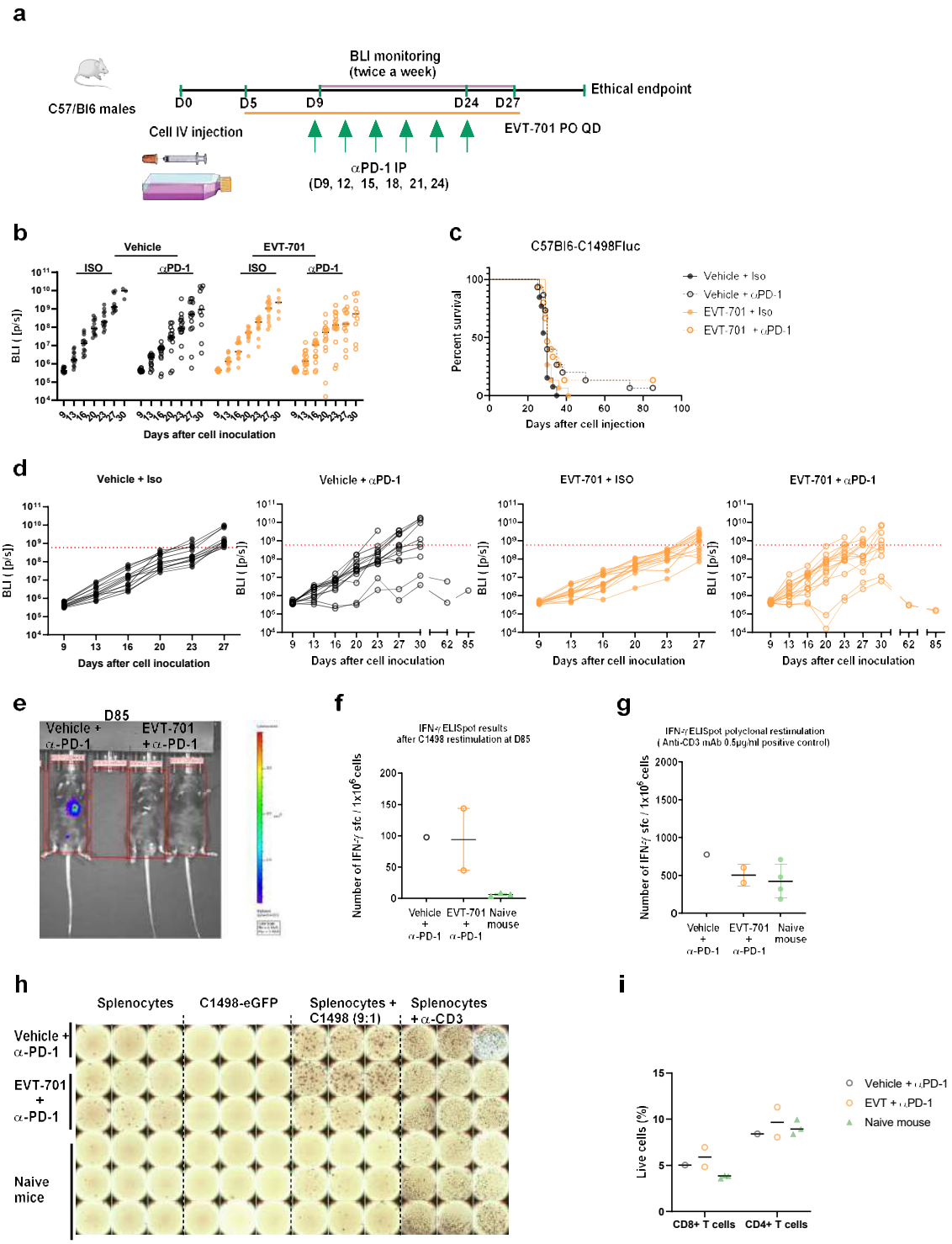


Figure 48. EVT-701 in combination with immune checkpoint therapy in C1498 syngeneic model. . a) Schematic view of the study plan. b) Bioluminescence signal progression upon Vehicle, EVT-701, anti-PD-1 or isotype and their combination. c) Kaplan-Meier survival curve. d) Individual BLI signal progression over time in each group. e) BLI image of vehicle + anti-PD-1 or EVT + anti-PD-1 treated mice at D85. f) ELISpot results after C1498 re-estimulation of splenocytes at D86. g) ELISpot positive control, consisting on splenocytes stimulated with anti-CD3 monoclonal antibody at a concentration of 0.5 µg/mL. h) ELISpot image. i) T cell percentage in the spleen of the mice used for the ELISpot study. Difference are ns unless otherwise specified.

4. Discussion and perspectives.

4.1. EVT-701 targets ETCI to exploit metabolic vulnerabilities in cancer: OxPhos subtypes, LKB1 deficiency and chemoresistance as suitable contexts.

EVT-701 is a new, safe and selective ETCI inhibitor

This thesis was part of the preclinical development of EVT-701 as therapeutic agent in cancer. Starting just after selection of this optimized lead compound, the studies presented here altogether aim to characterize potential of EVT-701 to become a clinical asset. In brief, selectivity, distribution, safety and efficacy. First, we showed that EVT-701 is a selective compound, since even at the high dose of 10 µM, activity of only one out of 198 kinases was affected by more than 30% (MLK2). However, as mentioned before, we do not expect any issue *in vivo* due to the high dose needed to modulate activity of this kinase. When assessing propensity of EVT-701 to compete with binding of reference ligands to their receptors as part of Cerep SafetyScreen®, we identified that only A2AR ligand binding was inhibited by more than 50% at the high dose of 10 µM EVT-701. Despite not being a concentration achieved *in vivo*, it may be an attractive off-target effect, due to the potential of A2AR antagonists in cancer treatment (Sitkovsky, 2020). On the distribution and bioavailability side, compound is widespread thoughout the body but in brain. Regarding safety, EVT-701 showed promises as no emesis issue was observed in ferret, as opposed to BAY 87-2243. One limitation of the compound is its interaction with efflux pumps, which makes it unsuitable for chronic QD regimens. However, this could be good to limit to maximum or even prevent any possible adverse effect, like lactic acidosis, which can be observed upon strong ETCI inhibition (Kwong and Brubacher, 1998; Misbin, 2004; Yendapally et al., 2020).

We characterized the effect of EVT-701 on metabolism in human and murine lung cancer, DLBCL and AML cell lines, leading to the conclusion that it induces a metabolic shift toward glycolysis in AML and DLBCL cells, increasing glucose consumption and lactate production while decreasing mitochondrial ATP production, and the cellular NAD^+/NADH ratio. In addition, EVT-701 decreased *in vitro* proliferation of lung cancer, DLBCL cell lines, as well as of AML cells, where it was evaluated both as a single agent and in combination with AraC. Succinate OCR recovery experiment, as well as reduction of exogenous ETCI activity in incubation with the compound shows that EVT-701 exerts its action by blocking specifically the first complex of the ETC.

