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Metabolic reprogramming in chronic lymphocytic leukemia: role of peroxisomal fatty acid β -oxidation as an energy supplier

Mariana Tannoury

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Metabolic Reprogramming in Chronic Lymphocytic Leukemia: Role of Peroxisomal Fatty Acid β -Oxidation as an Energy Supplier

La Reprogrammation Métabolique dans la Leucémie Lymphoïde Chronique : Rôle de la β -Oxydation Peroxysomale des Acides Gras comme Source d'Énergie

Thèse de doctorat de l'université Paris-Saclay

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Thèse soutenue au Centre de Recherche des Cordeliers,
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Title : Metabolic Reprogramming in Chronic Lymphocytic Leukemia: Role of Peroxisomal Fatty Acid β -Oxidation as an Energy Supplier

Keywords : Fatty acid metabolism, Peroxisomes, Mitochondria, Chronic lymphocytic leukemia, ACOX1, Anti-tumor therapy

Abstract : Chronic lymphocytic leukemia (CLL) is the most common form of adult leukemia in Western countries. It is characterized by the accumulation of CD5⁺ monoclonal B lymphocytes in peripheral blood, bone marrow, and secondary lymphoid organs. CLL prognosis depends on the clinical stage and biological markers, including IGHV mutational status, cytogenetic abnormalities such as del11q and del17p, or karyotype complexity. Despite excellent clinical results obtained with Bruton tyrosine kinase (BTK) or BCL-2 inhibitors, drug resistance due to the emergence of genetic mutations (e.g. mutations in BTK, BCL-2, del8p, etc.) remains a major cause of treatment failure. Indeed, no complete remission is guaranteed, so ongoing scientific exploration is necessary to develop innovative therapeutic strategies.

Studies of cancer cell metabolism have recently gained significant relevance with the recognition of “metabolic reprogramming” as an essential hallmark of the tumor phenotype. A major part of research is focused on ATP, the cell’s energy currency, which orchestrates essential cellular functions (e.g. proliferation, macromolecules biosynthesis, etc.). Deciphering metabolic adaptations can then reveal potential vulnerabilities specific to tumor cells. Selectively disrupting this tumor reprogramming opens up promising avenues for cancer treatment. Furthermore, targeting metabolic vulnerabilities in combination with existing therapeutic drugs appears to be a compelling strategy for improving treatment efficacy. Hence, my thesis project aims to identify a metabolic specificity characterizing malignant CLL B-lymphocytes, focusing on a potential metabolic pathway that could be key to ATP generation in these tumor cells.

I thus demonstrated an increase in mitochondrial oxidative phosphorylation (OXPHOS) in CLL CD5⁺ B-cells compared to healthy CD5⁻ B-cells suggesting a predominance of mitochondrial ATP generation. The use of glucose as a metabolic intermediate for OXPHOS was excluded and an initial mRNAseq approach revealed overexpression of genes involved in fatty acid β -oxidation (FAO). In particular, ACOX1, a key enzyme in peroxisomal FAO (pFAO), is overexpressed in CLL B-lymphocytes. A complementary metabolomic approach revealed high levels of C2-C6 carnitines, the metabolic end products of pFAO. It should be noted that these peroxisomal carnitines will be modified in the mitochondria to generate NADH and FADH₂ used by the mitochondrial electron transport chain to produce ATP. I finally demonstrated that the pharmacological inhibition of ACOX1 induces caspase-dependent cell death in CLL cells, but not in CD5⁻ B cells or in immune cells involved in cell death clearance. Interestingly, the combination of ACOX1 and BTK inhibitors at low doses induces apoptosis synergistically in the CLL B-lymphocytes, including those presenting del17p, del11q, or a complex karyotype.

Overall, my PhD work identifies ACOX1-mediated peroxisomal FAO as a metabolic signature of CLL cells. It also provides evidence for the contribution of pFAO in CLL mitochondrial ATP generation. Targeting this metabolic vulnerability, alone or as part of a combination therapy, could therefore offer new insights for innovative therapeutic strategies.

Titre : La Reprogrammation Métabolique dans la Leucémie Lymphoïde Chronique : Rôle de la β -Oxydation Peroxysomale des Acides Gras comme Source d'Énergie

Mots clés : Métabolisme des acides gras, Peroxysomes, Mitochondries, Leucémie lymphoïde chronique, ACOX1, Thérapie anti-tumorale

Résumé : La leucémie lymphoïde chronique (LLC) est la forme la plus courante de leucémie adulte dans les pays occidentaux. Elle se caractérise par une accumulation de lymphocytes B monoclonaux CD5+ dans le sang périphérique, la moelle osseuse et les organes lymphoïdes secondaires. Le pronostic de la LLC dépend du stade clinique et des marqueurs biologiques, dont le statut mutationnel IGHV, les anomalies cytogénétiques caractéristiques comme la del11q et la del17p, et la complexité du caryotype. Malgré des résultats cliniques exceptionnels obtenus avec les inhibiteurs de la Bruton tyrosine kinase (BTK) ou de BCL-2, la résistance aux drogues reste une cause majeure d'échec thérapeutique en raison de l'apparition de nouvelles résistances (par exemple, mutations dans la BTK, BCL-2, del8p, etc.). En effet, aucune rémission complète n'est garantie, il est donc nécessaire de poursuivre les explorations scientifiques afin de développer de nouvelles approches thérapeutiques.

Les études sur le métabolisme des cellules cancéreuses ont connu une renaissance récente avec la reconnaissance de la « reprogrammation métabolique » comme une composante essentielle du phénotype tumoral. Une partie centrale de la recherche se concentre sur l'ATP, la monnaie énergétique de la cellule qui orchestre les fonctions cellulaires essentielles (par exemple la prolifération, la biosynthèse des macromolécules, etc.). L'identification d'adaptations métaboliques peut révéler des vulnérabilités propres aux cellules tumorales. Ainsi, la perturbation sélective de ce métabolisme ouvre des voies prometteuses dans le traitement du cancer. En outre, cibler les vulnérabilités métaboliques en combinaison avec les molécules thérapeutiques existantes apparaît comme une stratégie convaincante pour améliorer l'efficacité du traitement. Ainsi, mon projet de thèse vise à identifier une spécificité métabolique caractérisant les lymphocytes B de la LLC, en se concentrant sur une voie métabolique qui pourrait être cruciale pour la génération d'ATP dans ces cellules tumorales.

Ainsi, j'ai démontré une augmentation de la phosphorylation oxydative mitochondriale (OXPHOS) dans les cellules B CD5+ de LLC par rapport aux cellules B CD5-B saines, suggérant une prédominance de la génération d'ATP par la mitochondrie. L'utilisation du glucose comme intermédiaire métabolique pour l'OXPHOS a été exclue et une approche mRNAseq a révélé en premier lieu la surexpression de gènes impliqués dans la β -oxydation des acides gras (FAO). En particulier, ACOX1, une enzyme clé de la FAO peroxysomale (pFAO), est surexprimée dans les cellules B de LLC. Une approche métabolomique complémentaire a dévoilé une augmentation des C2-C6 acylcarnitines, les produits métaboliques finaux de la pFAO. A noter que ces carnitines peroxysomales seront modifiées dans les mitochondries pour générer du NADH et du FADH₂, utilisés par la chaîne de transport d'électrons mitochondriale pour produire de l'ATP. Au final, j'ai démontré que l'inhibition pharmacologique de ACOX1 induit la mort cellulaire caspase-dépendante dans les cellules de LLC, mais pas dans les cellules B CD5- ou dans les cellules immunitaires impliquées dans l'élimination des cellules. De façon intéressante, la combinaison des inhibiteurs d'ACOX1 et de BTK à faibles doses induit l'apoptose de manière synergique dans la LLC, y compris dans les cellules avec del17p, del11q ou de caryotype complexe.

Mon projet de thèse identifie la pFAO médiée par ACOX1 comme une signature métabolique des cellules LLC. Il apporte également la preuve de la contribution de cette voie dans la génération d'ATP mitochondrial dans la LLC. Cibler cette vulnérabilité métabolique, seul ou en thérapie combinée, pourrait offrir de nouvelles perspectives pour des stratégies thérapeutiques innovantes.

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List of Abbreviations

A

AA: amino acid
AAG: alkyl-acylglycerol
ABC: ATP-binding cassette
ACOX1: acyl-CoA oxidase
ACS: acyl-CoA synthetase
ADCC: antibody-dependent cell-mediated toxicity
ADP: Adenosine diphosphate
AGP: 1-O-Alkyl-glycerol-3-phosphate
AGPS: alkylglycerone phosphate synthase
AIF: apoptosis-inducing factor
ALDP: adrenoleukodystrophy protein
ALDR: adrenoleukodystrophy-related protein
AMACR: Alpha-methylacyl-CoA racemase
AMP: adenosine monophosphate
AMPK: AMP-activated protein kinase
APAF-1: Apoptotic protease activating factor 1
APRIL: A Proliferation Inducing Ligand
Arg: arginine
ASS: argininosuccinate synthase
ATM: Ataxia Telangiectasia Mutated
ATP: adenosine triphosphate

B

BA: bile acid
BAFF: B-cell Activating Factor of the TNF Family
BAK: Bcl-2-antagonist/killer
BAX: Bcl-2-associated X protein
BCFA: branched chain fatty acid
BCL-2: B-Cell Lymphoma-2
BCR: B-cell receptor
BH3: BCL-2 homology 3
BIRC3: Baculoviral IAP repeat-containing protein 3
BLCFA: branched long chain fatty acid
BR: Bendamustine-Rituximab
BSCFA: branched short chain fatty acid
BTK: Bruton's tyrosine kinase

C

CA: cholic acid
CA: chromosomal abnormalities
Ca²⁺: calcium
CACT: carnitine-acylcarnitine translocase
cADPR: cyclic ADP-ribose
CAT: catalase
CAT-X: cationic amino acid transporter
CCL22: C-C motif chemokine 22

CD: Cluster of differentiation
CD40L: CD40 ligand
CDC: complement-dependent cytotoxicity
CDCA: chenodeoxycholic acid
C_H: constant heavy
CK: complex karyotype
C_L: constant light
CLL: chronic lymphocytic leukemia
CLP: common lymphoid progenitor
CMP: common myeloid progenitor
CoQ: ubiquinone
CPT: carnitine palmitoyltransferase
CRAT: Carnitine acetyltransferase
CROT: Carnitine octanoyltransferase
CXCLX: C-X-C motif chemokine ligand X
CXCRX: C-X-C chemokine receptor type X
CypA: Cyclophilin A
Cyt c: cytochrome c

D

DAG: diacylglycerol
DHA: docosahexanoic acid
DHAP: dihydroxyacetone phosphate
DHCA: 3 α ,7 α -dihydroxycholestanoic acid
DHEA: Dehydroepiandrosterone
DLBCL: diffuse large B cell lymphoma
DLEU2: deleted in lymphocytic leukaemia 2
DLP1: dynamin-like protein 1
DNA: Deoxyribonucleic acid

E

EndoG: endonuclease G
eNOS: endothelial NO synthase
ER: endoplasmic reticulum
ERK: Extracellular Signal-Regulated Kinase
ETC: electron transport chain

F

FA: fatty acid
FABP: fatty acid binding protein
FADH₂: flavin adenine dinucleotide
FAO: fatty acid β -oxidation
FAR: fatty acyl-CoA reductase
FATP: fatty acid transport protein
FCR: fludarabine-cyclophosphamide-rituximab
FDA: food and drug administration
Fis1: fission 1

FISH: fluorescence in situ hybridization

G

G3P: glycerol 3-phosphate
G3PDH: glycerol 3-phosphate dehydrogenase
GLN: glutamine
GLS: glutaminase
GLU: glutamate
GLUD1: glutamate dehydrogenase
GLUT: glucose transporter
GNPAT: glyceronephosphate O-acyltransferase
GPx: Glutathione peroxidase
GSH: glutathione

H

H⁺: protons
H₂O₂: hydrogen peroxide
HCK: high-complex karyotype
HCX: calcium hydrogen exchanger
HDR: homology-directed repair
HIF: hypoxia inducible factor
HO[•]: hydroxyl radical
HSC: hematopoietic stem cells

I

IDH: isocitrate dehydrogenase
Ig: Immunoglobulin
IGF1R: insulin-like growth factor 1 receptor
IGHV: Immunoglobulin heavy chain variable region
IL-6: interleukin-6
iNOS: inducible NO synthase
InsP3: inositol-1,4,5,-triphosphate
INSR: insulin receptor
ITAM: Immunoreceptor Tyrosine-based Activation Motifs

J

JAK2: Janus kinase 2

L

LCFA: long chain fatty acid
LD: lipid droplets
LDCA: long dicarboxylic acid
LDT: Lymphocyte doubling time
lncRNA: long non-coding RNA
LPL: lipoprotein lipase
Lyn: Lck/Yes Novel tyrosine kinase

M

MAC: membrane attack complex
MAPK: Mitogen-Activated Protein Kinase
MBL: monoclonal B cell lymphocytosis
MCFA: medium chain fatty acid
MCL-1: Myeloïd Cell Leukemia-1
MCU: mitochondrial calcium uniporter
MDA: malondialdehyde
MDR: minimal deleted region
mFAO: mitochondrial fatty acid β -oxidation
Mff: mitochondrial fission factor
MHC-I: major histocompatibility complex class I
M-IGHV: mutated-IGHV
MnSOD: manganese superoxide dismutase
MOMP: mitochondrial outer membrane permeabilization
MPP: multipotent progenitor
mRNA: mitochondrial RNA
MSC: mesenchymal stromal cells
mtDNA: mitochondrial DNA
mTOR: Mammalian target of rapamycin
mtROS: mitochondrial ROS
MUFA: monounsaturated fatty acid

N

NAC: N-acetylcysteine
NAD⁺: nicotinamide adenine dinucleotide
NADH: nicotinamide adenine dinucleotide
NADPH: nicotinamide adenine dinucleotide phosphate
NAM: nicotinamide
NCLX: sodium/calcium/lithium exchanger
NFAT: nuclear factor of activated T cells
NF- κ B: Nuclear Factor Kappa B
NGS: Next-Generation Sequencing
NH₄⁺: ammonium
NHEJ: nonhomology end-joining
NK: natural killers
NLC: neural-like cells
NO: nitric oxide

O

O₂^{-•}: superoxide anion
OCR: oxygen consumption rate
OGDH: oxoglutarate dehydrogenase
OS: overall survival
OXPHOS: oxidative phosphorylation

P

PBMC: peripheral blood mononuclear cell
pBTK: phosphorylated BTK
PC: phosphatidylcholine

PCD: programmed cell death
PCR: polymerase chain reaction
PD-1: Programmed cell death protein 1
PD-L1: Programmed death-ligand 1
PE: phosphatidylethanolamine
PEX: peroxin
pFAO: peroxisomal fatty acid β -oxidation
PGC-1 α : proliferator-activated receptor- γ coactivator 1- α
PI3K: Phosphatidylinositol 3-Kinase
PIP2: phosphatidylinositol-4,5-diphosphate
PKC: protein kinase C
PL: phospholipid
PLC γ : Phospholipase C Gamma
PMP: peroxisomal membrane protein
PPAR: peroxisome proliferator-activated receptor
PPP: pentose phosphate pathway
ppV: peroxisome precursor vesicles
PRDX3: peroxiredoxin 3
PRDX5: peroxiredoxin 5
pSYK: phosphorylated SYK
PTP: permeability transition pore
PTS: peroxisomal targeting signal
PUFA: polyunsaturated fatty acid

Q

QH₂: ubiquinol

R

RB1: retinoblastoma 1
RCDP: rhizomelic chondrodysplasia punctate
RNA: Ribonucleic acid
ROR1: receptor tyrosine kinase like orphan receptor 1
ROS: reactive oxygen species
RT: Richter transformation

S

SC: supercomplex
SFK: Src-family kinase
SGLT: sodium-glucose linked transporter
SH2: Src homology 2
SHM: somatic hypermutation
SLC: solute carrier
smIg: surface membrane immunoglobulin
SOD: superoxide dismutase
SR: sarcoplasmic reticulum
STAT3: signal transduction and activator of transcription 3
sTK1: Serum Thymidine kinase 1
SYK: Spleen tyrosine kinase
s- β 2M: Serum beta2-microglobulin

T

TAG: triacylglycerol
TCA: tricarboxylic acid
TCR: T-cell receptor
Tfam: transcription factor A mitochondrial
THCA: 3 α ,7 α ,12 α -trihydroxycholestanic acid
TIGAR: TP53-induced glycolysis and apoptosis regulator
TK: Thymidine kinase
TLR-9: Toll-like receptor 9
TNF: tumor necrosis factor
TP53: tumor protein 53
TRAIL-R: tumour necrosis factor-related apoptosis-inducing ligand-receptor
TTFT: time to first treatment

U

UM-IGHV: Unmutated-IGHV

V

VDAC: voltage-dependent anion channel
V_H: Variable heavy
V_L: Variable light
VLCFA: very long chain fatty acid
VLDL: very-low density lipoproteins

X

X-ALD: X-linked adrenoleukodystrophy
XPO1: exportin-1

Z

ZAP-70: Zeta-associated Protein of 70 kDa

Other

$\Delta\Psi_m$: mitochondrial membrane potential
8-oxo-dG: 8-oxo-2'-deoxyguanosine
 α -KG: alpha-ketoglutarate
2-HG: 2-Hydroxyglutarate

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Introduction

Chapter I: Chronic Lymphocytic Leukemia

I. Overview

Chronic lymphocytic leukemia (CLL) is an extremely heterogeneous disease, both biologically and clinically: while some patients never require therapy, others display an aggressive course with poor response to therapy and death within months. Over the past years, significant advancement in understanding the biology of this disease led to the development of targeted therapies. However, despite the drastic enhancement in CLL treatment opportunities, drug resistance remains an issue¹. In this chapter, CLL pathophysiology, clinical outcomes as well as treatment options will be discussed in detail.

a. Definition and Risk Factors

CLL is a disease characterized by clonal expansion of mature CD5+ B lymphocytes that accumulate in the blood, bone marrow, lymph nodes, and spleen¹. A genetic risk factor has been recognized in CLL, with 10% of CLL cases being associated with a family history of CLL or other lymphoproliferative malignancies². While an inverse association was observed between CLL cases and exposure to sun, other factors like living on farms or being exposed to Hepatitis C virus might potentially increase the risk of developing CLL³.

b. Epidemiology

The incidence of CLL is higher in people from European countries (Caucasians), whereas it is lower in Hispanics and Asians. Globally, around 4 to 5 new CLL cases occur among every 100,000 inhabitants each year^{4,5}. In France, more than 4,000 new cases of CLL are reported every year with a higher incidence among males (59.3% of the total cases). CLL is considered an elderly disease with a median age of 71 years old among men and 73 among women⁵.