Targeting OxPhos dependency in cancer with EVT-701

OxPhos DLCBL was previously shown to be transcriptionally and phenotypically enriched in OxPhos and ETC subunits (Monti et al., 2005; Caro et al., 2012), therefore the rationale to target the first component of the ETC was evident. NSCLCs tumors were reported to show increased TCA cycle activity than non cancerous lungs (Hensley et al., 2016; Fan et al., 2009), and to depend on mitochondria for tumorigenesis in the case of KRAS-driven lung cancer (Weinber et al., 2010). In addition, upon LKB1 deficiency, energetic imbalance has been previously indentified as the driver of enhanced ETCI sensibility (Shackelford et al., 2013). This is due to the inability of cells harbouring this defect to activate AMPK upon low energy status, impairing therefore glycolytic enhacement to compensate for mitochondrial respiration inhibition. We confirmed this in our settings using H460 cells as LKB1-deficient model (Dong et al., 2012). In addition, we identified that transcriptomes of TCGA-lung cancer patients with deficiency or low expression levels of LKB1 were enriched for OxPhos and mitochondrial gene signatures, suggesting an enhanced OxPhos function for these patients. Several studies support this finding, since upregulation of OxPhos and mitochondria genes upon LKB1 deficiency have been already reported (Kaufman et al., 2014), along with enhanced TCA metabolite levels (Faubert et al., 2014), increased mitochondrial membrane potential and respiration (Whang et al., 2015). If this is the case, then LKB1 deficiency would elicit a synthetical lethal vulnerability with OxPhos inhibition, due to increased reliance of mitochondrial respiration in parallel with an abrogated energy stress response in such cells. However, literature regarding impact of LKB1 deficiency in metabolism is diverse since other studies have linked LKB1 loss to mitochondrial defects in cancer (Shackelford et al, 2013) and in murine hematopoietic stem cells (Gan et al., 2010). Overall, we have re-confirmed that certain conditions, including LKB1 deficiency in lung cancer (Shackelford et al., 2013) or increased reliance on OxPhos metabolism in Eμ-myc OxPhos clones of Non-Hodking lymphoma in murine context and its human equivalent OxPhos-DLBCL (Chiche et al., 2019), make these tumors vulnerable to ETCI inhibition, as shown by the decrease in tumor weights and increased survival observed upon EVT-701 treatment. Interestingly, LKB1 deficiency is often associated with mTOR activation (Dong et al., 2012; Shackelford et al., 2013; Faubert et al., 2014; Momcilovic and Shackelford, 2015), since functional LKB1 induces phosphorylation of TSC2 by AMPK to block mTORC1 activity (Inoki et al., 2003; Shaw et al., 2004; Gwinn et al, 2008). Remarkably, DLBCL-GAPDH^{low} expression samples also showed mTORC1 activation (Chiche et al., 2019). Therefore, mTOR inhibitors can also be of interest to target the metabolic vulnerabilities induced by LKB1 mutation, and combination studies with ETCI inhibitors could improve trial outcomes by targeting metabolic flexibility.

Targeting chemoresistance in AML with EVT-701

Another interesting setting to test EVT-701 is, without doubt, chemotherapy resistance. There is an emergency to find treatment options for patients with refractory or relapsed disease. One of the approaches to do so, is through the eradication of chemoresistant cancer cells by targeting specific

metabolic vulnerabilities. We showed that the genetic signatures of shorter survival groups within TCGA-AML and Metzeler cohorts are enriched in OxPhos and mitochondrial metabolism gene sets, underscoring the involvement of mitochondrial metabolism in disease progression and at relapse. In addition, we identified that AraC-resistant murine leukemic cells have increased oxygen consumption rate, mitochondrial mass and mitochondrial membrane potential, their transcriptomes are enriched for OxPhos gene signatures, as well as they overexpress ETC components compared to AraC-sensitive counterparts. Therefore, in summary, we have demonstrated that the OxPhos phenotype induced by metabolic adaptation to chemotherapy is a common feature of murine and human AML cells (Farge et al., 2017) as well as in other leukemias such as chronic myeloid leukemia (Kuntz et al., 2017), B-lymphoblastic leukemia (Dobson et al., 2020) and many other types of cancer (Haq et al., 2013; Vazquez et al., 2013; Vitiello et al., 2017; Fischer et al., 2019; Zhang et al., 2019b; Masoud et al., 2020). Targeting OxPhos in leukemia, in combination with other therapies such as AraC or venetoclax, has emerged in the recent years as a rather safe and promising strategy to improve treatment outcome of AML patients. With that aim, we developed and characterized the C1498 syngeneic model, showing evidence of its robustness and reproducibility. Nevertheless, we could not find the appropriate conditions in which EVT-701, nor IACS, could work in vivo in the C1498-AML syngeneic model. We tried different EVT-701 treatment onsets (day 10 vs day 5 after cell injection), different dosing schedules (priming with EVT-701 before AraC treatment, or beginning EVT-701 treatment concomitant to AraC administration), as well as we evaluated the combination with lower doses of AraC in case that any synergistic effect of the combination could be masked by high AraC doses. One of the factors that can have influenced this result is that, as it will be discussed in the next chapter of the discussion, C1498 was the EVT-701 “more resistant” cell line in terms of proliferation in vitro. Maybe other AML syngeneic murine models, such as the AML1/ETO9a or the MLL/ENL could have responded differently (Zuber et al., 2009), what would open the door to future exploration. We also tried to establish L1210 syngeneic model but, as reported in the results section, despite the maintenance of both BLI and GFP signal along *in vitro* passages, the disease was not established *in vivo* and BLI signal tended to decrease *in vivo* as time passed by, probably due to immune response directed towards GFP-positive cells. The use of other clones, maybe less immunogenic, can be evaluated for further development of the model.