II. Pathophysiology of Chronic Lymphocytic Leukemia

a. Hematopoiesis and B Lymphopoiesis

All blood cells derive from hematopoietic stem cells (HSCs) by a mechanism called hematopoiesis that takes place in the bone marrow (**Figure 1**). Every hour, HSCs generate $\sim 1 \times 10^9$ red blood cells indispensable for oxygen transport, $\sim 1 \times 10^8$ white blood cells including myeloid and lymphoid cells that act as defenders against infections, and platelets necessary for coagulation and tissue repair^{6,7}.

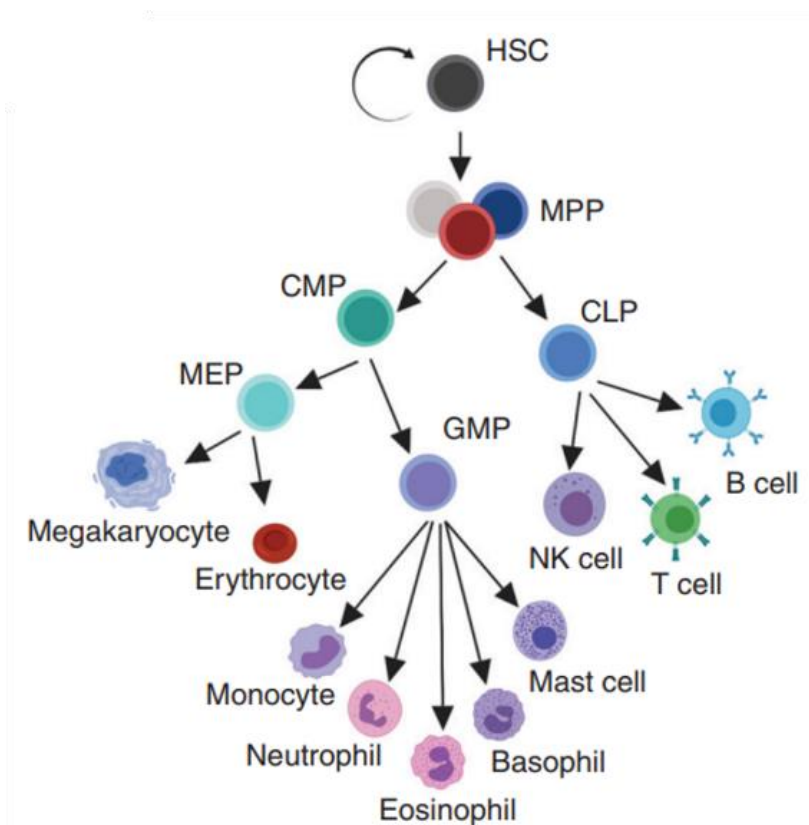


Figure 1: An illustration of hematopoiesis. Hematopoietic stem cells (HSC) generate multipotent progenitors (MPP) that differentiate into common myeloid progenitors (CMP) and common lymphoid progenitors (CLP), which eventually become mature blood cells. GMP: granulocyte-macrophage progenitor; MEP: megakaryocyte-erythroid progenitor; NK: natural killers (Reproduced from Olson et al, 2020)⁶.

CLL development begins by an accumulation of CLL-like B cells in the peripheral blood, causing a monoclonal B cell lymphocytosis (MBL). This condition is asymptomatic, but the accumulation of genetic lesions and epigenetic changes, the activation of some signaling pathways, such as the B-cell receptor (BCR) signaling pathway, as well as the effect of the microenvironment are all responsible for its progression to CLL⁸.

b. Role of BCR Signaling in CLL Development

BCR signaling is a normal signaling cascade that occurs in B cells upon antigen recognition, subsequently causing B cell activation and antibody production. The receptor is composed of a surface immunoglobulin, called IgM, responsible for antigen binding, coupled with an Ig α /Ig β heterodimer.

The signaling pathway is initiated by antigen binding, causing a direct phosphorylation of Immunoreceptor Tyrosine-based Activation Motifs (ITAMs) by Src-family kinases (SFKs), like LYN, FYN, and BLK. SYK kinase is then recruited via their tandem Src homology 2 (SH2) domains and activated by the phosphorylated ITAMs, causing a subsequent activation of Bruton's tyrosine kinase (BTK) and other downstream effectors including the PI3K/Akt/mTOR, NF- κ B, and ERK/MAPK pathways^{9,10}. Indirectly, the Janus kinase 2 (JAK2)/signal transduction and activator of transcription 3 (STAT3) signaling pathway is activated by interleukin-6 (IL-6), with the latter production being induced by the NF- κ B pathway¹¹. JAK2/STAT3 is constitutively activated in CLL, and known to be a regulator of anti-apoptotic genes transcription such as *BCL-2* and *MCL-1*, both overmodulated in the CLL malignant cells¹² (**Figure 2**). Compared with healthy B cells, CLL cells have a higher activity of the BCR signaling cascade that is crucial for their proliferation and survival. While exhibiting low levels of surface IgM (sIgM), CLL cells show elevated levels of BTK/pBTK compared to healthy lymphocytes as well as high levels of phosphorylated SYK (pSYK), especially in the lymph nodes¹³. CLL BCR can recognize self-antigens, such as apoptotic cells, or foreign antigens. Furthermore, its interaction with the microenvironment can also initiate this signaling pathway¹⁴.

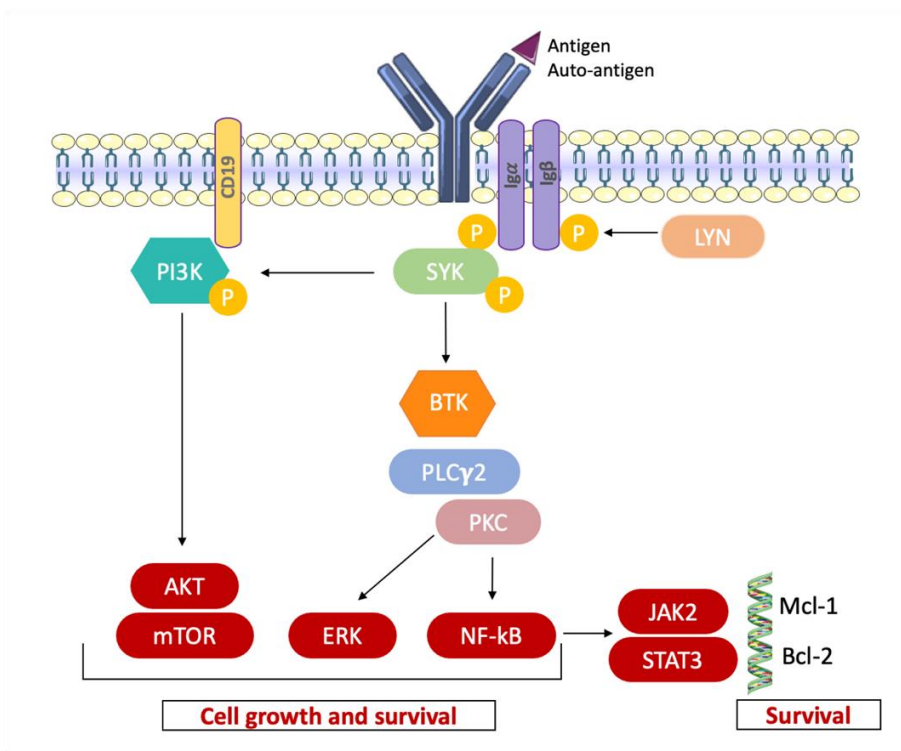


Figure 2: BCR signaling. Antigen or autoantigen binding to surface IgM causes the phosphorylation of ITAMs by LYN (or other Src-family kinases). Syk is then recruited and activated, activating in its turn: PI3K and its downstream effectors Akt/mTOR and BTK and the downstream pathways ERK and NF- κ B. NF- κ B can activate JAK2/STAT3 pathway, a regulator of *MCL-1* and *BCL-2* gene transcription. The activation of all these signaling pathways plays a major role in CLL cell growth and survival.

c. Tumor Microenvironment

When cultured *in vitro*, CLL cells undergo rapid spontaneous cell death, suggesting that the cellular microenvironment plays a role in protecting CLL cells from apoptosis. The pioneering evidence that CLL cells rely on the microenvironment for survival derived from two studies from the nineties that pointed out the role of human bone marrow stromal cells in preventing apoptosis of the leukemic cells^{15,16}. Indeed, the CLL tumor microenvironment is made up of different components, including mesenchymal stromal cells (MSCs), neural-like cells (NLCs), T cells, and natural killers (NK) cells, each cell type playing a role in CLL activation and/or survival¹⁷.

- **Mesenchymal Stromal Cells (MSCs)**

MSCs are a subset of stem cells present in adult bone marrow, adipose tissue and birth derived tissues such as the placenta and the umbilical cord¹⁸. MSCs have the capacity to differentiate into other cell types to generate and repair damaged tissues. MSCs can also secrete diverse factors essential for hematopoietic support but also implicated in immunomodulation and cell's protection¹⁹. MSCs can interact with CLL cells in the bone marrow or the secondary

lymphoid tissues. This contact is mediated by the secretion of a chemokine called CXCL12 by the stromal cells, which attracts circulating malignant CLL cells via CXCR4 receptor²⁰ (**Figure 3**). This receptor is overexpressed and functional in CLL B cells compared to healthy B cells²¹. Apart from CXCL12 role in stimulating the lymphocytes migration, MSCs from bone marrow cause the upregulation in CLL of ZAP70 and CD38, two old biomarkers of aggressive forms of the disease²². Furthermore, CXCL12 released by bone marrow MSCs induces the internalization of CXCR4²³ and the B-lymphocytes specific CD20 antigen in CLL cells, resulting in resistance to anti-CD20 therapy (rituximab)²⁴.

- **Nurse-like Cells (NLCs)**

NLCs adopt this name because they resemble thymic feeder cells. *In vitro*, NLCs are large round cells that differentiate from monocytes, and similar to MSCs, they confer protection against CLL spontaneous mortality^{24,25}. NLCs can be found in the spleen and secondary lymphoid tissues where CLL cells are homed via CXCL12 and CXCL13 chemokine secreted by NLCs. NLCs also promote CLL cells survival via TNF family members, BAFF and APRIL (**Figure 3**), or by direct BCR activation²⁶.

- **T-cells and Natural Killers (NK)**

In CLL, the interaction between T cells and B cells is bidirectional (**Figure 3**). On the one hand, T cells activate and contribute to the survival of CLL B cells via the interaction between the CD40 expressed on B cells and the CD40 ligand (CD40L) expressed on CD4+ helper T cells. *In vitro* studies showed that CLL cells attract CD4+ helper T cells by releasing CCL22²⁷. On the other hand, an increased number of both CD4+ and CD8+ T cells in CLL is observed with a differential increase in CD8+T cells comparing to CD4+ cells especially in advanced stages of the disease. Despite this increase in T-cell numbers, CLL T-cells are referred to as "exhausted T-cells" because they show a decrease in functionality²⁸. This is mainly due to the interaction between PD-1 overexpressed on activated T cells and PD-L1 overexpressed on B lymphocytes, leading to inhibition of T cell function. In this way, CLL cells can avoid the anti-tumor immune surveillance^{22,29}.

A similar loss of activity is observed in NK cells of patients with CLL. Normally, NK cell function depends on an activating or inhibiting receptor-ligand interaction with the target cell. In CLL, reduced NK cell activity is due to a lower expression of activating NK receptors and a higher expression of inhibitory NK receptors. Additionally, CLL cells can evade NKs by

releasing NK-specific activating ligands into plasma or overexpressing inhibitory ligands, resulting in an impaired NK-induced cytotoxicity³⁰.

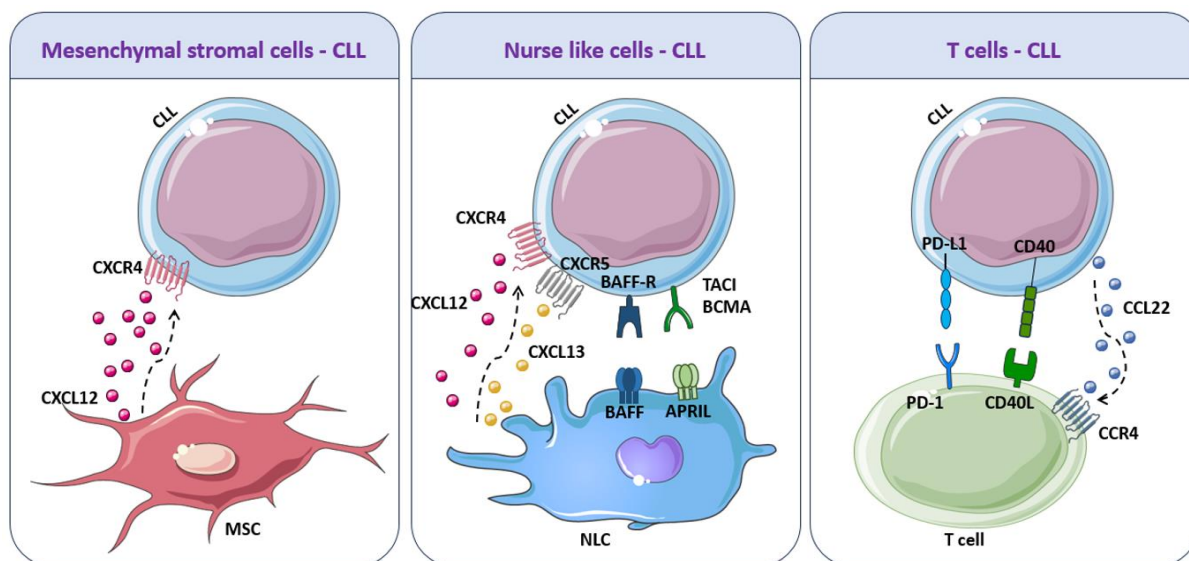


Figure 3: Schematic representation of the interaction between CLL and different microenvironment components. *Left*, MSCs can stimulate CLL cells migration by releasing CXCL12 chemokine that interacts with its receptor CXCR4 expressed on CLL cells. *Middle*, NLCs can attract circulating CLL cells via CXCL12 and CXCL13 secretion, but they can also promote CLL survival by a direct ligand-receptor interaction. *Right*, CLL cells attract T cells by releasing CCL22. The direct CD40-CD40L interaction contributes to CLL survival. Effectors T cells function can be inhibited by PD-1/PD-L1 interaction.

III. Diagnosis and Staging

a. Clinical presentation

CLL is generally an asymptomatic disease, often discovered during a routine blood count. However, some patients show symptoms such as fatigue, weight loss and night sweats. Lymph nodes in the neck, under the arms or in the groin may get swollen, and the spleen might enlarge. Moreover, disease complications might happen. For example, people with CLL are more prone to infections, mostly bacterial ones, affecting the respiratory tract. This is mainly due to reduced immunoglobulin levels. In addition, autoimmune diseases may develop in 25% of CLL cases, and the incidence of secondary cancers may increase as well³¹.

A histopathological and biological change occur in 2-10% of CLL cases, causing the development of an aggressive lymphoma, in most cases a diffuse large B cell lymphoma (DLBCL). This development is called Richter transformation (RT)³².

b. Morphology and Cell Count

CLL is primary diagnosed by the presence of $\geq 5 \text{G/L}$ (5×10^9 B-lymphocytes/mL) in the peripheral blood, sustained for over three months. If suspected, CLL can be identified in a

blood smear in which lymphocytes appear small with a large dense nucleus and partially aggregated chromatin. Cells can rupture during the blood smear, causing the appearance of smudge cells also called Gumprecht nuclear shadows. **Figure 4** represents an example of CLL blood smear. This is one of CLL morphological characteristics; however, CLL diagnosis should be confirmed with immunophenotyping⁴.

The presence of $<5\text{G/L}$ of clonal B-cell lymphocytes with a CLL phenotype, in the absence of clinical manifestation, corresponds to an MBL that progresses to CLL at a rate of 1%–2% per year³³.

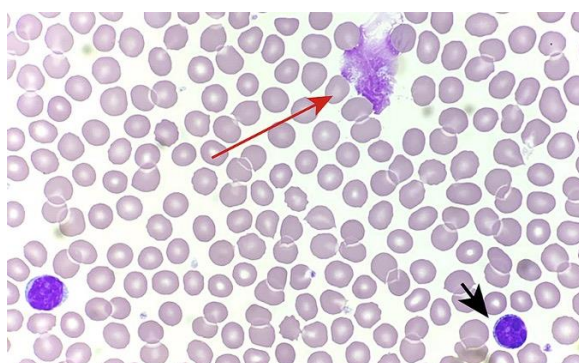


Figure 4: CLL blood smear. Black arrow represents a CLL B lymphocyte with aggregated chromatin. Red arrow represents a Gumprecht nuclear shadow (Figure from Maitre et al, 2019)³³.

c. Immunophenotyping

To confirm CLL diagnosis, immunophenotyping is performed by flow cytometry. Several markers known as clusters of differentiation (CD) are expressed in CLL tumor cells and detected by specific antibodies, notably CD5 and CD23. Low expression of CD20 and CD79b also marks CLL, as well as the low level of surface membrane immunoglobulin (smIg). The Matutes score is commonly used in France and the UK. As shown in **Table 1** below, one point is assigned to each characteristic marker detected in the CLL B lymphocytes. If the score is 4 or 5, the diagnosis of CLL is confirmed. More recently, additional markers have been added to the diagnosis of CLL such as CD43, CD81, CD200 and CD10^{5,34,35}.

Marker	Score 1	Score 0
CD5	Positive	Negative
CD23	Positive	Negative
2CD20	Low	High
CD79b	Low	High
smIg	Low	High

Table 1: Matutes scoring system for the diagnosis of CLL (Adapted from Moreau et al, 1997)³⁵.

d. Staging Systems

Physicians can determine the stage of the disease according to two classifications, Rai and Binet, which are based on a physical examination of the patient complemented by a blood count. These classifications help the physician identify the severity of the disease and decide whether the patient should be treated or simply monitored³⁶.

- **Rai Classification**

In 1975, Rai et al. established a staging system that classifies CLL according to disease risk, ranging from low (stage 0), to intermediate (stages I and II), and high risk (stages III and IV). They defined lymphocytosis in the blood or bone marrow as stage 0, additional enlargement of the lymph nodes as stage I, and further enlargement of the spleen and liver as stage II. In the event of anemia (hemoglobin <11g/dL), the patient is considered to be in stage III of the disease. Eventually, if the patient is also thrombocytopenic with platelets count less than $100 \times 10^9/L$, they are included in the high-risk Stage IV (**Table 2**). Interestingly, Rai et al. found this staging system correlated with the overall survival (OS) of patients³⁷.

Rai Stage	Risk	Finding
0	Low	Lymphocytosis
I	Intermediate	Lymphocytosis + enlarged lymph nodes
II	Intermediate	Lymphocytosis + enlarged lymph nodes/spleen/liver
III	High	Lymphocytosis + anemia +/- enlarged lymph nodes/spleen/liver
IV	High	Lymphocytosis + thrombocytopenia +/- enlarged lymph nodes/spleen/liver +/- anemia

Table 2: Characteristics of the different stages considered in the Rai classification (Adapted from Rai et al, 1975)³⁷.

- **Binet Classification**

Few years after Rai, Binet et al. set up a new staging system³⁸ commonly used in Europe (Rai system is mainly used in USA)⁵. Binet's classification, simpler than Rai's, takes into account the number of enlarged lymph nodes and organs, and the presence or absence of anemia (hemoglobin <10g/dL) or thrombocytopenia (platelets <100x10⁹/L). The enlarged areas included in the analysis are head, neck, axillae, groins, spleen, and liver³⁶. Binet's stages are classified into three categories: A, B, and C. A description of each stage is shown in **Table 3** below:

Stage	Enlarged areas number	Anemia	Thrombocytopenia
A	<3	Absent	Absent
B	>3	Absent	Absent
C	indifferent	Present	Present

Table 3: Characteristics of the three stages included in the Binet classification (Adapted from Binet et al, 1981)³⁸

IV. Prognostic Factors

The staging systems described above are of great help to physicians, but they have their limitations. For example, they cannot predict disease progression in its early stages (Binet A and Rai 0)³⁹. For this reason, intensive research has been carried out over the last years to discover representative CLL prognostic factors. The evaluation of IGHV mutation status, the identification of mutations/aberrations/overexpressions of certain genes, the assessment of karyotype complexity, and the discovery of serum markers associated with disease progression now help physicians assess whether or not a CLL patient needs treatment⁴⁰.

a. IGHV Mutational Status

Thanks to their ability to identify intruders' antigens, immunoglobulins (Ig), part of the B cell receptor, are surface proteins essential for immune defense. They are organized as a heterodimer of two heavy chains (H) joined to two identical light chains (L). The constant antibody region corresponds to the C-terminal moiety, while the variable region (V) is the N-terminal moiety of the H and L regions⁴¹. The heavy chain is formed by the random rearrangement of three genes: variable (V), diversity (D) and joining (J). Other gene rearrangements form the kappa or lambda Ig light chains. Finally, the functional

rearrangements of both the heavy and light chains form a complete Ig receptor. Ig rearrangements happen during B-cell development. However, upon antigen exposure, a further heavy Ig chain (IgH) gene rearrangement takes place in the germinal center of the lymph nodes, causing complementary somatic mutations. This mechanism is called somatic hypermutation (SHM) and is essential for the generation of the antibody diversity⁴² (**Figure 5**). In CLL, Ig heavy chain variable region (IGHV) status is a crucial component when assessing patients' risk levels and is categorized into two distinct subgroups according to the SHM status:

- Unmutated-IGHV (UM-IGHV): $\leq 2\%$ gene sequence variation from its germline analog.
- Mutated-IGHV (M-IGHV) : $>2\%$ gene sequence variation from its germline analog⁴³.

The IGHV mutational status is commonly determined by DNA sequencing. Briefly, DNA is extracted from the collected sample, followed by polymerase chain reaction (PCR) for gene amplification. The amplified DNA can then be sequenced by a Next-Generation Sequencing (NGS) approach, and the resulting sequence is compared and interpreted to identify the percentage of similarity to its germline analog⁴¹.

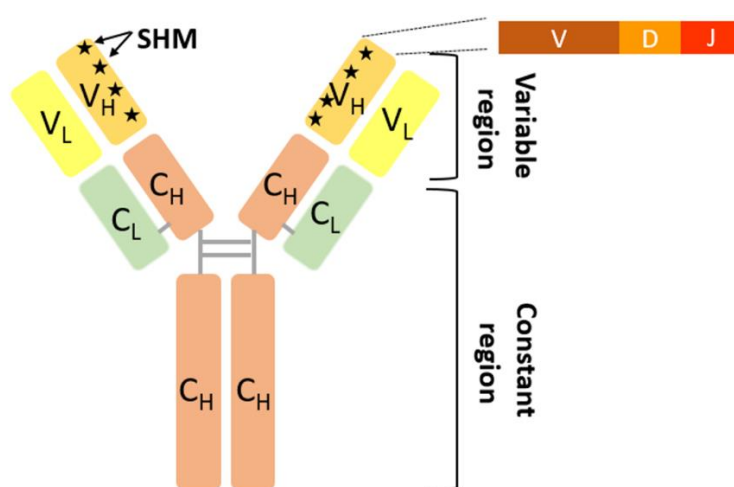


Figure 5: Schematic representation of an Immunoglobulin structure. The constant part is composed of constant light (CL) and constant heavy (CH) regions. The variable part is composed of variable light (VL) and variable heavy (VH). The latter is formed by the random rearrangement of the three genes: variable (V), diversity (D) and joining (J). Stars represent somatic hypermutations (SHM).

UM-IGHV CLL have a more progressive disease, with a shorter OS compared with M-IGHV CLL cases⁴⁴. It is suggested that the total number of mutations in IGHV gene is positively correlated to a better prognosis. As described previously, Ig, which are components of the BCR, can be stimulated by self-antigens. The difference in the disease aggressiveness between M-IGHV and UM-IGHV status may be due to a lower capacity of the mutated form

to recognize self-antigens. Because of this, less BCR signaling pathways are activated, and this might contribute to their indolent clinical course⁴⁵. Additionally, a link between the cells' proliferation rate and the DNA repair system was proposed to explain the appearance of the mutated IGHV in half of the CLL cases. For instance, in highly proliferating cells, the homology-directed repair (HDR) system becomes more active, minimizing the number of mutations and leading to UM-IGHV cells. Whereas, in slowly or non-proliferating cells, the nonhomology end-joining (NHEJ) repair system is usually predominant but is believed to have a reduced repair capacity, causing an accumulation of mutations and generation M-IGHV cells⁴⁶.

b. Cytogenetic Abnormalities

Since the 1970s, many chromosomal abnormalities (CA) have been identified in CLL⁴⁷. These are present in more than 80% of the patients. Some of the recurrent CA have clear pathogenic importance because they have an impact on the course of the disease and are used in routine clinical practice to identify patients at high risk of disease progression and poor survival. The hierarchical prognostic model of Döhner *et al*, published in 2000, is based on the fluorescence in situ hybridization (FISH) detection of the four most recurrent CA and defines five risk-stratifying categories. As shown in **Figure 6** below, these five categories are in the following order, from low risk to high risk: deletion in the long arm of chromosome 13 (del13q) as a unique abnormality, a normal FISH with no detected abnormalities, trisomy 12, deletion of the long arm of chromosome 11 (del11q), and deletion of the short arm of chromosome 17 (del17p). This stratification was validated when immunochemotherapy was the gold standard treatment for CLL^{39,40}. The prognostic value of these CA has to be evaluated in patients treated with targeted therapies, the most prescribed drugs nowadays⁵.

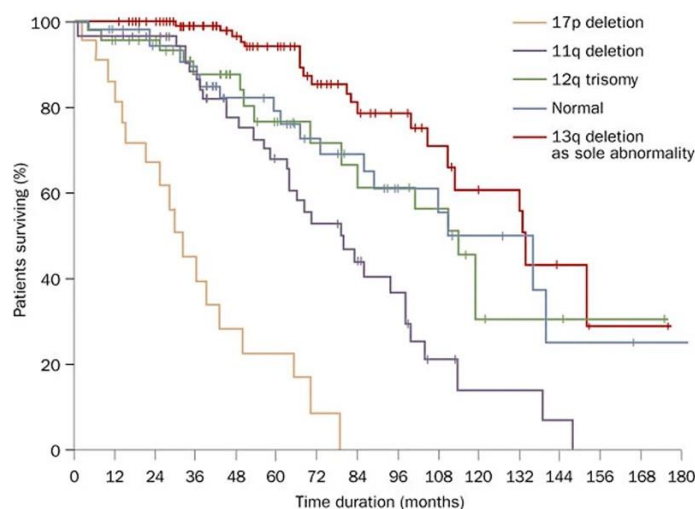


Figure 6: OS curve of the five CLL groups categorized by Döhner et al. The probability of survival is depicted among patients based on the presence of the four most common chromosomal aberrations (Reproduced from Cramer et al, 2010)⁴⁰.

- **Del13q**

Deletion in the long arm of chromosome 13 is frequently detected in CLL (~50-60%). When isolated, it is considered a good prognostic factor, and when treated, patients respond remarkably well to the current anti-CLL therapies. The deletion can be monoallelic or biallelic, and it can happen in different positions of the chromosome. Although the size of the deletion is heterogeneous across patients, the minimal deleted region (MDR) located in the long arm of chromosome 13 at position 14 (13q14) contains the deleted in lymphocytic leukaemia 2 (DLEU2) locus, which encodes the long non-coding RNA (lncRNA) DLEU2, and the microRNA cluster MIR15A–MIR16-1. This cluster is a tumor suppressor⁴⁸ that can down-regulate the anti-apoptotic protein BCL-2. The deletion of this cluster causes, therefore, an increased expression of BCL-2 in CLL⁴⁹. In some patients, the size of the del13q is larger, and the tumor suppressor gene retinoblastoma 1 (*RBI*) gene is also deleted. The clinical outcome of the *RBI* loss is still controversial, with some studies showing a poorer response to treatment and survival, while others attributing no prognostic value to this loss⁵⁰. Despite this, most studies confirm the relationship between del13q and good prognosis/clinical outcome⁴⁸.

- **Trisomy 12**

The presence of three chromosomes 12 is the second most frequent genetic abnormality in CLL cells (15-20% of cases). Moreover, 70% of trisomy 12 CLL cases present this cytogenetic aberration alone⁵¹, which is usually associated with an intermediate prognosis and a 9.5 years median OS³⁹. Despite this, patients with trisomy 12 have twice the risk to develop

additional genetic lesions and become in high-risk than patients with a normal karyotype or patients with del13q only⁵². This anomaly is also present in 50% of CLL cases evolving towards a Richter syndrome. Interestingly, 30 to 40% of trisomy 12 cases are associated to NOTCH1 mutation⁵¹. Despite its recurrence and prognostic importance, the mechanisms by which trisomy 12 contributes to CLL pathogenesis remain unknown⁴.

- **Del11q**

Deletion in the long arm of chromosome 11 is another common cytogenetic abnormality in CLL. In early stages and before any treatment, 10% of patients present this deletion, with the percentage increasing to 25% in advanced forms of the disease. As shown previously in **figure 6**, del11q is associated with a decreased OS but also with a rapid disease progression in patients treated with immunochemotherapy⁵³. However, the del11q has no adverse impact with targeted therapies^{54,55}. Clinically, patients with del11q have enlarged lymph nodes, and usually have an UM-IGHV. Genetically, the deletion size can be variable, with large deletions being more common than small ones. In most cases, the MDR affects *ATM*, a gene that encodes an essential protein in DNA repair. *ATM* deletion occurs in 36% of del11q cases and leads to a worse prognosis⁵⁶. Another frequently deleted (80% of del11q cases) gene is *BIRC3*, which encodes a negative regulator of the NF- κ B signalling pathway. A recent *in vivo* study showed an increase in leukemic cell proliferation promoted by *BIRC3* deletion⁵⁷.

- **Del17p**

Deletion of the short arm of chromosome 17 is a less frequent, poor-prognosis alteration found in 5-8% of untreated CLL patients. Unlike the deletions described above, del17p corresponds, in most cases, to a complete loss of the short arm of the chromosome. The most important consequence of del17p is the loss of the gene encoding the tumor suppressor *TP53*. Deletion of *TP53* is always mono-allelic, and in 80% of cases, CLL cells present an inactivating mutation in the second *TP53* allele. It is worth nothing that, without del17p, *TP53* mutations rarely occur alone in CLL. If identified, this genetic lesion is correlated to a decreased time to first treatment (TTFT), an increased resistance to anti-CLL treatments (including targeted therapy), and a lower OS^{4,58,59}. Due to the clonal evolution of CLL cells, the del17p clone increases by 40-50% in patients with refractory CLL. This is mainly due to the development of del17p subclones during the relapse period. Consequently, it is necessary for physicians to identify the presence of *TP53* aberrations before choosing a treatment⁵⁹.

- **Other Chromosomal Abnormalities**

Less frequent deletions can occur in CLL. For example, deletion in the long arm of chromosome 6 (**del6q**) is observed in 3-10% of CLL cases and is considered an intermediate risk factor with no effect on survival⁶⁰. Deletion of the short arm of chromosome 8 (**del8p**) is another less frequent deletion (5% of CLL cohort)⁶¹ and is considered a poor prognosis factor. This deletion causes the loss of the gene encoding the tumor necrosis factor-related apoptosis-inducing ligand-receptor (TRAIL-R) (also called TNFRFS10A/B). As a consequence, TRAIL induced apoptosis will be reduced, and this can lead to the BTK inhibitor ibrutinib drug resistance⁶².

In addition to chromosomal deletions, CLL cells can gain chromosomal parts. For example, a rare abnormality causes the gain of the short arm of chromosome 2 (**gain 2p**). It usually occurs in patients with poor prognosis in late stages of the disease (~15%) and comes associated with other high risk abnormalities, like del11q and UM-IGHV⁶³. Two minimal regions of gain are identified. The first one includes two oncogenes: *XPO1*, coding for exportin-1, a protein essential for nucleic export, and *REL*. The other one encompasses the oncogene *MYCN*^{64,65}. Another example is the gain of the long arm of chromosome 8 (**gain 8q**) observed in 3-5% of CLL cases. It is usually associated with a complex karyotype (that will be further discussed below), with other high-risk cytogenetic alterations like *TP53* and *ATM* loss, and with a shorter OS. The gain includes *MYC*⁶⁶, a gene that encodes the oncogene c-MYC, a transcription factor that regulates many cellular mechanisms, like cell cycle progression and cell differentiation/growth/death⁶⁷.

All these abnormalities and their combination perfectly illustrate the genetic heterogeneity of CLL.

- **Karyotype Complexity**

The complexity of the cell karyotype is also taken into account by physicians when designing treatment for patients with CLL. A complex karyotype (CK) was first defined as the presence of 3 or more chromosomal abnormalities in the CLL cells. It was associated with a faster time to relapse and a decreased OS. Recently, scientists and physicians have begun classifying CLL in an additional subgroup called “high-CK” (HCK). An HCK is characterized by the presence of 5 or more chromosomal abnormalities excluding karyotype with trisomy 12 and trisomy 19 related to a good prognosis⁶⁸. HCK is considered as an independent poor

prognostic factor in patients receiving targeted therapies or chemoimmunotherapies with shorter OS, regardless of the IGHV and the TP53 status⁶⁹.

c. Protein Expressions as a Biological Marker of CLL

• CD38 Expression

In 1999, Damle et al. identified CD38 as a new prognostic indicator for CLL⁷⁰. CD38 is a transmembrane protein usually highly expressed on the surface of B cells progenitors and B cells within secondary lymph nodes. The expression is lower in mature B cells. The protein is also expressed on other immune cells, like the T lymphocytes or the NK cells. It has an enzymatic role in converting nicotinamide adenine dinucleotide (NAD⁺) to cyclic ADP-ribose (cADPR) releasing nicotinamide (NAM). cADPR and its product ADPR are second messengers essential for calcium (Ca²⁺) release, and Ca²⁺ is a second messenger of many survival cellular processes⁷¹. In B cells, CD38 is generally localized close to other molecules, such as the CXCR4 or the BCR⁷². In CLL, both CD38 and CXCR4 receptor can be implicated in CXCL12 mediated chemotaxis⁷³. *In vitro* studies showed a faster and better response to BCR triggering in CD38⁺ cells⁷². This link between CD38 and BCR was also highlighted in another study carried out in a mouse model, showing higher CLL cytotoxicity and reduced tumor growth when an anti-CD38 was combined with a targeted therapy of the BCR pathway⁷⁴. Interaction between CD38⁺ cells and the microenvironment exists. CD38 can bind to its nonenzymatic ligand CD31, mainly expressed on endothelial cells, activating ERK1/2 signaling pathway and facilitating cells' chemotactic migration to secondary lymph nodes and bone marrow^{75,76}. Some components of the microenvironment, such as nurse like cells, express elevated levels of CD31 that can interact with CD38⁺ CLL cells and contribute to their survival and proliferation⁷⁷.

Clinically, CD38 overexpression is an adverse prognostic factor that correlates to aggressive forms of CLL. More specifically, the flow cytometry detection of more than 30% tumor CLL cells overexpressing CD38 correlates to a decreased OS, TTFT and enlarged lymph nodes⁷⁵. It should be noted that CD38 expression could change during the course of the disease, due to expansion of the clone. CD38, therefore, appears to be a dynamic marker that can be used to predict a patient's clinical course⁷².

- **ZAP-70 Expression**

Zeta associated protein 70 or ZAP-70 is a protein tyrosine kinase expressed only in UM-IGHV CLL cells, and is usually associated with higher expression of CD38⁷⁸. ZAP-70 is typically a T cell protein, not expressed in mature B cells, and is implicated in the activation of various downstream pathways, like Ras-MAPK and PLC γ 1, through T-cell receptor (TCR) signaling⁷⁹. ZAP-70 also plays a crucial role in the interaction of malignant B cells with their microenvironment⁸⁰. An association between ZAP-70 expression and an enhanced BCR signaling was observed in CLL, with an increased phosphorylation/activation of the SYK kinase⁷⁸. Other studies demonstrated that ZAP-70 acts more as an adaptor protein instead of a kinase in regulating the BCR signaling pathway^{81,82}. Interestingly, a recent study has suggested a role of ZAP-70 that goes beyond BCR signaling. Indeed, in the absence of BCR stimulation, ZAP-70 positively modulated cell survival by contributing to a constitutive regulation of gene expression essential for CLL cell interaction with T cells⁸³. Additionally, ZAP-70 expression seems to favor the migration capacity of CLL cells in the bone marrow after CXCR4 stimulation⁸⁴, and the Toll-like receptor 9 (TLR-9) anti-apoptotic signals appear highly dependent on ZAP-70 expression⁸⁵.

d. Other Biomarkers

- **Lymphocyte Doubling Time (LDT)**

LDT is a simple routine measurement to assess the number of CLL B cells in the blood overtime. It is defined as the number of months needed for lymphocytes to double in number. If the cells take more than a year to double, a patient has a great prognosis outcome. However, if it takes less than a year for the CD5+ CLL B cells to double, the patient's prognosis is poorer due to the rapid progression of the disease. In this case, clinical treatment is required⁸⁶.

- **Serum Beta2-Microglobulin (s- β 2M)**

β 2M protein is a component of the major histocompatibility complex classe I (MHC-I). It can be detected in the serum of CLL patients especially in advanced bone marrow infiltration cases. Patients with high levels of s- β 2M at diagnosis are more likely to have a shorter remission time after treatment and a general lower survival rate⁸⁷.