As mentioned above, IACS-010759 was not able to induce any anti-leukemic effect in our C1498-AML syngeneic model. This is globally aligned with results from Molina and colleagues, who evaluated IACS both in syngeneneic (C57/Bl6 injected with MLL/ENL KRASG12D, p53null; MLL/ENL KRASG12D, p53wt and AML1/ETO9a, KRASG12D, p53null) and immunodeficient AML mice models (NSG mice OCI-AML3 and PDX). While IACS-010759 treatment significantly improved the survival in OCI-AML3 and PDX immunodeficient mice models (from 30 days -35 days as OS for vehicles to 70 days for IACS treated mice), IACS effect was marginal if any, increasing mice survival by maximum 2 days in the three immunocompetent models tested (Molina et al., 2018). These results, in parallel with ours, may suggest

the importance of lactate in the immune response in the context of AML. As described in the introductory chapter "1.3.2.3.2.iii. Lactate", lactate has been reported to abrogate immune function by several mechanism and targets, which can be an issue for anti-tumor efficacy approaches that target ETCI inhibition in patients. In fact, lactate increase without associated metabolic acidosis has been reported in IACS phase I clinical trials (Yap et al., 2019). It is worth to underscore that one of the risks of targeting ETCI is the induction of lactic acidosis. Phenformin was removed from the market for this reason (Kwong and Brubacher, 1998). With metformin, lactid acidosis cases are very few, and they are linked to pathological conditions such as heart failure or hypoxia (Misbin, 2004; Yendapally et al., 2020). All new ETCI inhibitors should carefully consider this potential adverse effect. Another factor is that immune landscape in AML is considered to be immunosuppressive (Isidori et al., 2014), and maybe the impact of lactate cooperates more efficiently in the disease, abrogating any beneficial effect of ETCI inhibition in AML-syngeneic models. That's why the assessment of drugs to impair lactate efflux, such as MCTs inhibitors or LDHA inhibitors, may be of great interest in combination with ETCI inhibitors.

Combination of EVT-701 with immunotherapy would require further optimization

Regarding the combination with immunotherapy, and assuming that lactate does not drive a completely immunosuppressive landscape, testing lower doses of EVT-701, different administration schedules (on- and off-treatment periods), and even more importantly, different onset of EVT-701 administration can be determinant factors, since, ETCI inhibition can boost T cell response after clonal expansion has taken place, otherwise, inhibiting OxPhos can impair T cell activation and expansion due to lack of energy to support these processes (Pearce et al., 2009). Therefore, optimization of the conditions could potentially result in a more positive outcome. Another limitation regarding the in vivo response to EVT-701 in AML is the lack of biomarker specific for EVT-701 activity on tumor cells, since systemic lactate denotes a global organism response, but not directly target engagement on tumor cells. In solid tumors, the evaluation of the glycolytic ATP content in harvested and dissociated tumors is easier than in AML, where the cancer cells are more "disseminated".

Future directions: further evaluation of EVT-701 in other models of anti-cancer therapy resistance and different contexts of increased OxPhos dependency

Overall, it seems clear that EVT-701, or other ETC inhibition approaches, should be evaluated in context of high OxPhos metabolism and OxPhos dependency since, as demonstrated by us and others (Chiche et al., 2019), ETCI inhibition in cancer cells with glycolytic phenotypes did not result in improved outcome. One of these interesting contexts where EVT-701 could be further evaluated is in lung cancer with mutated components of the chromatin remodeling SWI/SNF complex. These mutations, for instance in SMARCA4 or ARID1A, have been shown to increase OxPhos both transcriptomically and phenotipically while they blunted transcriptional response to energy stress (Lissanu Deribe et al., 2019). The use of other cancer models of chemoresistance previously reported to depend on OxPhos could be appropriate settings to further evaluate combination therapies with EVT-701. Other scenario could be NSCLC resistance to

cisplatin (Wangpaichitr et al., 2017; Cruz-Bermúdez et al., 2019), chemotherapy resistance in breast cancer (Lee et al., 2017b) or gemcitabine resistance for high OxPhos pancreas tumors (Masoud et al., 2020). In addition, resistance to targeted therapy, such as to EGFR or BRAF inhibitors in EGFR- driven lung adenocarcinoma or melanoma respectively, are also interesting contexts to further evaluate EVT-701, since resistance to both inhibitors is characterized by enhanced OxPhos (Haq et al., 2013; De Rosa et al., 2015; Martin et al., 2016).

4.2. Metabolic mechanism of action of EVT-701

EVT-701 induces a glycolytic shift and decreases intracellular levels of aspartate and serine

Among the changes in the metabolite content induced by EVT-701, there are several remarks. On the one hand, the increase in lactate secretion and glucose uptake is not surprising since glycolysis acts as a compensatory mechanism upon OxPhos inhibition and this mechanism has been previously described with other ETCI inhibitors such as metformin, phenformin and IACS (Scotland et al., 2013; Molina et al., 2018; Vangapandu et al., 2018, Veiga et al., 2018). As previously mentioned, a fall in aspartate levels has been observed with IACS-010759 (Molina et al., 2018). This can be explained by the drop in NAD^+/NADH ratio levels induced upon ETCI inhibition, which impairs mitochondrial malate dehydrogenase (MDH2) activity, preventing OAA formation and thus, mitochondrial aspartate biosynthesis (Birsoy et al., 2015; Sullivan et al., 2015). To compensate this, reductive carboxylation of glutamine by ATP-citrate lyase is put in place, by which citrate lyase converts Gln-derived citrate into OAA that subsequently will generate cytosolic asparte by GOT1 action. However, aspartate levels obtained by this reductive biosynthetic pathway are lower when compared to the classical mitochondrial route, and not even enough to maintain cell proliferation without external supply of NAD^+ source, which induces a fall in aspartate levels (Birsoy et al., 2015; Sullivan et al., 2015). We also observed a decrease in serine levels upon EVT-701 treatment. The drop in NAD^+/NADH levels induced by ETCI inhibiton, this time with rotenone, has been shown to abrogate serine biosynthesis (Diehl et al., 2019). The authors also showed that upon rotenone treatment, serine biosynthesis could be restored by supplementation with an exogenous electron acceptor that regenerated NAD^+, underscoring the role of NAD^+ and of functional ETC for serine biosynhtesis (Diehl et al., 2019). In the same line, it's been reported that upon several respiration inhibitors (metformin, phenformin and rotenone for ECT I, antimycin for ETC III and oligomycin for ETC V), mitochondrial serine catabolism via folate pathway mediated by MTHFD2 becomes the main contributor to NADH generation, since NADH production by TCA cycle is inhibited upon respiration inhibition, but not serine catabolism (Yang et al., 2020). In the same work, authors found that serine biosynthesis nearly ceased upon metformin treatment. Therefore, decreased serine and aspartate levels have been previously reported with other ECT I inhibitors. Interestingly, it resulted in auxotrophy for these amino acids as well as in diminished purine or pyrimidines (respectively)

biosynthesis (Birsoy et al., 2015; Sullivan et al., 2015; Diehl et al., 2019; Yang et al., 2020), providing a mechanism by which ETCI inhibition results in decreased proliferation.

Reductive carboxylation may explain why TCA intermediates, with the exception of succinate, are not affected by EVT-701

Perhaps one of the most unexpected metabolomic results was not to observe a global decrease in TCA cycle intermediates upon EVT-701 treatment, as reported in literature for other ETCI inhibitors (Andrzejewski et al., 2014; Janzer et al., 2014; Molina et al., 2018). However, some studies have shown different effects of ETCI inhibition in TCA intermediates. In cancer cells, it has been reported that metformin or rotenone induced no changes in TCA cycle metabolites, due to the use glutamine-derived reductive carboxylation of α-KG via NADPH-dependent isocitrate dehydrogenase (IDH) to generate citrate, acting as the carbon source to maintain TCA cycles intermediates (Mullen et al., 2011). More recent studies have proposed a bidirectional TCA cycle upon mitochondrial disfunction (upon mutations, drugs or hypoxia) that requires both oxidative and reductive pathways (Mullen et al., 2014). The oxidative pathway produces reducing equivalents at the level of α-KGDH and nicotinamide nucleotide transhydrogenase, required for NADPH generation and therefore, for IDH "reverse" function (Mullen et al., 2014). Interestingly, it's been found that succinate accumulation was not a "general" indicator of reductive carboxylation in cells (Mullen et al., 2014) as it was previously thought (Mullen et al., 2011). Upon ETC III inhibition with antimycin, or upon hypoxia, reductive carboxylation of glutamine-derived α-KG induced succinate accumulation (Mullen et al., 2014). However, an important contribution of this work is that they showed that succinate production through reductive carboxylation of glutamine requires functional ECT I (Mullen et al., 2014). Mechanistically, the reduction of fumarate to succinate catalyzed by reverse SDH constitutes the final step in the reductive carboxylation, and it requires the transference of reducing equivalents from NADH to ETC I, from here to CoQ and eventually to ETC II (SDH) to catalyze the reduction, highlighting the role of a functional ETCI for the succinate generation upon glutamine reductive carboxylation (Mullen et al., 2014). Therefore, it is to expect that cells with impaired ETCI have lower succinate levels, which is what we observed with EVT-701. Interestingly, Scotland and colleagues suggested that reductive carboxylation of glutamine-derived α-KG was the mechanism responsible for TCA intermediate replenishment upon metformin treatment in MOLM14 (Scotland et al., 2013). Interestingly, they observed that metformin had opposite effects on TCA intermediate levels in U937 and MOLM14 cells, since it decreased TCA metabolites in U937 whereas in MOLM14 they were augmented (Scotland et al., 2013). They explained the divergent response by stressing differences in the basal energetic status of both cells, as well as by the preferential use of glutamine-derived reductive carboxylation of α-KG to fuel TCA cycle by MOLM14. In spite of the compatibility of our results with glutamine reductive carboxylation, metabolomics tracing experiment with labelled glutamine should be performed to confirm if that is the case.

Overall, we showed that EVT-701 is a specific ETCI inhibitor, that metabolically induces a glycolytic shift as well as other metabolic changes resulting from the specific inhibition of ETC I.

EVT-701 decreases UDP-GlcNAc, which could explain the decreased membrane expression of PD-L1 and CD39

Remarkably, the intracellular levels of UDP-GlcNAc were decreased upon EVT-701treatment. UDP-GlcNAc is a sugar nucleotide that constitutes an essential building unit of glycosaminoglycans, proteoglycans, glycolipids, and for protein modifications by O- and N-linked protein glycosylation (Tran et al., 2020). UDP-GlcNAc synthesis requires glucose, sharing the two first steps of glycolysis, branching at F6P formation. At this point, the rate limiting enzyme glutamine fructose-6-phosphate amidotransferase (GFAT) converts F6P and glutamine to glucosamine-6-phosphate (GlcN6P) and glutamate. Alternatively, GlcNAc kinase (GNK) can generate GlcN6P from glucosamine entering the cell. Subsequently, GlcN6P and acetyl-CoA generate N-acetylglucosamine-6-phosphate (GlcNAc-6P) in a reaction catalyzed by the enzyme glucosamine-phosphate N-acetyltransferase (GNPNAT). GlcNAc-6P is isomerized into GlcNAc-1-P by the GlcNAc phosphomutase PGM3/AGM1. Lastly, UDP-N-acetylglucosamine pyrophosphorylase generates UDP-GlcNAc from GlcNAc-1P and UTP (Akella et al., 2019; Tran et al., 2020). This pathway is known as the hexosamine biosynthetic pathway, and since it requires serveral macromolecules for UDP-GlcNAc synthesis, it's being seen as a sensor of energy availability and nutritional status in the cells. As previously mentioned, UDP-GlcNAc is indispensable for O-GlcNAcylation, taking place in cytosol, nucleous and mitochondria and being catalyzed by O-GlcNAc transferase (Biwi et al., 2018); as well as for the O- and N-linked protein glycosylations that take place in the endoplasmic reticulum and the Golgi apparatus (Akella et al., 2019). N-glycosylation is an essential process for protein stability and maturation, especially for membrane proteins as well as for cellular protein quality control (Moremen et al., 2012; Lee et al., 2015; Chandler and Costello, 2016). N-linked glycosylation has been reported to stabilize PD-L1 and prevent its proteasomal degradation, as well as to enhance interaction with its ligand PD-1 on T cells (Li et al., 2016; Hsu et al., 2018). Therefore, a defect in PD-L1 glycosylation directs PD-L1 for ER-associated degradation. In the case of CD39, it's been published that only the fully glycosylated protein is active and locates to the membrane, therefore (Zhong et al., 2001), impairment in N-glycosylation prevents CD39 localization and activity. We thought that potentially, lower levels of UDP-GlcNAc induced by EVT-701 could impact PD-L1 and CD39 glycosylation state and therefore their stability. Aiming to test that, development of a proteomics-based assay has been initiated. Protein-specific peptides (for both native and glycosylated forms) have been characterized from pure protein digestion. We now need to be able to detect and quantify those from cell homogenates and then apply this to treated cells. Hopefully something to be completed in the near future.

Future directions

A remarkable observation is that EVT-701 seems to be more potent in human than in murine leukemic cells. At the same EVT-701 dose, OCR is decreased by 70% in murine leukemic cell lines, whereas in the

human cell lines, only MV4-11 inhibition is lower than 90% (75%). OCR inhibition exerted by EVT-701 reached to 95% in MOLM13, 98% in THP1, and 90 % in U937. Therefore, EVT-701 exhibits greater inhibitory capacity of oxygen consumption rate in human AML cell lines than in murine counterparts. In proliferation the same pattern is repeated. EVT-701 elicited a stronger inhibition of proliferation in human AML cell lines than in murine leukemic cell lines, C1498 being the most "resistant" (% of proliferation inhibition: 47% C1498 < 66% L1210 < 70% U937 and MOLM13 < 76% MV4-11 and THP1).