- **Serum Thymidine Kinase 1 (sTK1)**

Thymidine kinase (TK) is an enzyme essential for DNA synthesis. One of its isoforms, TK1, can be detected in CLL patients' serum. A high sTK1 level is usually detected in patients with an UM-IGHV form as well as in those who express high levels of CD38 and ZAP-70⁸⁸. However, this serological marker is not evaluated for CLL diagnosis⁵.

- **Gene mutations**

A few gene mutations are identified in CLL and some of them are considered as prognostic and predictive markers of the disease. This includes NOTCH1 and TP53 mutations⁸⁹. NOTCH1 is a transmembrane protein that serves as a transcription factor. Once activated by ligand binding, its intracellular domain is internalized to the nucleus, causing a transcriptional activation of target genes implicated in cell differentiation, proliferation and downregulation of tumor suppressors⁹⁰. NOTCH1 gain-of-function mutations are associated to a faster disease progression⁹¹ as well as poorer outcome. These mutations can also be associated to resistance to anti-CD20 immunotherapy⁹². Moreover, identifying mutations in the tumor suppressor gene TP53 is an important step in CLL diagnosis as this biomarker is a predictor of a worse disease outcome. For instance, these mutations impair the effectiveness of chemoimmunotherapy⁵⁹ and patients carrying them are directly given ibrutinib or venetoclax (in case of ibrutinib contradiction) as a first-line treatment⁵.

V. Treatment Options

The decision of treating a patient as well as the type of treatment depends on many factors. Patients with progressive Binet stage A or B and patients with Binet stage C should be treated. The progression criteria are i) development or aggravation of anemia and/or thrombocytopenia, ii) massive, progressive or symptomatic splenomegaly, iii) significantly enlarged, symptomatic, or progressive lymphadenopathies, iv) progressive lymphocytosis, with an increase of more than 50% over a period of 2 months or a LDT of less than 6 months, v) presence of constitutive symptoms such as fever, weight loss, and fatigue⁵. If the treatment is required, many factors are taken into consideration for treatment choice including the general fitness situation of the patient, the IGHV mutational status, as well as the genetic profile, especially the TP53 status^{4,5}.

a. Conventional Chemotherapy Agents

• Alkylating Agents

Alkylating agents were proposed as the first CLL treatment in the 1950s. These chemicals have the capacity to crosslink with the DNA strands, causing DNA damage and cellular toxicity (**Figure 7**). **Chlorambucil**, **cyclophosphamide** and **bendamustine** are examples of alkylating agents, which nowadays, are used with other therapeutic agents. These combinations will be further discussed in this section⁹³.

• Purine Analogs

In the 1980s, purine analogs were introduced as another treatment option for CLL. These agents have the capacity to incorporate the DNA and inhibit DNA replication by blocking the activity of DNA polymerase, ribonucleotide reductase, DNA ligase, and DNA primase (**Figure 7**). **Cytarabine** and the more widely used **fludarabine** are examples of purine analogs, also mostly used in combinational therapy⁹³.

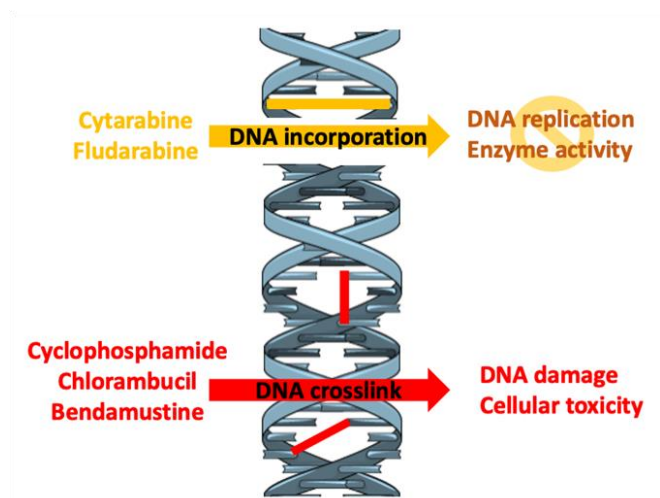


Figure 7: Mechanism of action of alkylating agents and purine analogs

b. Monoclonal Antibodies

In 1997, the anti-CD20 antibody **Rituximab** was FDA approved for the treatment of CLL. CD20 is a transmembrane phosphoprotein that serves as a calcium channel regulating B cells activation, differentiation and proliferation⁹³. There are basically three mechanisms of action by which Rituximab seems to exert the cytotoxic effect⁹⁴: 1) A direct apoptotic induction triggered by the activation of some deleterious signaling pathways⁹⁵ 2) A complement-dependent cytotoxicity (CDC) which recruits complement proteins, resulting in the formation

of a membrane attack complex (MAC) that leads to cell lysis 3) An interaction with NKs via the anti-CD20 Fc region of the antibody, which enables an NK cytotoxic response activating in turn the antibody-dependent cell-mediated toxicity (ADCC) that includes perforin and granzyme, both responsible for the activation of caspase-dependent programmed cell death. These mechanisms of action are illustrated in **Figure 8**. The second generation of the anti-CD20 antibodies includes **Ofatumumab** and **Obinutuzumab**⁹⁴.

Other monoclonal antibodies include the FDA approved **Alemtuzumab** that targets CD52, a surface antigen expressed on malignant B cells. This drug show effectiveness in cases with *TP53* mutation/deletion⁹⁶ and can be given to relapsed and refractory cases; however, its use is limited by an increased infection risk as a side effect⁹⁷.

Novel monoclonal antibodies targeting other B cell surface antigens, like CD37, CD200, and the receptor tyrosine kinase like orphan receptor 1 (ROR1) are currently being tested in clinical trials⁹⁸.

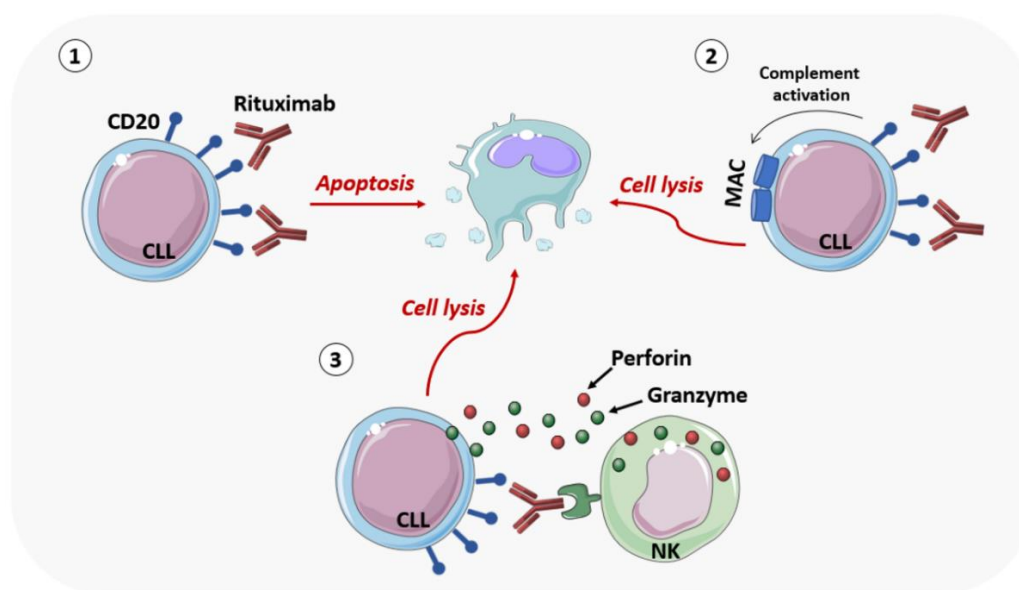


Figure 8: Rituximab mechanisms of action

c. Immunochemotherapeutic Options

- **Fludarabine-Cyclophosphamide-Rituximab (FCR)**

CLL patients aged below 65 years old, physically fit and not having comorbidities could be treated with the tri-therapy **fludarabine-cyclophosphamide-rituximab (FCR)**. Two additional conditions must be met: a mutated IGHV status and the absence of *TP53* mutation/deletion^{5,99}. Different clinical trials demonstrated the effectiveness of this therapy in increasing the overall response rate, the complete response rate as well as the progression free

survival (PFS). FCR can cause side effects including myelosuppression and infections¹⁰⁰. However, relapse, defined by the reappearance of the disease after at least 6 months of remission, can occur. 6% of CLL patients treated with FCR relapse within 6 months to 1 year, while 14% of cases relapse within 2 years¹⁰¹.

- **Bendamustine-Rituximab (BR)**

This bi-therapy is a treatment option for patients who are aged above 65 and have other health problems and diseases, together with CLL. This type of therapy can be used in the absence of *TP53* alterations and leads to similar PFS results with less adverse effects than the FCR¹⁰⁰.

- **Obinutuzumab-chlorambucil**

This bi-therapy is another treatment option for unfit patients aged above 65; with coexisting diseases and no *TP53* alteration⁵. It gives a better OS rate than chlorambucil given alone. It is worth noting that chlorambucil is a standard treatment option for old patients with comorbidities¹⁰².

The above-reported immunochemotherapies are gradually being replaced by the targeted therapies described below.

d. Targeted Therapies

- **BTK Inhibitors**

BTK is a cytoplasmic enzyme that catalyzes the phosphorylation of proteins and plays a central role in the BCR signaling pathway. BTK transmits the BCR activation signal to downstream targets via phospholipase C gamma 2 (PLC- γ 2) phosphorylation, leading to the activation of crucial survival pathways such as NF- κ B, MAPK and NFAT¹⁰³, as shown previously in **Figure 2**. B cells have a higher death rate in the absence of this enzyme, highlighting its importance for B cells survival¹⁴. Because of the constitutive activation of BCR in CLL and the important role of BTK in this signaling pathway, BTK inhibitors (BTKi) have been developed (**Figure 9**).

Ibrutinib, is a first generation developed BTKi, initially approved by FDA as a treatment for relapsed and refractory CLL cases and as a frontline treatment for patients with

17p deletion/*TP53* mutation. Concerning its mechanism of action, ibrutinib binds irreversibly to the cysteine 481 residue (C481) of BTK blocking its catalytic activity^{5,104}. However, it is associated with diverse side effects like bleeding, hypertension and cardiac arrhythmia. This is due to its off-target effect on other cellular kinases¹⁰⁵. Another problem encountered with the use of this drug is the acquired mutations causing drug resistance (due to long treatments associated to CLL). 19% of patients treated with ibrutinib relapse within 4 years of treatment and about 85% of them relapse because of a mutation in BTK or PLC- γ 2 proteins¹⁰⁶. Woyach et al. identified a cysteine to serine substitution mutation in BTK that diminishes the binding capacity of ibrutinib rendering it reversible, as well as substitution gain-of-function mutations in PLC- γ 2 causing downstream pathways activation independently of BTK¹⁰⁷.

Ibrutinib adverse side effects, as well as the acquired mutations leading to drug resistance led to the development of second generation more specific BTKis. For example, **Acalabrutinib** and **Zanubritinib** are irreversible BTK inhibitors with higher selectivity and specificity towards BTK than ibrutinib. They are both approved for treatment and they show less adverse effects than ibrutinib¹⁰⁵.

To avoid potential future problems (e.g. induced-mutations) with the irreversible BTKis, **Pirtobrutinib**, a reversible BTKi was more recently developed. This drug doesn't bind to BTK via C481 so it can be a good treatment option for patients resistant to an irreversible BTKi¹⁰⁸.

Because of their efficacy, it's important to note that BTKis are increasingly used as first-line therapy for all CLL patients needing a treatment.

- **BCL-2 Inhibitors**

B-cell lymphoma-2 (BCL-2) is an oncogene that, among others, works as a prosurvival anti-apoptotic protein. Apoptosis is mainly triggered when BAX and BAK, two pro-apoptotic proteins, are activated and create pores in the outer mitochondrial membrane, resulting in the loss of mitochondrial transmembrane potential, the release of cytochrome c and the subsequent formation of the apoptosome (together with APAF-1 and caspase-9) leading to cell death. Very schematically, cellular regulation of cell death also involves anti-apoptotic proteins such as BCL-2, which, through its interaction with BAX and BAK, inhibits their apoptogenic effect. To complicate matters, BCL-2 activity can in turn be inhibited by the BH3 family of proapoptotic proteins. Overall, the balance between prosurvival and proapoptotic effectors will determine the fate of the cell¹⁰⁹.

CLL malignant cells have an increased expression of BCL-2 protein rendering the cells more resistant to apoptosis. This overexpression can be due to a hypomethylation of the *BCL-2* gene¹¹⁰ and to a reduced levels of miR-15/16. These microRNAs decrease the levels of BCL-2 by targeting BCL2 mRNA. miR-15/16 are usually lost with the 13q deletion, as mentioned previously¹¹¹.

Venetoclax, a highly selective BH3 mimetic drug, is used as a BCL-2 antagonist for CLL treatment (**Figure 9**). 75 to 80% of refractory CLL cases respond positively to venetoclax treatment. It shows effectiveness even in the presence of negative prognostic factors like *TP53* alterations or *NOTCH1* mutations, and is associated with a prolonged remission when given as a monotherapy¹⁰⁹. Despite this, relapse can occur especially when venetoclax is given as a second line treatment. In this case, resistance mechanisms associated to mutations in *BCL-2* (e.g., GLY101VAL) decrease the binding affinity of the drug and, consequently, increase the BCL-2 prosurvival activity¹¹². To avoid these problems, second-generation BCL-2 inhibitors are currently under development¹¹³.

- **PI3K Inhibitors**

Phosphoinositide 3-kinase (PI3K) are lipid kinases that constitute a downstream part of the BCR signaling cascade and are key players in the PI3K/AKT/mTOR signaling pathway. They are usually overexpressed in UM-IGHV compared with mutated cases¹¹⁴. In 2014, the FDA approved the use a PI3K inhibitor (PI3Ki) **Idelalisib** as a treatment for CLL. *In vitro* studies showed the effectiveness of the drug in blocking the BCR signaling and inducing cell death¹¹⁵, by a caspase-dependent path, in the presence or not of del17p or del11q. Additionally, it reduces the synthesis of chemokins by nurse like cells causing less CLL migration to the secondary lymph nodes. Remaining in the peripheral blood, CLL cells are subsequently more prone to undergo apoptosis¹¹⁶. Recently, two mechanisms of resistance to idelalisib were identified and they involve the activation of the MAPK pathway¹¹⁷ and the upregulation of insulin-like growth factor 1 receptor (IGF1R)¹¹⁸. The toxicity of idelalisib restricts its use, and it is now reserved for BTKi/venetoclax refractory patients, in combination with rituximab¹¹⁹. However, in BTKi refractory patients, rituximab+idelalisib is unlikely to induce a prolonged response¹²⁰. Second generation PI3Ki include **Alpelisib** and **Duvelisib**, both FDA approved, but also related to many adverse effects¹²¹ (**Figure 9**).

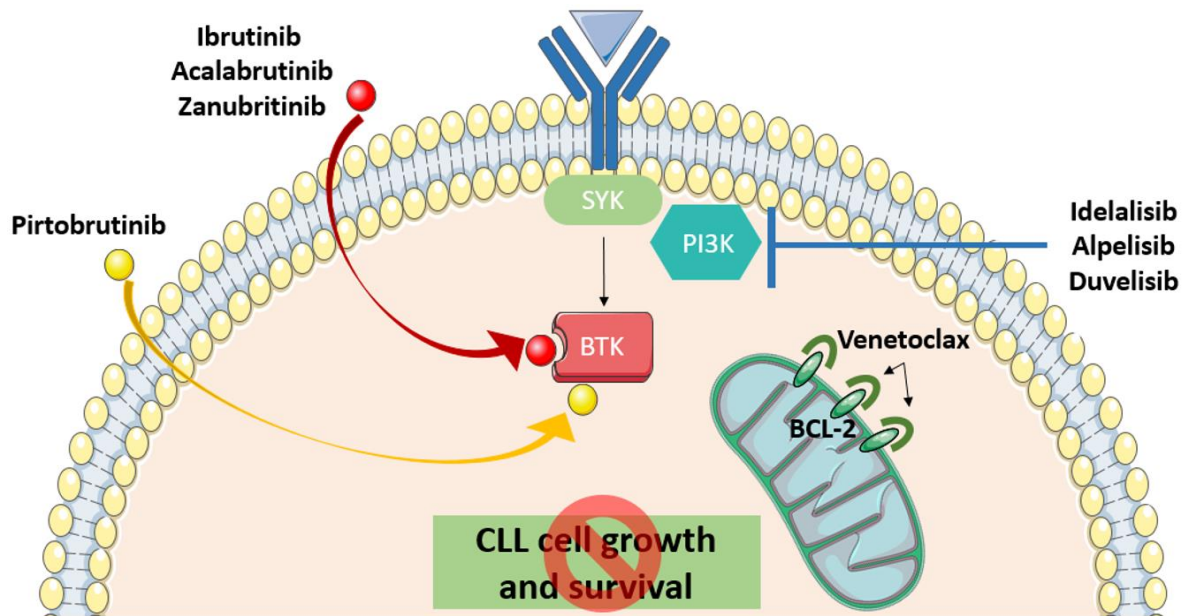


Figure 9: Developed targeted therapies against CLL. Irreversible BTK inhibitors include ibrutinib, acalabrutinib and zanubritinib that bind to BTK via C481. Pirtobrutinib, a reversible BTK inhibitor does not bind to the same site. Other targeted therapies include PI3K inhibitors such as Idelalisib, and venetoclax, a BCL-2 inhibitor.

Chapter II: Metabolic reprogramming in CLL

I. Introduction

a. Overview

Metabolism or *metabolē* in Greek, means “to change”, and corresponds to the set of biochemical reactions that occur in the cell to ensure its viability. It is divided into two branches: anabolism and catabolism. Anabolism corresponds to the synthesis, from simple components, of macromolecules such as lipids, proteins, polysaccharides, and nucleic acids. On the contrary, catabolism is the breaking down of these macromolecules into small compounds such as CO₂, H₂O, and NH₃. In general, catabolism releases energy and anabolism consumes it¹²².

Metabolic reprogramming is a term used to describe the metabolic rewiring that tumor cells exhibit to support their high demand of fuels needed to support their survival and enhanced proliferation¹²³. In 2011, it was recognized as one of the hallmarks of cancer¹²⁴.

b. Energy metabolism in health and cancer

Within the cell, energy is mainly stored in the highly charged molecule adenosine triphosphate (ATP). This molecule has three-bonded negatively-charged phosphates responsible for storing energy. Biochemically, a fundamental interconnection between energy and metabolic reactions exists. For instance, in anabolic reactions, ATP is consumed and a phosphate bond is broken down releasing energy to support the synthetic reactions. On the other hand, complex molecules break down in catabolic reactions, and release energy and this energy is stored by the synthesis of ATP from adenosine diphosphate (ADP)^{122,125}. Glycolysis, which is the breakdown of glucose, is the primary metabolic pathway that can directly produce ATP from its biochemical reactions. However, most ATP production requires the oxidative phosphorylation (OXPHOS) that takes place inside the mitochondria and is made up of two closely connected components: the electron transport chain and chemiosmosis¹²⁶. Conversely, under limited oxygen circumstances, such as during intense exercise, cells have the capacity to produce ATP outside mitochondria by transforming glucose into lactate by a series of reactions defined as anaerobic glycolysis¹²⁷. This way the cell can rapidly support the high demand for ATP that cannot be met alone by mitochondrial OXPHOS¹²⁸.