Moreover, the additive effect of the EVT-701 + AraC combo to decrease viability of human AML cells is encouraging, since the response obtained with the combination of both is much greater than with the single agents. This would allow to reduce the concentration of the drugs as single agents in the combination without losing efficacy, what is of great relevance to avoid therapy toxicity and morbidity that usually appear at high doses of compounds and could make elderly people eligible to such AraC-based combinations.

Overall, these results may suggest that EVT-701 could be more potent in the human pathology than in the murine one, opening a path to future research with the compound.

4.3. Emerging immunomodulatory role of ETCI

AraC and ETCI inhibition exert opposed effects on PD-L1 and CD39 membrane levels in leukemic cells

We showed that AraC increased CD39 and inducible PD-L1 levels at cell surface, while EVT-701, as single agent decreased membrane expression levels of both of these proteins and, when used in combination with AraC, neutralized the increase induced by the chemotherapeutic agent *in vitro*. In addition, we showed that the decrease of immune checkpoints is not exclusive to EVT-701, since other ETCI inhibitors, such as IACS and, as reported in literature, metformin elicited similar responses (Cha et al., 2018; Li et al., 2018). Since NAD^+ supplementation abrogated the decrease of inducible PD-L1 expression but not that of CD39 upon ETCI inhibition, and considering that IFN-γ only modulates inducible PD-L1 expression without affecting CD39 (Lee et al., 2006; Garcia-Diaz et al., 2017; Castro et al., 2018; Jorgovanovic et al., 2020), we propose that different regulatory mechanisms concur to achieve modulation of these two immune checkpoint components.

Inducible PD-L1 expression may be regulated by OxPhos

PD-L1 expression has been described to be regulated by several factors. Maybe one of the most characterized is the IFN regulatory factor 1 (IRF1). IRF1 is the one that binds to PD-L1 promoter, but it is involved in a bigger axis, constituted by IFN-γ-JAK1/JAK2-**STAT1**/STAT2/**STAT3**-IRF1 (Lee et al., 2006; Garcia-Diaz et al., 2017). In PD-L1 promoter, regulatory elements responsive to STAT3 have also been described (Song et al., 2018; Zerdes et al., 2019; Parackova et al., 2020). STAT1 and STAT3 have

been reported to be upregulated by IL-27 signaling, which results in increased PD-L1 expression (Parackova et al., 2020). In similar settings, expression of CD39 as well as of other immunomodulatory molecules such as IDO was increased in dendritic cells, while differentiation of naive T cells into effector T cells was decreased (Mascanfroni et al., 2013). By the way, PD-L1 inducible expression has been reported to be upregulated through IFN-γ activation of the NF-κB pathway (Gowrishankar et al., 2015). Interestingly, recent work identified ETCI function to be essential for IFN-γ signaling. Indeed, genetic or pharmacological abrogation of ETCI function was sufficient to impair IFN-γ signaling as denoted by decreased levels of PD-L1 and CD40 (Kiritsy et al., 2020), whereas manipulation of other ETC complexes had no effect. This study suggests a direct link between NAD metabolism, ETCI and IFN-γ signaling. In addition, it has been recently observed that pancreatic stem cells grown in galactose and therefore, with an increased OxPhos, had increased expression of PD-L1 compared to stem cells grown in glucose, suggesting a direct relationship between PD-L1 upregulation and OxPhos function (Valle et al., 2020). In NSCLC resistance to cisplatin it has also been observed an increase in PD-L1 that correlated with higher OxPhos reliance as well as with increased epithelial mesenchymal transition capacity of cells resistant to therapy (Wangpaichitr et al., 2017). Overall, these studies suggest that OxPhos, and especially ETC I, sustains upregulated PD-L1 expression.

NAD could be involved in the regulation of PD-L1 expression through the modulation of SIRT1 and or/TET1

Recent works have established a more direct connection between NAD^+ metabolism and PD-L1 expression, however, their conclusions are not aligned. Lv and colleagues proposed that NAD^+ pool maintains Tet1 expression and activity by sustaining α-KG levels. In parallel, IFN-γ signaling induces Stat1 phosphorylation and activation. Phospho-Stat1 stabilizes Tet1 binding to Interferon regulatory factor 1 (Irf1) promoter, which is subsequently demethylated and leads to downstream PD-L1 expression on tumors (Lv et al., 2020). Therefore, an impairment in NAD synthesis resulted in decreased PD-L1 tumor expression via low Tet1 activity, while increasing the NAD pool resulted in an enhanced PD-L1 expression in presence of IFN-γ (Lv et al., 2020). Later on the same year, Li and colleagues reported opposite observations in glioblastoma. They showed that NAD^+ depletion induced by the NAMP inhibitor GMX1778 upregulated PD-L1 expression in glioblastoma cells (Li et al., 2020), but, surprisingly, they reported increased recruitment of CD8 and CD4 T cells as well as a reduction of M2-macrophages and $FOXP3^+$ Tregs within the tumors (Li et al., 2020). Usually, PD-L1 is associated with immunosuppression, since the binding to its ligand, PD-1, on T cells induces apoptosis of antigen-specific T cells (Blank et al., 2004; Barber et al., 2005; Ostrand-Rosenberg et al., 2014) and it promotes Treg development and function (Francisco et al., 2009), therefore, the results of Li and colleagues are unexpected. Nevertheless, one interesting remark, is that Lv and colleagues focused their study on inducible PD-L1, thus, upon IFN-γ stimulation. However, Li's work is focused on constitutive PD-L1 expression, as they clearly highlighted the absence of any cytokine produced by immune cells within their study. Therefore, one can consider the

presence or absence of IFN-γ (physiologically produced by immune cells upon activation) can be a key factor for the regulation of PD-L1 expression exerted by NAD^+. Remarkably, NAD^+ is a necessary cofactor for the activity of the class III histone deacetylase sirtuin 1 (SIRT1, Etchegaray and Mostoslavsky, 2016). Resveratrol is a SIRT1 agonist that has been reported to stimulate ETCI activity in order to increase NAD^+/NADH ratio and activate SIRT (Desquiret-Dumas et al., 2013). In literature, different effects of resveratrol on PD-L1 expression have been reported. However, several studies described that resveratrol upregulated PD-L1 expression (Gowrishankar et al., 2015; Yang et al., 2021). In breast and colon cancer cells, it's been reported that PD-L1 expression was upregulated upon resveratrol treatment via NF-κB (Lucas et al., 2018), and as previously mentionted, PD-L1 inducible expression has been reported to be upregulated through IFN-γ activation of the NF-κB pathway (Gowrishankar et al., 2015). Considering that EVT-701 is an ETCI inhibitor that alters NAD^+/NADH ratio, both an impairment of TET1 and/or of SIRT1 activity could potentially explain the PD-L1 decrease we observe in the presence of EVT-701 upon IFN-γ and the rescue of PD-L1 levels when NAD^+ is available.

It is worth to highlight that some studies have shown that mammalian cells are able to uptake NAD^+ and that it can rescue them from cell death. Billington and colleagues showed that exogenous NAD^+ could rescue NIH-3T3 fibroblasts from death upon NAD depletion induced by the nicotinamide phosphoribosyltransferase (NAMPT) inhibitor FK866 (Billington et al.; 2008). In addition, it has been recently shown that NAD^+ supplementation was able to rescue MCF-7 breast cancer cells from blocking of DNA repair resulting from PARP1 inactivation induced by FK866 NAD-depletion (Wilk et al., 2020).

These studies support the idea that NAD^+ can be uptaken by cells and exert same cellular actions as endogenous NAD^+. In addition, the mammalian NAD^+ mitochondrial transporter MCART1/ SLC25A51 has been recently identified by different teams (Kory et al., 2020; Luongo et al., 2020). It has also been shown that mitochondrial NAD^+ transport is essential for ETCI activity (Kory et al., 2020), and, previously, it was shown that exogenous NAD^+ could access mammalian mitochondria, and increase respiration (Davila et al., 2018). Therefore, supplementation with NAD^+ could potentially affect intracellular NAD^+/NADH ratio changes induced by ETCI inhibition.

Interestingly, epigenetic modulation of PD-L1 expression has been previously reported to be linked to TET activity. TET1 repression induced by NF-kB activation has been associated with increased tumor-infiltrating immune cells in several cancer types, such as basal-like breast cancer, melanoma, ovarian, lung and thyroid cancer (Collignon et al., 2018). Mechanistically, Collignon and colleagues showed that NF-kB activation induced p65 binding to TET1 promoter and this mediated TET1 repression, resulting in increased tumor-infiltrating immune cells (Collignon et al., 2018). In addition, glioma patients carrying IDH mutations have been described to show lower PD-L1 expression levels accompanied by higher PD-L1 gene promoter methylation (Berghoff et al., 2017; Mu et al., 2018). These results may suggest the inhibition of TET function exerted by 2-HG derived from mutant IDH. (Figueroa et al., 2010; Xu et al.,

2011). Supporting this hypothesis, it has been recently reported that PD-L1 levels in IDH1 mutant tumors were increased to the same levels observed in WT-IDH gliomas upon mutant IDH inhibition, and therefore, decreased 2-HG (Kadiyala et al., 2021). A similar phenotype could be expected from AML patients carrying IDH mutations (Mardis et al., 2009; Ward et al., 2010), despite not having been reported up to date. Considering all the above mentioned, a decrease in NAD^+/NADH induced by ECT I inhibition could affect TET1 activity and mediate the decrease of PD-L1 levels.

Irregular glycosylation through decreased levels of UDP-GlcNAc may explain the drop in CD39 membrane expression upon ETCI inhibition

As previously mentioned, ETCI inhibitors such as metformin have been reported to boost immune response, despite the lack of description of the molecular mechanisms. Cha and colleagues linked the enhanced PD-L1 degradation in breast cancer to increased ER-associated protein degradation (ERAD). This enhanced PD-L1 degradation was due to phosphorylation of PD-L1 at S195 by AMPK, which leaded to abnormal PD-L1 glycosylation and accumulation of the protein in the ER (Cha et al., 2018). One of our hypothesis is indeed that the decrease in UDP-GlcNAc levels may impact PD-L1 and CD39 glycosylation and result in abnormal glycosylated proteins that would not reach the cell membrane. However, this hypothesis only partially fits our results since it would not explain the differences observed upon NAD^+ supplementation in the IFN-γ -inducible-expression of PD-L1 and the IFN-γ-independent expression of CD39.

ETCI inhibition could decrease CD39 expression by impairing hypoxic signaling

Hypoxia has also been reported to upregulate both CD39 (Eltzschig et al., 2003; Eltzschig et al., 2009) and PD-L1 expression (Barsoum et al., 2013; Noman et al., 2014; Cubillos-Zapata et al., 2017). Considering that ETCI inhibitors such as IACS (Molina et al., 2018; Rytelewski et al., 2020), metformin (Zhou et al., 2015; Guimarães et al., 2016; Kocemba-Pilarczyk et al., 2020), and EVT-701(Meneyrol, 2016) have been reported to impair HIF-1α stabilization, this could be another potential mechanism by which ETCI inhibition reduces both CD39 and PD-L1 membrane expression.

Future directions

Identification and characterization of the mechanisms involved in the downregulation of PD-L1 and CD39 cell membrane expression upon ECT I inhibition will deserve further studies, since this question can boost the therapeutic interest of both immunotherapy and ETCI inhibitors.

5. Conclusion

In summary, this thesis describes the mitochondrial, immunomodulatory and therapeutic effects of targeting ETCI with EVT-701 in cancer, which is summarized in the next figure (**figure 49**).