A hundred years ago, a pioneering discovery made by Otto Warburg introduced metabolic reprogramming to the field of cancer research. He discovered the capability of cancer cells to increase their glucose uptake and transform it into lactate even in the presence of oxygen. This observation is referred to as aerobic glycolysis or the Warburg effect¹²⁹. Despite the lower ATP yield from aerobic glycolysis compared to glucose metabolism through OXPHOS (2 ATP versus 36-38 ATP respectively), the first one generates ATP approximately 100 times faster than the mitochondria meeting the high cell energy demand¹³⁰. Additionally, aerobic glycolysis metabolic intermediates can contribute to de novo biosynthesis of other macromolecules essential for cancer cell proliferation, growth, and survival¹³¹. The extracellular acidity derived from lactate causes direct effect on the tumor microenvironment that can promote metastasis, angiogenesis, and drug resistance (**Figure 10**). Controversy, Warburg proposed a defective mitochondrial activity as a driver for this metabolic shift towards aerobic glycolysis¹³².

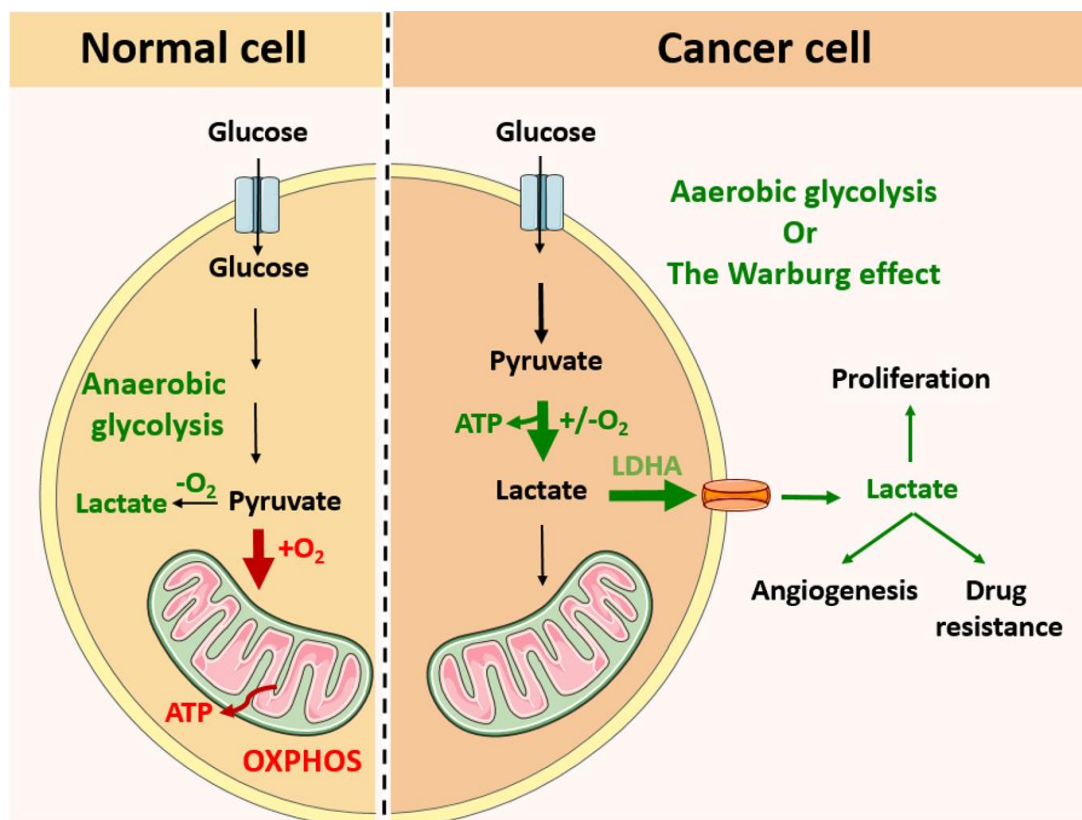


Figure 10: The Warburg effect. Normal cells mostly rely on glucose metabolism to fuel mitochondrial OXPHOS. In the absence of oxygen, the cells have the capacity to generate ATP outside the mitochondria, by a mechanism called anaerobic glycolysis. The Warburg effect suggests that cancer cells rely on lactate production outside the mitochondria even in the presence of oxygen. This metabolic shift does not only serve for ATP generation but also gives the cancer cells many advantages such as proliferation, angiogenesis and drug resistance.

Despite being accepted for decades, Warburg hypothesis began to be questioned by scientists toward the 1950s, and up until now many researchers suggest a sustained normal

mitochondrial activity even in the presence of the Warburg effect¹³⁰. In CLL, it is strongly believed that despite having a functional glycolysis, the cells do not follow the Warburg effect¹³³. Jitschin et al. reported an increased mitochondrial OXPHOS but no enhancement in aerobic glycolysis when comparing CLL cells to healthy B cells, suggesting a mitochondrial contribution to CLL metabolism¹³⁴. The mitochondria role in CLL as well as the metabolic pathways identified in this disease will be detailed in the upcoming sections.

II. Mitochondria's role in ATP production

a. Mitochondria structure and homeostasis

Mitochondria are double membrane organelles present in the cytoplasm of most eukaryotic cells that, as indicated above, generate ATP by aerobic respiration. They often are structured in a communication network within the cell. The outer mitochondrial membrane, which encloses the entire organelle, has a similar composition to the plasma membrane and contains key transport proteins such as the voltage-dependent anion channel (VDAC) or essential fatty acids metabolic enzymes. The mitochondrial intermembrane space is the space between the outer and inner membranes. As the outer membrane is highly permeable, its composition of small molecules is very similar to that of the cytosol. The mitochondria's inner membrane, composed of an unusual phospholipid called cardiolipin, is highly impermeable to most molecules that require specific transporters to cross it. This is key to creating the electron path needed to generate ATP¹³⁵. The mitochondria's inner membrane has inward folds called cristae and the internal compartment enclosed by the inner membrane is called the matrix¹²². They have their own DNA called mitochondrial DNA (mtDNA) present in small amounts in the matrix and that encode proteins essential for the functioning of the mitochondrial electron transport chain and, thus, for mitochondrial OXPHOS¹³⁶. Another specific characteristic of this special organelle is the presence of little mitoribosomes (39S/28S subunits) that translate the mitochondrial RNAs (mRNAs) encoded by the mtDNA¹³⁷ (**Figure 11**).

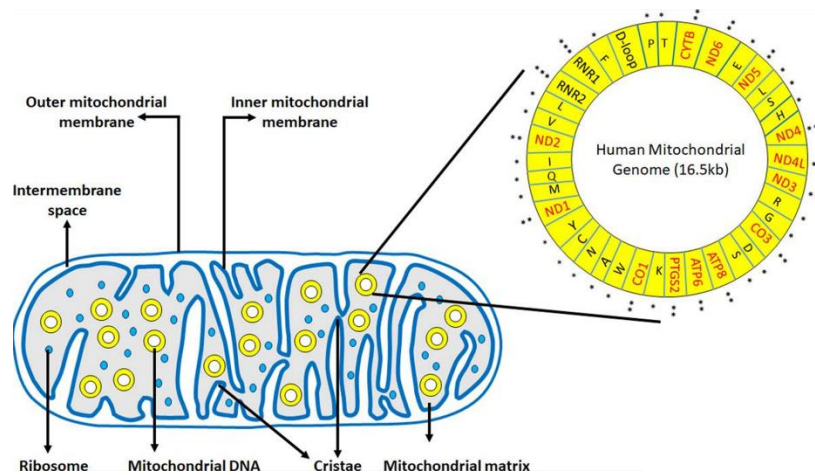


Figure 11: Mitochondria structure and composition. The figure illustrates the mitochondrial outer and inner membrane that folds into cristae. The two membranes are separated by an intermembrane space. The matrix contains ribosomes and mtDNA. This DNA is composed of 37 genes including 13 genes encoding for OXPHOS proteins and represented in red (*Reproduced from Adeboya et al, 2022*)¹³⁸.

Mitochondrial number and homeostasis are maintained in the cell by a balance between fusion and fission, a process called mitochondrial dynamics. This mitochondrial characteristic is important for the cell's energy requirements. For instance, a low performance of mitochondrial respiratory function results in mitochondrial fusion in order to mix and exchange chemical content for enhanced metabolic and respiratory activity. In parallel, mitochondrial fission is necessary to maintain the number of mitochondria and the correct distribution of organelles during cell division. Mitochondrial homeostasis is also maintained by mitophagy¹³⁸, a mechanism triggered to eliminate damaged mitochondria. It begins with the recruitment and activation of mitophagy receptors or ubiquitin–autophagy adaptors to the mitochondrial surface, followed by the formation of an autophagosome around the mitochondria. Autophagosome is then fused to lysosomes where the damaged mitochondria are subject to enzymatic degradation¹³⁹.

Finally, as indicated above, cells have the capacity to generate new mitochondria in response to an increased energy need for cell development or proliferation. This mechanism relies mainly on the transcription factor peroxisome proliferator-activated receptor- γ coactivator 1- α (PGC-1 α) that activates both mitochondrial and nuclear DNA transcription and leads to the synthesis of proteins involved in mitochondrial structure¹⁴⁰.

b. Mitochondrial oxidative phosphorylation

Mitochondria are widely described as being the powerhouse of the cell. ATP generation by the mitochondrial OXPHOS is directly linked to the electron transport chain (ETC) and is crucially dependent on a previous series of mitochondrial metabolic reactions known as the

TCA cycle or Krebs cycle. Briefly, metabolic substrates such as glucose, glutamine, and fatty acid derivatives produce metabolic intermediates that join the TCA cycle inside the mitochondrial matrix. The byproducts of this cycle, mainly nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH_2), provide the electrons for the ETC for ATP generation¹⁴¹.

- **TCA cycle**

The TCA cycle consists of eight metabolic reactions that are central to cellular metabolism (**Figure 12**). It forms a loop in a way that the final product regenerates one of the initial products, oxaloacetate. The cycle depends mostly on acetyl-CoA, the final product of many metabolic pathways and the starting substrate of the cycle. The cycle produces energy in the form of reducing equivalents NADH and FADH_2 (three molecules and two molecules respectively) that are delivered to the ETC¹⁴².

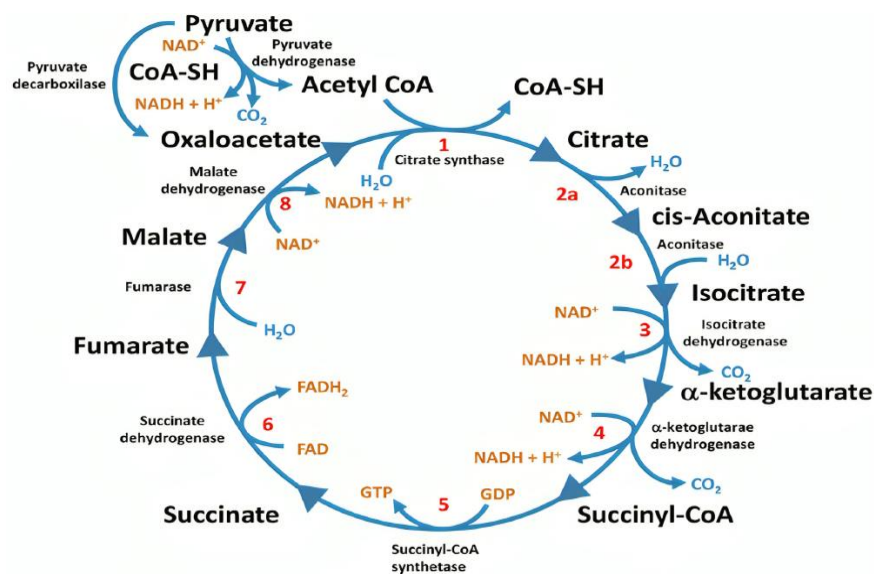


Figure 12: The TCA (or Krebs) cycle. The figure illustrates the eight metabolic reaction of the cycle, as well as the substrates, products, and enzymes of each reaction (Reproduced from Gasmí et al, 2021)¹⁴³.

- **The electron transport chain (ETC)**

The ETC is composed of five protein complexes assembled in the inner mitochondrial membrane. Its activity depends on two mobile electron carriers: ubiquinone (CoQ) and cytochrome c (Cyt c)¹⁴⁴ (**Figure 13**):

-Complex I, also known as NADH: ubiquinone oxidoreductase, constitutes the biggest component of the ETC machinery (44 subunits, seven of them encoded by the mtDNA). Its function includes a series of reactions that transfer electrons from NADH to CoQ, reducing it

into ubiquinol (QH₂); this is coupled to the pumping of four protons (H⁺) from the matrix to the mitochondrial intermembrane space.

-Complex II, or succinate dehydrogenase, has a dual role within the mitochondria. It catalyzes the oxidation reaction of succinate into fumarate within the TCA cycle and it reduces CoQ into QH₂ within the OXPHOS system.

-Complex III, or coenzyme Q: cytochrome c reductase, receives QH₂ produced by complex I and II and transfers its electrons by a series of reactions into Cyt c. This is coupled to a pumping of 4H⁺ in the intermembrane space.

-Complex IV, also referred to as cytochrome c oxidase, corresponds to the final electron acceptor in which the electrons are transferred from Cyt c to reduce oxygen into water. Similarly, two H⁺ are pumped for each water molecule formed. The process of consuming oxygen by the mitochondria is referred to as mitochondrial respiration¹⁴¹. The oxygen supply is crucial for mitochondrial activity. Notably, an inadequate oxygen supply also referred to as “hypoxia” causes mitochondrial dysfunction, cell death, and tissue damage¹⁴⁵. Finally, the difference in the distribution of protons between the matrix and the mitochondrial intermembrane causes a potential difference known as the mitochondrial membrane potential ($\Delta\Psi_m$).

-Complex V, or ATP synthase, utilizes the $\Delta\Psi_m$ created across the ETC and generated by the proton pumps (complexes I, III, and IV) to re-pump the protons back into the matrix and uses the energy dissipated to phosphorylate ADP into ATP¹⁴¹.

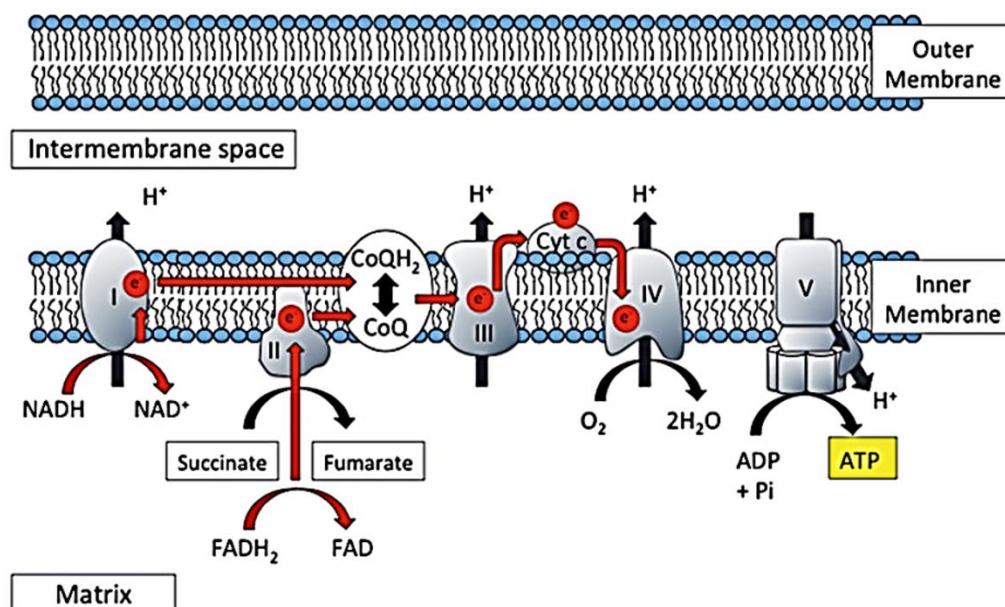


Figure 13: The mitochondrial ETC. Electrons are transferred from Complex I to Complex IV where they reduce oxygen into H₂O. The proton (H⁺) gradient generated causes a $\Delta\Psi_m$ used by complex V to repump H⁺ back into the matrix. This transfer provide the energy to generate ATP (Reproduced from De Villiers et al, 2018)¹⁴⁶.

Structurally speaking, and up until 2001, it was believed that the complexes were diffused independently across the mitochondrial inner membrane. This was referred to as the fluid model of the ETC. However, a solid model was proposed in which the complexes are organized in supramolecule structures called respirasomes or supercomplexes (SC). SCs can consist of complex I, III, and IV assembly, favoring electron transfer and minimizing electron loss¹⁴⁷.

c. Mitochondrial respiration in CLL

Mitochondria and mitochondrial OXPHOS have been largely studied in CLL. It is suggested that CLL primary cells have a higher mitochondria number compared to healthy B cells and the number of mitochondria is positively correlated to fludarabine resistance¹⁴⁸. Similarly, transcription factor A mitochondrial (Tfam), a protein involved in stimulating the transcription of mDNA and involved in mitochondrial biogenesis¹⁴⁹, is overexpressed in CLL¹³⁴. Interestingly, TP53 dysfunction increases mitochondrial biogenesis, probably as a compensatory response to the reduced expression of genes that encode for components of the ETC observed in CLL cells with del17p¹⁵⁰. In another study, Jitschin et al. work did not only confirm the increased biogenesis in CLL, but it also showed an increase in $\Delta\Psi_m$ and ATP levels, suggesting an enhancement in mitochondrial OXPHOS. The oxygen consumption rate (OCR), an indicator of the mitochondrial respiration activity, was significantly higher in CLL compared to healthy donors B cells when measured at the basal state of the cells^{134,151}. The basal OCR was found to be higher in CLL cells with poor prognostic factors (ZAP-70, and CD38 positive) but showed no variation related to the IGHV mutational status. Remarkably, basal respiration decreased post-ibrutinib treatment, while cells from relapsed patients (post-chemotherapy or ibrutinib) had no change in OCR compared to the untreated CLL¹⁵². This suggests a rewiring of mitochondrial metabolic activity developed with emerging resistance¹⁵³. *In vitro* pharmacological inhibition of the ETC complex I by metformin or IACS-010759 impairs CLL OXPHOS but demonstrates a shift towards aerobic glycolysis as a compensatory mechanism. Targeting OXPHOS as well as this metabolic shift (by a glycolysis inhibitor) induced significant toxicity in CLL^{154,155}.