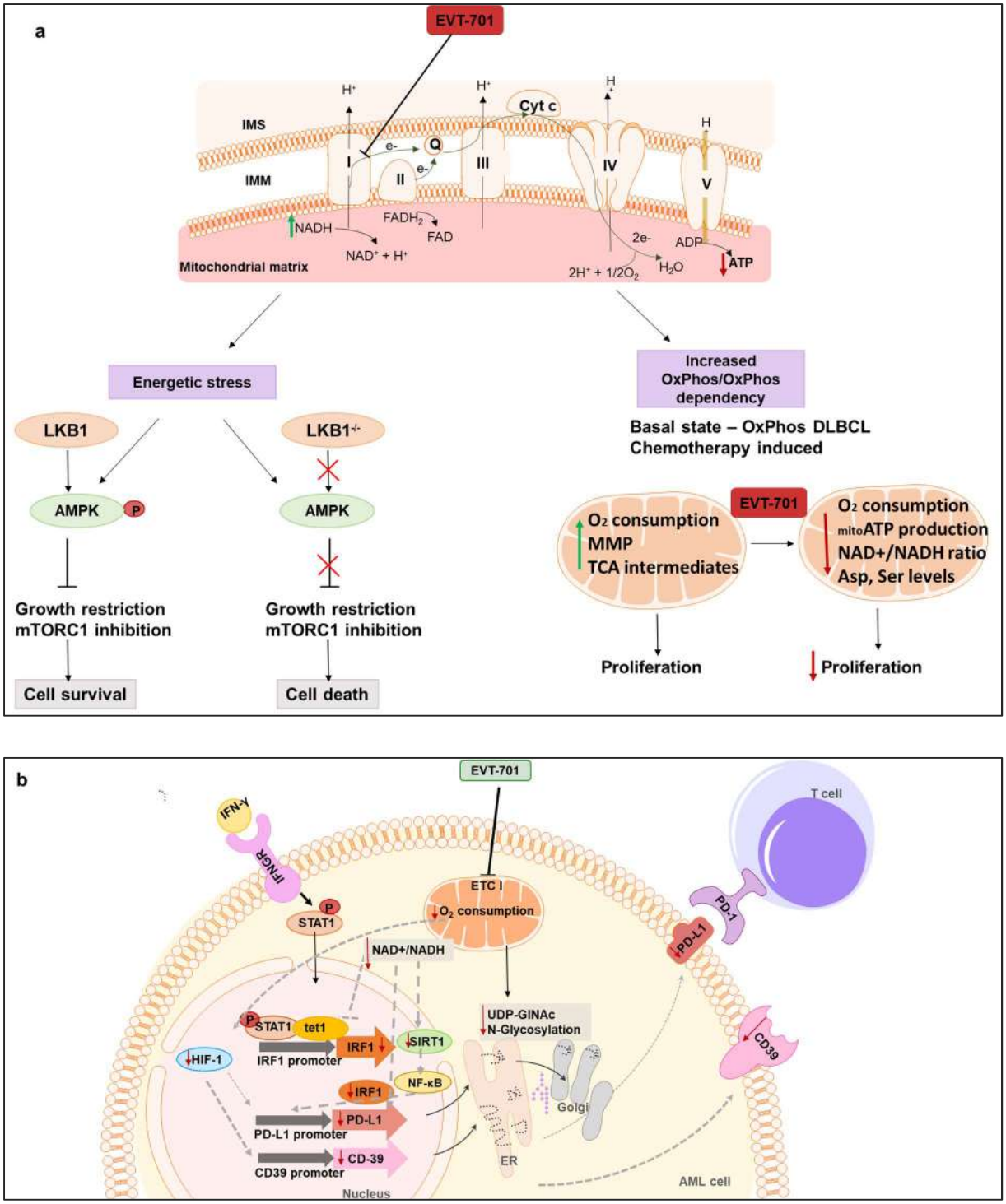


Figure 49. EVT-701 as therapeutic agent in cancer: mechanism of action, targeted vulnerabilities and immunomodulatory effects.

EVT-701 inhibits the first component of the ETC, the NADH dehydrogenase, blocking the oxidation of NADH into NAD^+. Thus, it impairs electron transference, as well as it dampens protons pumping to the IMM, resulting in decreased chemiosmotic gradient and therefore, in diminished levels of mitochondrial ATP production. ETCI inhibition is especially efficient as anti cancer approach upon LKB1 deficiency, since cells harbouring this mutation are not able to activate AMPK and die from energetic crisis, due to decreased mitochondrial ATP production in parallel to high energetic demand of the biosynthetic processes coordinated by mTORC1, which looses also its AMPK negative regulation. ETCI inhibition is also appropriate for cancers with increased OxPhos metabolism, intrinsic to the tumor phenotype, or developed in response to chemotherapy. These high OxPhos cancer cells present increased oxygen consumption, mitochondrial membrane potential and mitochondrial ATP production, as well as increased intermediate metabolites to support proliferation. ETCI inhibition with EVT-701 abrogates oxygen consumption and decreases mitochondrial ATP production, while it also diminishes NAD^+/NADH ratio and the levels of serine and aspartate, essential for nucleotide biosynthesis, impacting therefore tumor cell proliferation (**figure 49a**).

ETCI inhibition by EVT-701 decreases PD-L1 and CD39 expression in the membrane of leukemic cells (**figure 49b**). This can be potentially explained by several mechanisms. First, ETCI inhibitors decrease HIF-1 levels by increasing oxygen availability. Both PD-L1 and CD39 genes contain HIF response elements in their promoters, thus, a decrease in HIF-1 could explain a decrease in the transcription of these two immune checkpoints. EVT-701 decreases UDP-GclNAc levels, which can result in abrogation of N-glycosylation of membrane proteins including PD-L1 and CD39, whose N-glycosylation has been reported as essential for their membrane location and even for CD39 activity. Therefore, a defect in the N-glycosylation of PD-L1 and CD39 constitutes another potential mechanism by which EVT-701 can decrease these immune checkpoint expression. In the case of PD-L1, another potential regulatory mechanism involves NAD^+/NADH modulation of tet1 or sirtuin activity, since decreases in NAD^+ could impair either tet1 or sirtuin activation, leading to a downregulation of IRF1 or NF-kB responsive genes upon IFN-γ stimulation (**figure 49b**).

Overall, this work has presented the safe and selective profile the new ETCI inhibitor EVT-701, as well as its efficacy as therapeutic agent in the context of cancer, more concretely upon increased OxPhos dependency like OxPhos-DLBCL and lung cancer, including specific metabolic vulnerabilities like LKB1 deficiency. This work has also identified an increase in OxPhos as a common mechanism of resistance to therapy for human and murine leukemic cells, highlighting the potential of this approach to prevent chemoresistance, despite failing so far to identify an appropriate *in vivo* model. In addition, we report a metabolic characterization of EVT-701 effect on leukemic cells. Lastly, we unraveled a new role of ETCI as modulator of PD-L1 and CD39 expression, proposing several mechanisms to explain this interesting phenomena, and opening a promising area of research.

6. Annexes

6.1. Material and methods of un-published data

6.1.1. L120-Fluc-eGFP cell line generation

L1210 cell line transduccion was performed as described by Perkin Elmer with RediFect Red-Fluc-GFP Lentivirus particles (#CLS960003, Perkin Elmer , https://www.perkinelmer.com/lab-solutions/resources/docs/SPC_RediFect_Lentiviral_Particles_Protocol_of_infection.pdf).

Briefly, 50,000 cells in 500µl were seeded per well in plates of 24 wells. After 24h, RediFect lentiviral particles were added to the cells at Multiplicity Of Infection (MOIs) of 0, 25, 50, 75 and plates were incubated for 24h. The next day, the medium containing the virus was discarded, cells were washed with fresh medium, resuspended in 1mL of fresh complete culture media and incubated for 24h.

Then, cells were plated in two cell culture plates at 130 cells per plate in 150 ul of media per well (to have 1-2 cells/well) of mw96 plates, and media was changed twice a week. Wells with good cell growth were marked, and cultured and amplified. Then, once clones with good growth were selected, cells were in a dilution 1:1 with media and BLI positive clones were selected, expanded and collected in frozen vials. Cellular morphology was assessed by FACs as well as growth kinetics, to have a similar profile than with the parental cell line.