The microenvironment also appears to influence the energy metabolism of the malignant CLL cells. For instance, the basal OCR is increased when CLL cells are co-cultured with stromal cell lines such as NK.Tert (NK cell line), M2-10B4 (murine fibroblast cell line), and HS-5 (human bone marrow-derived cell line)¹⁵⁶. A recent study showed a higher mitochondrial number in CLL cells harvested from lymph node samples compared to

circulating CLL. They also demonstrated an increase in basal OCR when stimulating the BCR pathway by anti-IgM or when mimicking the interaction with the immune system by CD40L stimulation¹⁵⁷. These results strongly suggest a role of the microenvironment in enhancing mitochondrial activity and respiration.

III. Other mitochondrial functions and their deregulation in CLL

Despite being called the powerhouse of the cell and having OXPHOS as their core function, mitochondria have diverse functional activities such as regulation of reactive oxygen species (ROS), calcium homeostasis, or programmed cell death (PCD). It is also home to metabolic reactions implicated in the amino acid, lipid, and one carbon metabolisms¹⁵⁸.

a. Reactive oxygen species regulation

ROS is a name given to a group of oxygen-containing oxidant molecules, such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO\cdot$), and nitric oxide (NO). Mitochondrial ROS (mtROS) generation is a natural phenomenon by which ROS are produced due to electron leakage from the ETC. Physiologically speaking, 0.2-2% of electrons leak from the ETC and interact with oxygen molecules to form O_2^- ¹⁵⁹ (**Figure 14**). O_2^- production mainly happens in complexes I and II of the ETC, while less amounts of this anion are produced by complex III. However, O_2^- released by complex III can be transformed into H_2O_2 in a reaction catalyzed by superoxide dismutase 1 (SOD1). This oxidant can freely pass through the mitochondrial membrane into the cell cytoplasm¹⁶⁰. H_2O_2 can interact with metal ions such as Fe^{2+} , Cu^{2+} , Fe^{3+} , Ti^{4+} , and Co^{3+} to form $HO\cdot$ the most highly reactive and harmful free radical formed in the cell¹⁶¹.

It was initially believed that ROS play a harmful role within the cell. However recently, much evidence has emerged to support the beneficial role of ROS in regulating many physiological processes, including cellular signaling, gene expression, or hematopoiesis. A low level of ROS is important for maintaining quiescence and the differentiation potential of HSCs, whereas the level of ROS increases during hematopoietic differentiation¹⁶². For instance, H_2O_2 is essential for intracellular signaling by inducing oxidation of the cysteine residues of signaling proteins leading to changes in downstream biological events implicated in vital cell functions such as transcriptional regulation, cytoskeletal rearrangements, and cell replication¹⁵⁹. mtROS also plays an important role in eliminating many bacterial infections such as salmonella, mycobacterium, and listeria¹⁶³. For instance, mtROS generation is increased by phagocytes

after the intruder's recognition. Interestingly, ROS are implicated in the wound healing process. Apart from their role in protecting the wound site from bacterial infections, they also act as a messenger, stimulating signaling pathway essential for the recruitment of immune cells to the wound site and the tissue repair mechanism¹⁶⁴.

Conversely, excessive ROS production is harmful to the cell. Excessive ROS production causes oxidative stress and damage to cellular components such as proteins, lipids, and DNA all leading to cellular dysfunction¹⁶⁵. For example, ROS interaction with DNA leads to mutations that might increase the risk of developing cancers¹⁶⁶. ROS can also cause alterations in protein structure leading to the accumulation of damaged proteins in the brain tissue that contribute to the development of neurodegenerative diseases¹⁶⁷. Enhanced ROS production also contributes to an increased activation of inflammatory pathways promoting various inflammatory diseases¹⁶⁸. Of note that many environmental factors can trigger ROS production such as smoking, UV radiation, pollutants, pesticides, toxins, and many others¹⁶⁵.

Because ROS have a beneficial role within the cell, an antioxidant defense mechanism exists to prevent ROS overproduction and avoid its oxidative damage. The system includes both enzymatic and nonenzymatic antioxidant protective molecules. Numerous enzymes exist in the cell to convert reactive ROS into less harmful ones: 1) Superoxide dismutase (SOD) converts O_2^- into H_2O_2 . 2) Catalase (CAT) decompose H_2O_2 to H_2O and O_2 . 3) Glutathione peroxidase (GPx) detoxifies H_2O_2 by using glutathione (GSH) as a cofactor¹⁶⁹ (**Figure 14**).

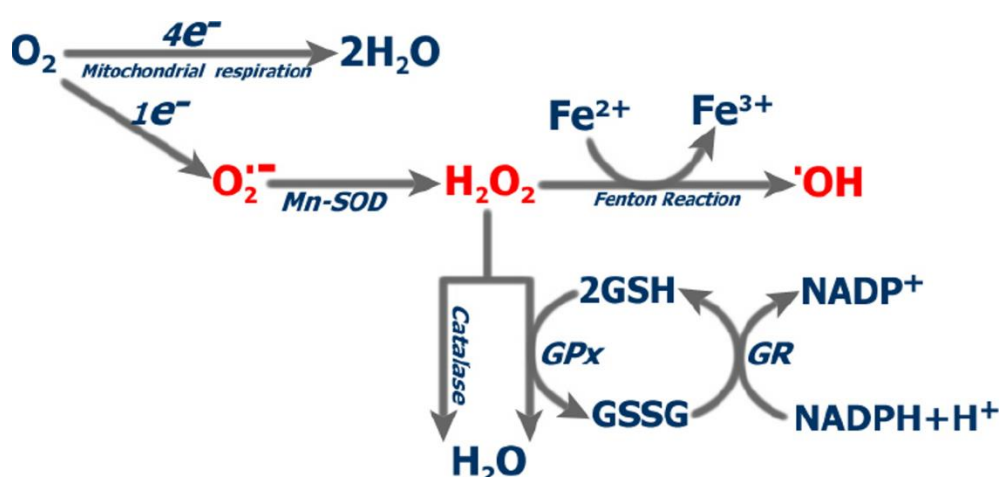


Figure 14: ROS production and detoxification. The superoxide anion (O_2^-) is produced from the interaction between an electron and O_2 . The latter can be transformed into H_2O_2 by a reaction catalysed by superoxide dismutase (SOD). A further interaction with Fe^{2+} transforms H_2O_2 into hydroxyl radical ($\cdot OH$). The detoxification of H_2O_2 into H_2O requires catalase or glutathione peroxidase (GPx). Glutathione reductase (GR) transforms the reduced form of glutathione (GSSG) back into glutathione (GSH) (Reproduced from Valle et al, 2010)¹⁷⁰.

Many vitamins also play an antioxidant role (**Table 4**). Vitamin A can directly bind free radicals while vitamin E can donate electrons to stabilize them, and both vitamins counteract

the damaging role of ROS on lipids, a mechanism called **lipid peroxidation**. Vitamin C can also scavenge free radicals and is a generator of vitamin E. In the end, ROS homeostasis is maintained by a balance between ROS and the cell antioxidants. Any deregulation in this balance causes an increase in the amounts of ROS, leading to oxidative stress that might be linked to the development of many diseases¹⁷¹.

Nutrients	Component (location in cell)	Function
Vitamin C	Ascorbic acid (cytosol)	Reacts with several types of ROS/RNS
Vitamin E	α -tocopherol (membranes)	Breaks fatty acid peroxidation chain reactions
β -carotene	β -carotene (membranes)	Prevents initiation of fatty acids peroxidation chain reaction
Selenium	Glutathione peroxidase (cytosol)	An enzyme that converts hydrogen peroxide to water
Copper and zinc	Superoxide dismutase (cytosol)	An enzyme that converts superoxide to hydrogen peroxide
Manganese and zinc	Superoxide dismutase (mitochondria)	An enzyme that converts superoxide to hydrogen peroxide
Copper	Ceruloplasmin (water phase)	An antioxidant protein, may prevent copper and iron from participating in oxidation reactions
Iron	Catalase (cytosol)	An enzyme (primarily in liver) that converts hydrogen peroxide to water

Table 4: List of vitamins and minerals with an antioxidant activity in mammals. For each nutrient, its cell localization and antioxidant function are described (Reproduced from McDowell et al, 2007)¹⁷².

Whether oxidative stress is one of the causes of CLL or simply a side effect is still a matter of debate. In either case, the oxidative stress characterizing CLL is mainly caused by high levels of OXPHOS-derived ROS¹⁷³. Indeed, CLL cells have high production of mtROS compared to healthy B lymphocytes. This is explained in the literature by an impaired antioxidant system. For example, the mitochondrial antioxidant manganese superoxide dismutase (MnSOD), is downregulated in CLL¹³⁴. Additionally, the reduced catalase expression in CLL, compared to healthy B cells, seems to explain the accumulation of H₂O₂ in the malignant CLL cells¹⁷⁴. In contrast, GPx activity and GSH levels are increased in stage A CLL suggesting an adaptation mechanism against oxidative stress. However, this increase is not sufficient to prevent it as malondialdehyde (MDA) and 8-oxo-2'-deoxyguanosine (8-oxo-dG) (two biomarkers of lipid peroxidation and DNA damage respectively) are still in elevated levels in these CLL B-lymphocytes¹⁷⁵. Interestingly, *in vivo* inhibition of ROS production by the antioxidant N-acetylcysteine (NAC) decelerated the CLL progression suggesting a role of

ROS in disease expansion¹⁷⁶. Conversely, ROS levels aren't always the same and differ according to treatment status and clinical stage. For instance, CLL cells isolated from patients treated with chemotherapy exhibited higher ROS levels than treatment naïve CLL cells. Also, ROS levels show a positive correlation with Rai stages (enhanced levels in advanced Rai stages)¹⁷⁷. Moreover, the microenvironment also plays an essential role in supporting ROS homeostasis as an adapting mechanism against oxidative stress¹⁷⁸. Transcriptomic analysis in CLL cocultured with HS-5 stromal cells revealed an increase in OXPHOS components as well as in ROS detoxifying antioxidants such as peroxiredoxin 3 (PRDX3)¹⁷⁹. Finally, another *in vitro* study showed that HS-5 stromal cells promote GSH synthesis within CLL cells and enhance their survival and resistance to fludarabine treatment¹⁸⁰.

b. Calcium homeostasis

Mitochondria is also a central player in maintaining the cell's calcium (Ca^{2+}) homeostasis. Under normal physiological conditions, Ca^{2+} concentration is around 100nM in the cytoplasm. However, under certain conditions (for example activation of intracellular signaling pathways), the cytosolic concentration of Ca^{2+} amplifies due to an increase in its transport across the plasma membrane or its release from intracellular stores like the endoplasmic reticulum (ER) or the sarcoplasmic reticulum (SR). This rise in calcium concentration stimulates its uptake by mitochondria to modulate the cytosolic levels¹⁸¹. Mitochondrial calcium uptake is regulated by three transport systems: (i) the mitochondrial calcium uniporter (MCU) is the dominant transport protein complex that is usually more active at higher cytosolic Ca^{2+} concentrations; (ii) the sodium/calcium/lithium exchanger (NCLX), and (iii) the hydrogen calcium exchanger (HCX) are bidirectional transporters that transport calcium inside the mitochondria in exchange of ions that are released outside¹⁸². Interestingly, mitochondrial Ca^{2+} has in turn an impact on mitochondrial function. Ca^{2+} modulates the activity of some TCA cycle enzymes such as the isocitrate dehydrogenase (IDH) and the oxoglutarate dehydrogenase (OGDH) increasing the reducing equivalents (NADH and FADH_2) that feed the ETC and subsequently generate ATP¹⁸³. In contrast, when the mitochondrial matrix becomes overloaded with Ca^{2+} , it stimulates the opening of a permeability transition pore (PTP) that becomes involved in the release of cyt c and the initiation of caspase-dependent cell death¹⁸⁴ (**Figure 15**).

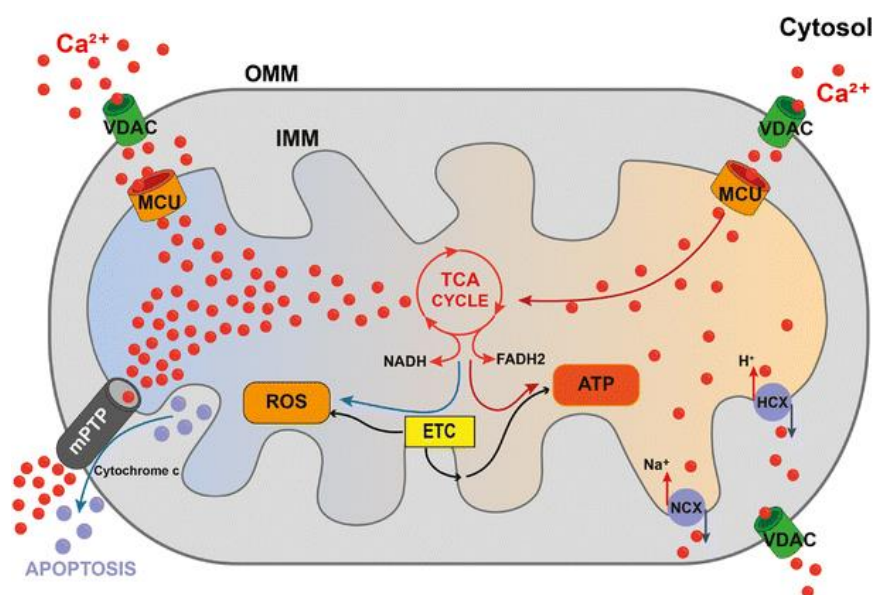


Figure 15: Influx and efflux mechanism of mitochondrial Ca^{2+} . Ca^{2+} pass through the mitochondrial outer membrane via the voltage-dependent anion channel (VDAC). It is then transported in the matrix by the mitochondrial calcium uniporter (MCU), in exchange of H^+ via hydrogen/calcium exchanger (HCX) or in exchange of Na^+ via the sodium/calcium exchanger (NCX). Ca^{2+} can modulate the activity of some TCA cycle enzymes increasing the reducing equivalents (NADH and FADH₂) that feed the ETC and generates ATP and ROS. In parallel, a Ca^{2+} overload causes the opening of mitochondrial permeability transition pore (PTP) that releases cytochrome c and consequently initiates apoptosis (Reproduced from Mammucari et al, 2018)¹⁸⁵.

Lastly, mitochondria play a crucial role in solving cell stress conditions, mainly in the ER. This stress is usually activated upon the accumulation of unfolded proteins in the ER. Morphological and spatial changes happen in the ER and the mitochondria to favor the exchange of Ca^{2+} between the two organelles. In the mitochondria, Ca^{2+} increases ATP production as described previously, and this energy is used in its turn to activate ER enzymes involved in activating the unfolded protein response essential to solve the stress generated¹⁸⁴.

In CLL, Ca^{2+} mediated induced apoptosis is investigated as a potential therapeutic strategy¹⁸⁶. Normally, B cells can induce Ca^{2+} mobilization via the BCR signaling pathway. Briefly, PLC γ 2 recruited by BTK activation, cleaves phosphatidylinositol-4,5-diphosphate (PIP₂) into diacylglycerol (DAG) and inositol-1,4,5,-triphosphate (InsP₃). The latter binds to its ER receptor INSP3R stimulating the release of Ca^{2+} ¹⁸⁷. Remarkably, BCL-2 has to capacity to bind to this ER receptor and inhibit Ca^{2+} release¹⁸⁸ (**Figure 16**). However, BCL-2 only blocks proapoptotic Ca^{2+} release but doesn't affect physiological Ca^{2+} signals. *In vitro* targeting of BCL2-INSP3R by a synthetic peptide causes a highly elevated Ca^{2+} release in CLL primary cells enabling a Ca^{2+} -dependent apoptotic cell death process¹⁸⁶.

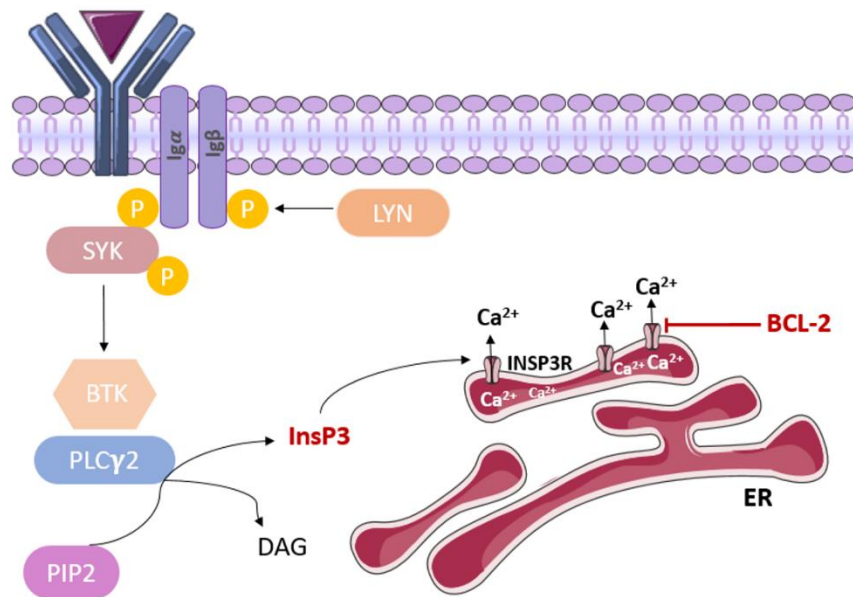


Figure 16: Ca²⁺ mobilization via BCR signaling. PLCγ2 is activated by BTK following BCR stimulation. It cleaves PIP2 into InsP3 and DAG. INSP3 binds to its ER receptor INSP3R, causing the release of calcium. ER Ca²⁺ release can be suppressed by the anti-apoptotic protein BCL-2.

c. Mitochondrial-mediated programmed cell death

In multicellular organisms, programmed cell death (PCD) is a genetically regulated mechanism that plays a crucial role in the control of normal development and in the regulation of tissue homeostasis and failure to accurately undergo PCD can cause severe anomalies, ranging from autoimmune diseases to cancer¹⁸⁹. In the last decades, much of the attention in the study of mitochondrial-mediated PCD was focused on caspases, a family of cysteine proteases specifically activated in apoptotic cells¹⁹⁰. However, mitochondrial-dependent PCD can still occur even when the caspase cascade is blocked¹⁹¹. This fact has revealed the existence of alternative mitochondrial caspase-independent PCD pathways¹⁹².

In classical apoptosis (caspase-dependent), mitochondrial outer membrane permeabilization (MOMP) induces the release of cytochrome c from mitochondria to cytosol to activate caspase-9. This activation happens in collaboration with ATP and the cytosolic factor APAF-1 (creating a complex called the apoptosome). Activated caspase-9 activates caspase-3 which in turn is responsible for the further cleavage of critical proteins and cellular components¹⁹⁰. **Figure 17** depicts the extrinsic (caspase-independent) and intrinsic (caspase-dependent) PCD.

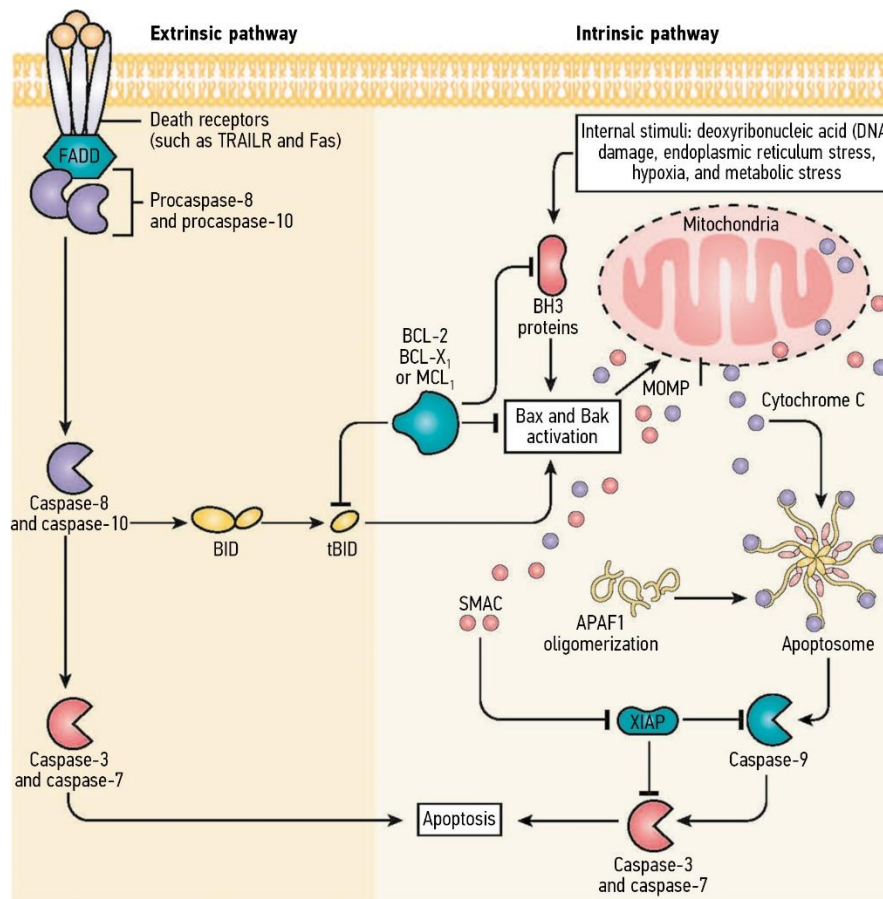


Figure 17: Extrinsic vs. intrinsic apoptotic pathways. In the extrinsic pathway, death receptor activation triggers the assembly of initiator caspases such as caspase-8 and caspase-10 by the help of the FAS-associated death domain protein (FADD). These caspases activate the effector caspases 3 and 7 leading to apoptosis. In the intrinsic pathway, internal stimuli activate BH3 proteins, leading to the activation of Bax and Bak that trigger the mitochondrial outer membrane permeabilization (MOMP). This causes cytochrome c release that assemble with APAF1 forming an apoptosome. The latter activates caspase-9 that in its turn activates caspase-3 and -7. The mitochondrial protein SMAC helps apoptosis by inhibiting the inhibitor X-linked inhibitor of apoptosis protein (XIAP). The interaction between the extrinsic and intrinsic pathway happens through the cleavage of BID into tBID that can trigger MOMP (Reproduced from Ichim et al, 2016)¹⁹³.

In other mechanisms implicating caspases and mitochondria, caspase-inhibitory factors are separated from their physiological partners by mitochondrial proteins such as Smac/DIABLO 1 or Omi/HtrA2, resulting in caspase activation¹⁹⁴. The intermembrane space of mitochondria also contains other proteins, such as apoptosis-inducing factor (AIF) and endonuclease G (EndoG), which can provoke PCD in a caspase-independent manner^{195,196,197} (**Figure 18**). Finally, Omi/HtrA2 can build a shuttle between caspase-dependent and -independent PCD through its serine protease activity¹⁹⁸. For example, AIF moves from the mitochondria to the nucleus where it induces chromatinolysis and cell death in cooperation with histone H2AX and cyclophilin A (CypA)¹⁹⁹. Therefore, mitochondria also appear as a weapon store where a cocktail of caspase-dependent or -independent proapoptotic proteins are sequestered until PCD is triggered. Of note that, mitochondrial-mediated PCD is regulated by an equilibrium between the pro- and anti-apoptotic members of the BCL-2 family of proteins.

This include BAX and BAK, two apoptogenic Bcl-2 family members, that assemble as oligomers and form pores in the mitochondrial outer membrane causing MOMP²⁰⁰.

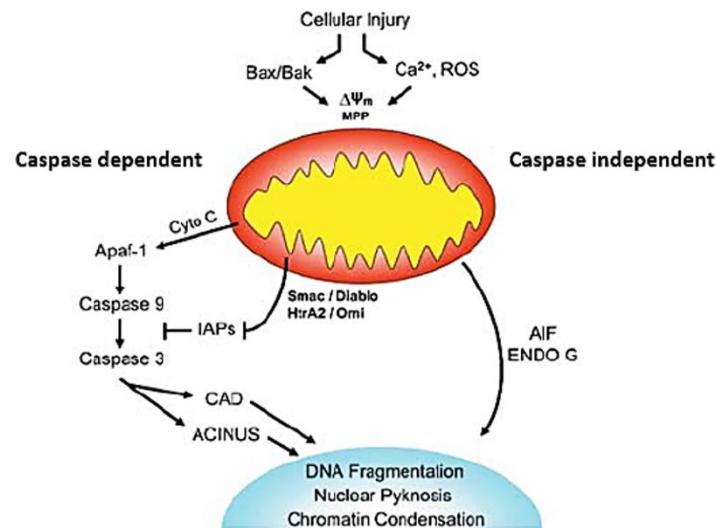


Figure 18: Mitochondrial proteins implicated in apoptosis. Many mitochondrial proteins are released after mitochondrial permeabilization. This includes SMAC/Diablo and HtrA2/Omi which inhibit the inhibitor of apoptosis proteins (IAPs). Therefore, the activation of caspase-3 can trigger the release of the caspase-activated Dnase (CAD) and the apoptotic chromatin condensation inducer (ACINUS) triggering DNA fragmentation and chromatin condensation respectively. Other caspase-independent proteins inducers of apoptosis include AIF and EndoG (Reproduced from Cregan et al, 2004)²⁰¹.

The PCD machinery is partially deregulated in CLL B-lymphocytes, and consequently the malignant cells accumulate within the peripheral blood²⁰². Most CLL patients have increased levels of the anti-apoptotic proteins BCL-2 and MCL-1 rendering the cells resistant to PCD favoring the disease development and modulating the response to therapy^{203,204}. *BCL-2* gene overexpression might be due to a DNA hypomethylation in the first major exon of the gene²⁰⁵. It can also be increased under the effect of microenvironmental stimuli, as observed in CLL-BM stromal cells *in vitro* cocultures²⁰⁶. *MCL-1* is overexpressed in response to therapy, and modulated by stromal interaction or BCR activation²⁰⁷. The deregulation of the mitochondrial apoptotic pathway because of these overexpressed prosurvival proteins contributes to the characteristics of CLL. Targeting these molecules is an important research area to provide therapeutic options²⁰³.

IV. Metabolic pathways and their implication in CLL

Eukaryotes utilize three main metabolic fuels including glucose, amino acids such as glutamine and arginine, and fatty acids. They are derived from the breakdown of carbohydrates, proteins, and fat coming from dietary intake¹²².

Just like the disease, metabolic rewiring is heterogeneous in CLL and depends on many factors such as genetic variants, clinical course, treatment received, as well as

microenvironment interactions^{133,151}. Each metabolic pathway as long as their implication in CLL will be discussed in the following sections.

a. Glucose metabolism

Glycolysis derived from the Greek word glykys (meaning sweet and lysis) was the first ever identified metabolic pathway. It is also known as the Embden–Meyerhof–Parnas pathway referring to the name of the scientists who contributed to its identification. It corresponds to a series of ten enzymatic reactions responsible for glucose breakdown²⁰⁸. Glucose is a polar molecule that cannot simply be diffused across the cell membrane but must be transported inside the cell by specific transporters. There are two types of glucose transporters: 1) sodium-glucose linked transporters (SGLTs) usually responsible for intestinal glucose absorption and 2) glucose transporters (GLUTs) divided into different subfamilies differentially expressed among cell types²⁰⁹. Once inside the cell, glycolysis takes place in the cytoplasm generating two ATP for each molecule of glucose. Its final product, pyruvate, is then transported to the mitochondria where it is transformed into acetyl-CoA to join the TCA cycle²⁰⁸ (**Figure 19**).

Other than its role in energy production through mitochondria, glycolysis generates intermediates that contribute to other metabolic pathways such as the pentose phosphate pathway (PPP). This pathway can be branched off glycolysis into the oxidative PPP or the non-oxidative PPP. The first branch is responsible for generating nicotinamide adenine dinucleotide phosphate (NADPH)²¹⁰, a reducing agent essential for the production of reduced GSH needed for H₂O₂ detoxification²¹¹. The final product of the oxidative PPP generates ribulose 5-phosphate that enters the non-oxidative branch. This branch generates ribose-5-phosphate, used as the backbone for DNA and RNA synthesis. Another product, erythrose 4-phosphate, is involved in various amino acid synthesis such as histidine²¹⁰ (**Figure 19**).

As previously indicated, CLL cells do not depend primarily on aerobic glycolysis (Warburg effect) to generate ATP¹³⁴. However, they have operational glycolysis¹³³ and even overexpress the glucose transporter GLUT4¹⁵⁴. A differential glycolysis dependency is observed between CLL subgroups, where UM-IGHV CLL show a higher glycolytic rate than the mutated form^{151,212}. Del11q-positive cells have increased sensitivity toward glucose uptake inhibition by Ritonavir (GLUT4 inhibitor) or glycolysis inhibition by 2DG²¹³, a glucose analog that inhibits glycolysis at its onset²¹⁴. Interestingly, insulin receptor (INSR) expression is elevated in CLL compared to healthy B-cells but is even higher within del11q+ CLL²¹⁵. INSR activation can promote glucose uptake via GLUT4²¹⁶. It is possible that the main function of

glycolysis here is to provide metabolic intermediates for the PPP²¹⁷. For instance, inhibiting PPP with a specific inhibitor called DHEA does not kill the cells but instead increases the mitochondrial activity suggesting a redirection of the glycolysis metabolites from the PPP to the mitochondria²¹³. TP53-induced glycolysis and apoptosis regulator (TIGAR) is known to inhibit glycolysis and promote the PPP. It is overexpressed in advanced Binet stages, as well as in UM-IGHV, s- β 2M+, CD38+, and independently of *TP53* status suggesting an uncoupled expression from *TP53*²¹⁸.

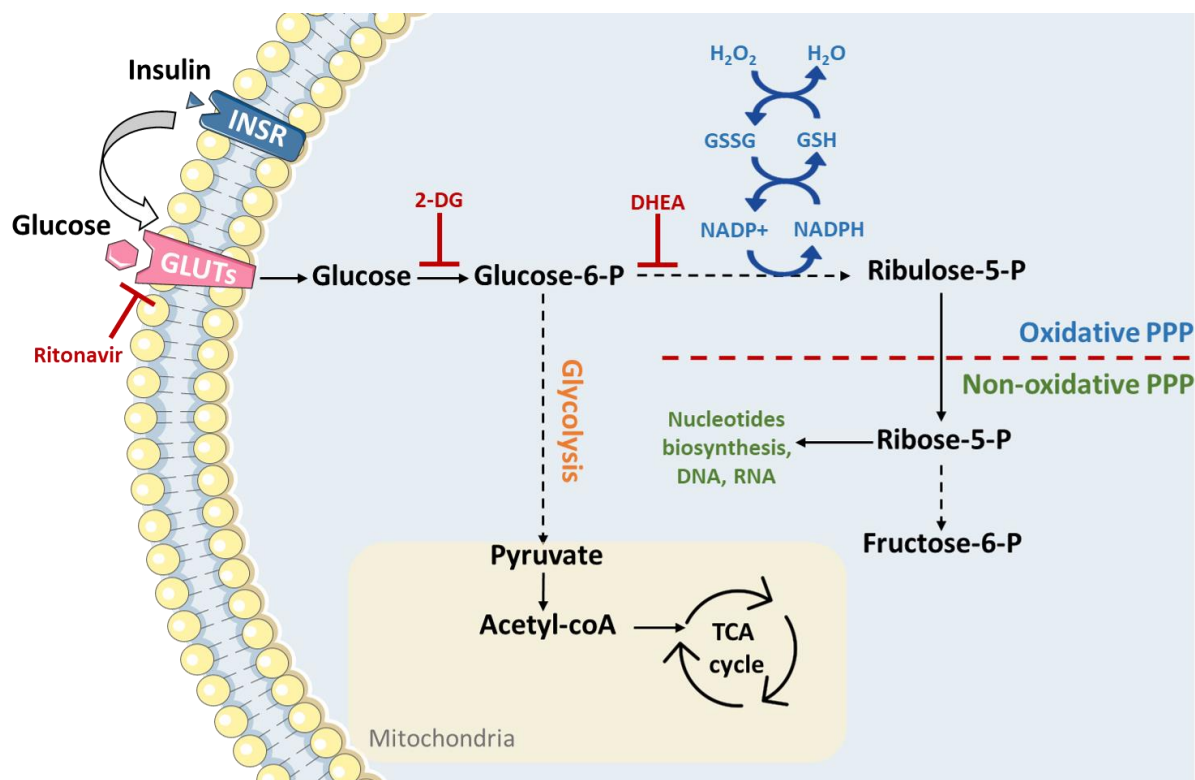


Figure 19: Glucose metabolism. Insulin receptor (INSR) activation stimulates the uptake of glucose by glucose transporters (GLUTs). Glucose can undergo glycolysis to produce pyruvate, further transformed to acetyl-CoA before joining the TCA cycle. Glucose intermediates such as glucose-6-P can join the pentose phosphate pathway (PPP) that contributes to NADPH generation in its oxidative branch, and ribose-5-P in its non-oxidative branch. The first one is essential for glutathione (GSH) synthesis and the second one is necessary for nucleotides synthesis.

The stromal microenvironment in the secondary lymph nodes and bone marrow might cause a metabolic reprogramming towards glucose dependency. For example, *in vitro* CLL cells co-culture with HS-5 bone marrow stromal cells increase aerobic glycolysis as well as the expression of the glucose transporter 3 (GLUT3) and some glycolytic enzymes. This metabolic shift is promoted by the NOTCH-c-Myc signaling²¹⁹. Moreover, recently emigrated CLL cells harvested from lymph nodes have a higher glucose uptake than circulating CLL cells. In addition, glucose-related metabolic pathways are upregulated in untreated cells when stimulated with CD40L or in previously ibrutinib-treated cells when BCR is stimulated with

anti-IgM. This suggests a relevant role of the microenvironment interaction in modifying CLL cell metabolism¹⁵⁷.

b. Amino acids metabolism

Amino acids (AAs) are the building blocks of proteins. Nine of them are essential and must be obtained from the diet because they cannot be synthesized by the body. Some AAs are semi-essential meaning they can be produced by the body, but in insufficient quantities, so a dietary intake is necessary. Finally, non-essential AAs can be synthesized by the body²²⁰. AAs can be generated by glycolysis and TCA cycle intermediates and some can be derived from other AAs through specific enzymatic reactions. Apart from their anabolic role as a precursor for protein synthesis, AAs can be catabolized within the cell. AAs degradation generates ammonium (NH_4^+) and a carbon skeleton that can be transformed into TCA cycle intermediates therefore also contributing to the cell ATP production. Moreover, some AAs act as neurotransmitters, some are essential for epigenetic processes such as DNA methylation and others are precursors for the synthesis of the antioxidant GSH²²¹. In CLL, amino acid metabolism can also be rewired in favor of the tumor cells. To date, two amino acid and their metabolic role were studied in CLL: glutamine (GLN) and arginine (Arg)²¹⁷.

- **Glutamine**

GLN is a non-essential amino acid that can be produced by the body under normal conditions. It is a water-soluble molecule that cannot simply diffuse through membranes but needs specific transporters to facilitate its movement. These transporters aren't usually exclusive for GLN but can also take other AAs and they include the solute carrier transporters SLC1, SCL6, SLC7, and SLC38 families²²². In the cytosol, GLN serves as a precursor for nucleotide synthesis. In the mitochondria, GLN is converted into another AA, Glutamate (GLU) by a reaction catalyzed by glutaminase (GLS) and called glutaminolysis. GLU can then be involved in both catabolic and anabolic roles within the cell. On one hand, it can be converted by glutamate dehydrogenase 1 (GLUD1) into alpha-ketoglutarate (α -KG), an intermediate of the TCA cycle, and therefore participates in the cell energy production. On the other hand, GLU can be transported back into the cytoplasm²²³ where it is used for the biosynthesis of GSH as well as many other non-essential amino acids such as arginine and proline²²⁴ (**Figure 20**).