6.1.2. C1498 leukemia syngeneic model

1 million of C1498-Fluc-eFGP cells were resuspended in 100 µL of PBS and injected in the tail vein of C57/Bl6 males. For the experiments with randomization, treatment was started at day 10 after cell injection, and randomization based on bioluminescent signal was performed at day 9 after cell injection. For early onset treatment experiments, compounds were administered on day 3 after cell injection.

Treatment administration: AraC (#PHR1787-500MG, Sigma Aldrich) was administered IP qd for 5 days at 100mg/kg (or 25 mg/kg, 50 mg/kg, as specified for each experiment) dissolved in PBS. EVT-701 was administered PO at 30 mg/kg q.d, dissolved in 30%PEG200, 5% Solutol HS15 and 65% H_2O. IACS-010759 was administered PO at 7.5 mg/kg q.d, dissolved in methylcellulose 0.5%. For α-PD-1 therapy, InVivoPlus anti-mouse PD-1/CD279(#BX-BP0146-50MG, EUROMEDEX) or InVivoPlus rat IgG2a isotype control (#BX-BP0089-50MG, EUROMEDEX) were diluted in PBS at a concentration of 10 mg/kg and injected IP at a dose of 10 mL/kg every 3-4 days, administering a final dose of 1 mg/mL.
Tumor assessment: Tumor load was assessed *in vivo* by bioluminescence (BLI) or by FACs by monitoring $eGFP^+$ cells at end-point. For BLI, mice images were taken twice a week, after IP injection of XenoLight D-Luciferin Potassium Salt (#122799, Perkin Elmer), using the IVIS Spectrum In Vivo

Imaging System (#124262, Perkin Elmer). Quantification of the bioluminescence signal was performed with Living Image software version 4.5.5 (Perkin Elmer). Blood cell counts were performed with VETSCAN® HM5 Hematology Analyzer or Procyte ProCyte Dx (from IDEXX laboratories). **FACS *ex vivo*:** When FACs was performed, organs were harvested and dissociated to single cell solution. Viability staining (#L34955, ThermoFisher) was used to gate on living cells. Blocking was performed by preparation of the staining buffer with PBS, 2% FBS, 2mM EDTA and CD16/CD32 (Fcγ III/II Receptor)-Purified Rat Anti-Mouse (#553141, Becton Dickinson France) at dilution 1/200. For FOXP3 intranuclear staining, nuclear transcription buffer set (#BLE424401, Ozyme) was used. Antibodies and their dilution used are gathered in **table 6**.

Table 6. Antibodies used in the study.

Antibody	Conjugated fluorochrome	Supplier	Catalog number	Dilution
α-CD45.2	APC-Cy7	Ozyme	BLE109824	1:25
Mouse IgG2a, κ Isotype Ctrl Antibody	APC-Cy7	Ozyme	BLE 400229	1:25
α-CD3	PE	Ozyme	BLE100308	1:100
Armenian Hamster IgG isotype control	PE	Ozyme	BLE400908	100
α-CD4	APC	Ozyme	BLE100412	1:100
at IgG2b, kappa Isotype control	APC	Ozyme	BLE400612	1:100
α-CD8	PE-Cy7	Ozyme	BLE100722	1:100
Rat IgG2a, kappa Isotype Control	PE-Cy7	Ozyme	BLE400522	100
α-CD25	PerCpCy5	Ozyme	BLE101912	1:100
α-FoxP3	AF700	Ozyme	BLE126422	1:25
Rat IgG2b, kappa Isotype control .	AF 700	Ozyme	BLE400628	1:25
α-CD11b	PerCpCy5	Ozyme	BLE101228	1:200
Rat IgG2b, kappa Isotype control .	PerCP Cy5.5	Ozyme	BLE400632	1:200
Ly6G	BV605	Ozyme	BLE127639	1:200
Rat IgG2a, kappa Isotype Ctrl	BV605	Ozyme	BLE400540	1:200
Ly6C	AF700	Ozyme	BLE128024	1:200
Rat IgG2c, kappa Isotype Ctrl	AF 700	Ozyme	BLE400729	1:200

6.1.3. ELISpot

Spleens were harvested in RPMI-1640 complete media (#21875-034, Gibco) supplemented with 10% FBS (#Sigma, F7524), 2mM Glutamax (#35050-038,Gibco,), 1mM Sodium pyruvate (#11360-039, Gibco); non essential aminoacids 1X (#11140-035, Gibco), containing 100µM Gly, Ala, Asn, Asp, Glu, Pro, Ser;

100µM β-Mercaptoethanol (Gibco 31350010) and Penicillin/Streptomycin at 100 IU/ml penicillin and 100 µg/ml streptomycin (#11074440001, Sigma). Spleens were dissociated with Gentle Macs technology (Gentle Macs C tubes #130-093-237 and Gentle Macs disociator #130-093-235). After 2 washes in comete media, lymphocytes where isolated using Lympholyte®-Mouse (#CL5031, Cederlane). Splenocytes and C1498 cells were counted and concentrations were adjusted to 10M cells/mL. In the mean time, plates mAB AN18 pre-coated (from the kit Mouse IFN-γ ELISpotPLUS (ALP), #3321-3PW, Batch: 1117, Mabtech) were washed with PBS and incubated for 1h with complete medium. Then, media was removed and splenocytes where added at a concentration of $0.9x10^6$ cells/well (except for the positive control a-CD3 stimulated, where to avoid saturation, only $0.2x10^6$ cells were added per well), C1498 cells where added at $0.1x10^6$ cells/well and for the positive control, Purified Hamster anti-mouse CD3e (clone 145-2C11)(# 553058, BD Biosciences) was added at a concentration of 0.5µg/ml. Then, plates were incubated in humidified incubator at 37°C with 5% CO_2 for 20 hours. After the incubation period, plates were washed 5 times with PBS and incubated with 1µg/ml R4-6A2-biotin (kit Mouse IFN-γ ELISpotPLUS (ALP), #3321-3PW, Batch: 1117, Mabtech) as detection antibody in PBS-0.5% FBS for 2h. Subsequently, plates were washed again with PBS and Streptavidine-ALP (kit Mouse IFN-γ ELISpotPLUS (ALP), #3321-3PW, Batch: 1117, Mabtech) was added in a 1/1000 dilution in PBS - 0.5% FBS. Finally, filtered BCIP/NBT-plus substrate solution (kit Mouse IFN-γ ELISpotPLUS (ALP), #3321-3PW, Batch: 1117, Mabtech) was added to the wells until spot development, when the reaction was stoped by washing plates extensively in tap water. After letting the plate dry, they were analized with the device Bioreader 6000 Pro-F β by Biosys and the software Eazyreader ® generation 18.9, by Bio-Sys GmbH.

7. References

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