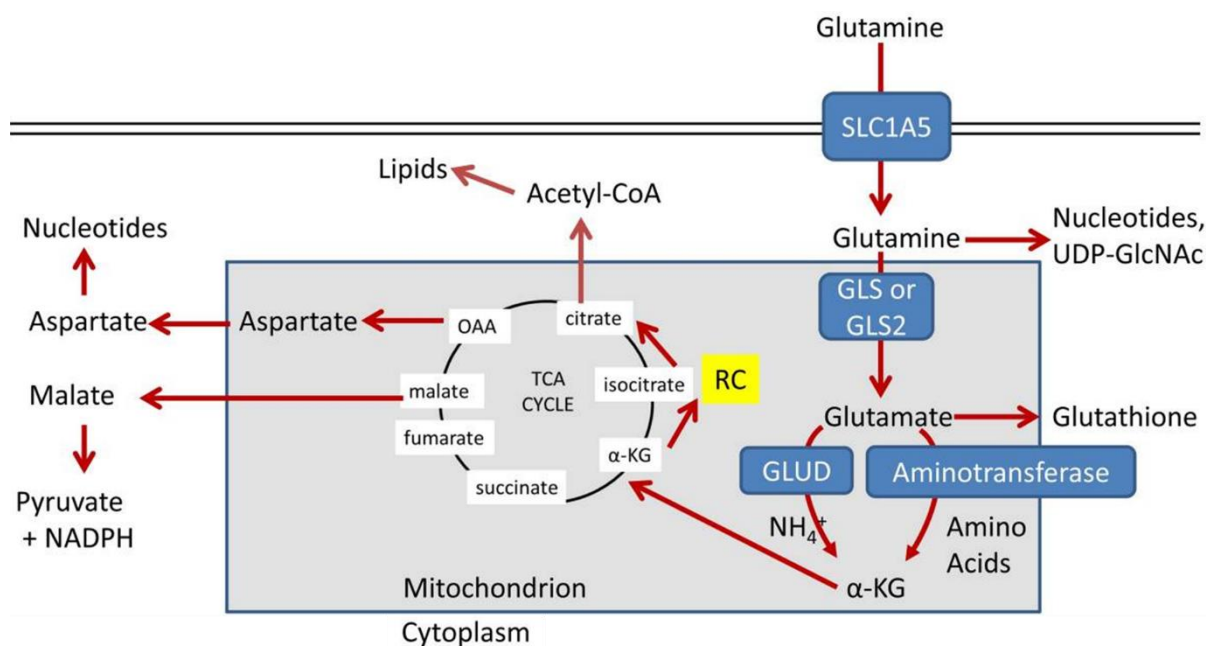


Figure 20: Glutamine Metabolism. Glutamine enter the cell via solute carriers such as SLC1A5. In the cytoplasm, it contributes to nucleotide and nucleotide sugar (UDP-GlcNAc) synthesis. In the mitochondria it is transformed into glutamate by glutaminase (GLS). Glutamate can be used for glutathione biosynthesis in the cytoplasm. In the mitochondria, it can be transformed into α -KG, an intermediate of the TCA cycle, under the effect of glutamate dehydrogenase (GLUD) or aminotransferase. The latter also produces other amino acids as by-products. A backward reaction can transform α -KG into citrate by a process called reductive carboxylation (RC). Citrate can act as a precursor for lipids synthesis. TCA cycle intermediates can contribute to other metabolic reactions such as nucleotides synthesis (*Reproduced from Altman et al, 2016*)²²⁵.

In CLL, GLS expression is increased compared to healthy group²²⁶ and is even higher in del11q CLL. NH_4^+ uptake is increased in the latter, suggesting de novo GLN synthesis in del11q CLL cells. Pharmacological inhibition of GLS with compound 968 only showed cytotoxicity towards del11q CLL, along with an increase in ROS levels and a decrease in the antioxidant GSH²¹³. Notably, a gene set involved in glutamine metabolism is upregulated when stimulating the BCR or mimicking the interaction with the microenvironment with CD40L stimulation. These cells show an increase in GLN uptake which contributes to both GSH synthesis and TCA cycle fueling. Pharmacological inhibition of GLN import in these cells decreases mitochondrial respiration and resensitize the cells to venetoclax, suggesting that GLN metabolism is a metabolic reprogramming triggered by venetoclax¹⁵⁷.

- **Arginine**

Arg is a semi-essential AA that could be synthesized by the body. However, in certain conditions such as early development and during infections, dietary uptake becomes essential to meet the body's needs. Arg transport inside the cell requires cationic amino acid transporters (CATs)²²⁷ and Arg de novo biosynthesis happens through the urea cycle (**Figure 21**) also known as the ornithine cycle²²⁸. The main purpose of this cycle is to convert toxic ammonia produced

by AA degradation into urea²²⁹. In endothelial cells, Arg is a precursor for NO synthesis via endothelial NO synthase (eNOS) action²²⁸. NO is a signaling molecule essential for regulating metabolic activity and necessary for a functional cardiovascular system²³⁰. It can be produced under stimulating conditions by inducible NO synthase (iNOS), the latter being expressed only after these stimulation conditions²³¹. Arginine can have direct functional effects within the cell. For example, it can activate mTOR complex 1 (mTORC1) signaling which is essential for cell growth²³². It can also directly affect the immune system, stimulate IgM production and enhance cytotoxic T cells function²³³.

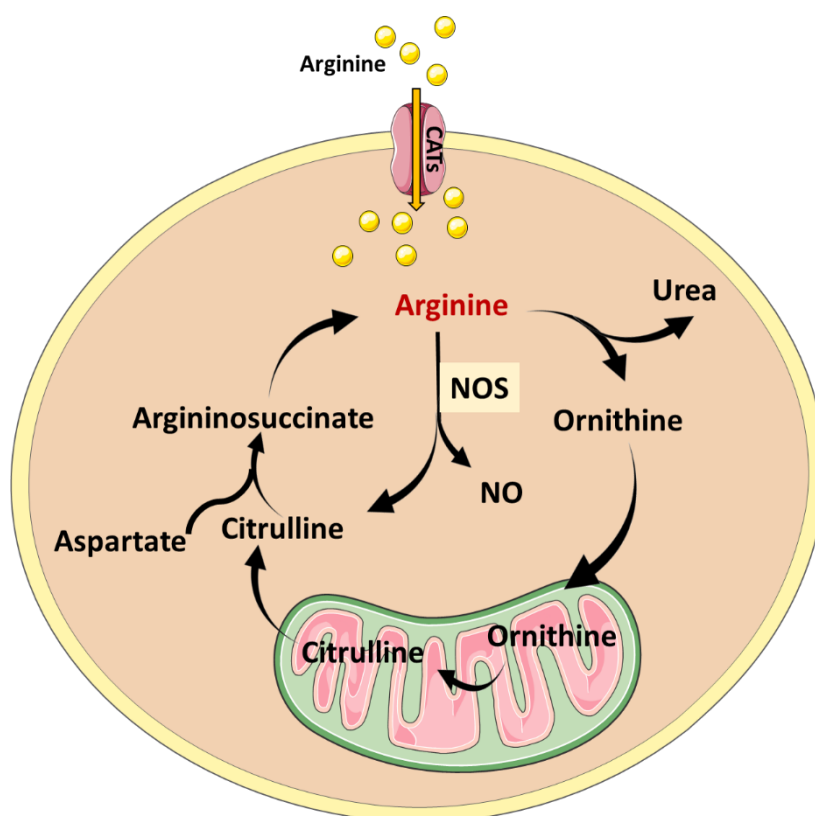


Figure 21: Arginine metabolism. Dietary arginine can be taken by the cell via CATs transporters. It can also be synthesized from citrulline and aspartate by the urea cycle. Arginine can also be metabolically degraded into urea and ornithine, or transformed into NO and citrulline by a reaction catalysed by NOS.

Arg uptake in CLL is mediated by CAT-1, the only Arg transporter expressed, but with distinct expression between the patients. CLL cells are entirely dependent on Arg uptake, as they do not express argininosuccinate synthase (ASS), the enzyme that catalyzes the Arg synthesis reaction. It is believed that tumor cells downregulate ASS expression in order to keep a high level of aspartate essential for nucleotide synthesis. Interestingly, Arg depletion abolishes CLL proliferation triggered *in vitro* by TLR-9 stimulation, and CLL cells cannot upregulate ASS expression as a compensation mechanism, rendering Arg a promising therapeutic target²³⁴. Furthermore, CLL cells express a functional iNOS, that when inhibited

causes cell death in CLL primary cells cultured *in vitro*. As iNOS requires Arg as a precursor for the reaction, this further validates the role of this AA in CLL²³⁵.

c. Fatty acid metabolism

Fatty acids (FAs) are the building blocks of lipids such as triacylglycerol (TAG) and phospholipids (PL). FAs have a hydrocarbon chain of different lengths with a carboxyl group (-COOH) at one end. They can be classified as saturated or unsaturated depending on the absence or presence of a double bond between carbon (C) atoms respectively. They are considered monounsaturated (**Table 5**) in the presence of one double bond and polyunsaturated in the presence of two or more.

# carbon atoms	Common name	IUPAC name	
Short-chain fatty acids (< 6 carbon atoms)			
2	Acetic acid		C2:0
4	Butyric acid	Butanoic acid	C4:0
6	Caproic acid/capronic acid	Hexanoic acid	C6:0
Medium-chain fatty acids (8–12 carbon atoms)			
8	Caprylic acid	Octanoic acid	C8:0
10	Capric acid/caprinic acid	Decanoic acid	C10:0
12	Lauric acid	Dodecanoic acid	C12:0
Long-chain fatty acids (14–20 carbon atoms)			
14	Myristic acid	Tetradecanoic acid	C14:0
16	Palmitic acid	Hexadecanoic acid	C16:0
18	Stearic acid	Octadecanoic acid	C18:0
20	Arachidic acid	Icosanoic acid/eicosanoic acid	C20:0
Very long-chain fatty acids (> 20 carbon atoms)			
22	Behenic acid	Docosanoic acid	C22:0
24	Lignoceric acid	Tetracosanoic acid	C24:0
26	Cerotate	Hexacosanoic acid	C26:0
Dicarboxylic acids [HOOC-(CH ₂) _n -COOH]			
6	Adipic acid	Hexanedioic acid	
8	Suberic acid	Octanedioic acid	
10	Sebacic acid	Decanedioic acid	

Table 5: List of monounsaturated FAs. FAs are divided in short, medium and long chain FAs according to the number of carbons in their chain. Dicarboxylic acids differ from FAs by the presence of two carboxylic groups (-COOH), one at each end (Reproduced from Adeva-Andany et al, 2018)²³⁶.

FAs can be obtained from dietary uptake in the form of TAG or synthesized in the body by a series of elongation cycles that begin with a molecule of acetyl-CoA and require NADPH, which can be produced by the pentose phosphate pathway^{237,238}. In the bloodstream, TAG is transported in the form of two lipoproteins: chylomicrons and very-low-density lipoproteins (VLDL). Lipoprotein lipase (LPL), an enzyme attached to the capillary endothelium, breaks down TAG into free FAs and glycerol, resulting in FAs uptake by surrounding cells²³⁹. FAs have the possibility to simply diffuse the plasma membrane into the cell or with the help of several membrane proteins. This includes the fatty acid transport proteins (FATP) family, the fatty acid translocase CD36, and the fatty acid binding protein (FABP). These transporters are

differently expressed between tissues and their activity can also depend on the carbon chain length²⁴⁰. Inside the cell, FA can be used for PL synthesis, which are major components of cellular membranes²⁴¹. They can also be stored in the form of TAG inside storage organelles known as lipid droplets (LDs). LDs are PL monolayer organelles formed in the ER that can establish direct contact with other cellular organelles to facilitate lipid exchange. They serve both as protectors against lipotoxicity in case of FAs accumulation, or providers of FAs in case of an increased demand for metabolic processes²⁴².

FAs can be oxidized in different cellular compartments such as mitochondria, peroxisomes and the ER. These reactions depend on the substrate specificity, length or as a backup pathway in case of a certain impairment in another one. **Table 6** below summarize the different types of fatty acid oxidation, as well as their substrate specificity. The table represent the most identified substrates (saturated FAs) for each pathway even though some exceptions can be included in some cases and some of them will be discussed later in the introduction.

Reaction	Organelle	Substrate	Product	Reference
α -oxidation	Peroxisomes	Phytanic acid (Branched chain FA)	Pristanic acid	243
β -oxidation	Mitochondria	Short, medium and long chain FAs	FADH ₂ , NADH, acetyl-CoA	244
β -oxidation	Peroxisomes	<ul style="list-style-type: none"> • Very long chain FAs • Pristanic acid 	<ul style="list-style-type: none"> • FADH₂, NADH, acetyl-CoA • H₂O₂, NADH, propionyl-CoA 	243,245
ω -oxidation	Endoplasmic reticulum	Short and medium chain FAs (when the β oxidation is impaired)	Dicarboxylic acid	244,246

Table 6: The three types of fatty acid oxidation pathways. The location of each pathway, its substrates and the products of each cycle are described in the table.

Importantly, FAs are used as a fuel source for the mitochondrial OXPHOS through a catabolic process known as mitochondrial fatty acid β -oxidation (mFAO)²⁴⁴. Prior to entering the mitochondria, FAs are thioesterified to coenzyme A to form fatty acyl-CoA by a reaction catalyzed by acyl-CoA synthetase (ACS)²³⁶. Different isoforms of ACS exist and have a tissue-specific differential distribution and a chain-length dependent FA substrate specificity²⁴⁷.

Mitochondria can handle saturated fatty acyl-CoA that have up to 20 carbons in their chain as well as long-chain unsaturated FAs with one or two double bonds²⁴⁸. Once inside the organelle, they undergo successive rounds of four biochemical reactions: oxidation, hydration, oxidation, and thiolysis. Each round shortens the acyl-CoA chain by removing two carbons and generates one acetyl-CoA, one NADH, and one FADH₂. These products join the TCA cycle and the ETC for ATP production²³⁶.

It is also worth noting that as the carbon number increases in the fatty acyl-CoA chain, they become less likely capable of simply diffusing across the mitochondrial membrane. This is mostly the case of long-chain fatty acyl-CoA with $C > 14$ ²³⁶. To enter the mitochondria, they must bind to a small hydrophilic molecule called carnitine by a reaction catalyzed by carnitine palmitoyltransferase 1 (CPT1) on the mitochondrial outer membrane. The generated acylcarnitine is then transported across the mitochondrial membrane by carnitine-acylcarnitine translocase (CACT). Once inside the matrix acyl-CoA is regenerated by palmitoyltransferase-2 (CPT2) and can enter the mFAO cycle. This transfer system is referred to as the carnitine shuttle²⁴⁹, illustrated in **Figure 22**.

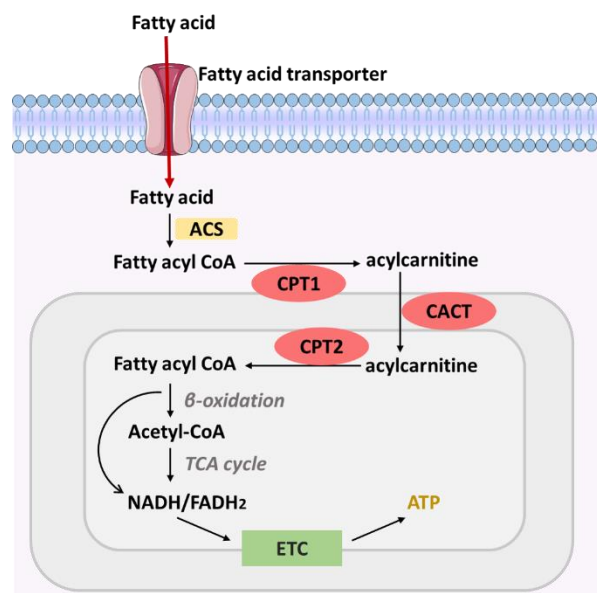


Figure 22: mitochondrial carnitine shuttle. Fatty acids are transported into the cell cytoplasm where they are transformed to fatty acyl-CoA by acyl-CoA synthetase (ACS). It is then bound to a carnitine to form a fatty acylcarnitine via a reaction catalyzed by CPT1. CACT transport the acylcarnitine to the mitochondrial matrix where it is transformed back into a fatty acyl-CoA by CPT2. Then β -oxidation occurs generating an acetyl-CoA for each cycle as well as NADH and FADH₂ that join the TCA cycle and the electron transport chain (ETC) respectively.

While there is no statistically significant difference in TG and VLDL levels between CLL and healthy individuals²⁵⁰, treatment naïve CLL fit patients have lower TG and VLDL levels compared to unfit patients. The latter usually have low OS following treatment²⁵¹. Furthermore, UM-IGHV have higher serum levels of VLDL compared to mutated CLL

cases²⁵². Interestingly, LPL, the enzyme that catalyzes the hydrolysis of TAG from VLDL is overexpressed in CLL cells both on the surface and in the cell cytoplasm. LPL transcription is mediated by STAT3²⁵³ and its expression is higher in UM-IGHV²⁵⁴, and is increased by BCR stimulation²⁵⁵. LPL-positive CLL cells show enriched expression of genes related to FAO²⁵⁶ and targeting this enzyme by orlistat results in efficient cytotoxicity for CLL cells, but not for healthy B cells, suggesting that the cells depend on fatty acids as an energy source²⁵⁵. Additionally, Ibrutinib treatment downregulates LPL levels indirectly by downregulating STAT3²⁵⁷. In 1999, Rutella et al. provided the first evidence of CD36 expression on CLL cells, which was positively correlated to advanced Rai stages and bone marrow infiltration²⁵⁸. Thus, CLL cells have an increased FAs uptake including palmitic acid and oleic acid^{253,259} that is mediated by CD36. The latter high expression is also driven by STAT3²⁶⁰. Moreover, proteomic and metabolomics data show an increase in medium and long chain FAs as well as FAO enzymes in CLL cells compared to healthy B cells, providing additional evidence of the crucial role of FAO in CLL^{226,261}. Targeting the carnitine shuttle gained attention due to its important role in modulating mFAO. For example, targeting both CPT1 and CPT2 with perhexiline demonstrated high cytotoxicity only on CLL cells but not on healthy B cells, even with HS-5 stromal coculture protection²⁶². The microenvironment could also trigger mFAO dependence of CLL cells. For example, *in vitro* inhibition of CPT1 by ST1326 induces higher cytotoxicity in stimuli-activated CLL cells than in quiescent circulating CLL cells²⁶³. Interestingly, while quiescent cells in the peripheral blood tend to sequester excess lipids in the form of LDs, the latter are degraded for use in the bone marrow and lymph nodes²⁶⁴. Finally, FAO upregulation may contribute to ibrutinib resistance. *In vitro* combination of ibrutinib and etomoxir, a CPT1 inhibitor, resensitized ibrutinib resistant cells to ibrutinib induced cell death²⁶⁵.

V. Metabolic Regulators in CLL

Metabolic regulation is crucial to maintain optimal cell, tissue, and organ function and to sustain overall health. Adaptive metabolic responses are triggered in response to nutrient requirements that highly depend on the cell type²⁶⁶ and are controlled by extracellular stimuli²⁶⁷.

1. Under hypoxic conditions, hypoxia inducible factor (HIF) is activated and mediates adaptive responses to cope with the limited oxygen supply. This includes a decrease in mitochondrial respiration and a metabolic shift to anaerobic glycolysis and lactate

production²⁶⁸. In CLL, HIF1 α , an important subunit of the transcription factor HIF, isn't overexpressed in the malignant cells compared with healthy B cells, but its high expression is found related to an unfavorable prognosis²⁶⁹ and is particularly observed in cells with a *TP53* alteration²⁷⁰. Under hypoxic conditions, CLL cells increase HIF1 α levels and can shift their metabolism towards anaerobic glycolysis²⁷¹.

2. At the intracellular signaling level, AMP-activated protein kinase (AMPK), an energy sensor enzyme, is activated in response to ATP levels decline and AMP levels increase. AMPK is activated by phosphorylation after allosteric binding of AMP, and can therefore restore energy balance by inhibiting anabolic processes and increasing catabolic reaction thus ATP production. Notably, in case of a drug-induced metabolic stress, CLL cells can reprogram their metabolism by a mechanism involving AMPK that leads to drug resistance²⁷².
3. A group of nuclear receptor proteins are also implicated in metabolic regulation, especially in glucose and lipid metabolism. They are called the peroxisome proliferator-activated receptors (PPAR) and work as transcription factors after ligand binding. There are three PPARs subtypes characterized by differential expression among cell types as well as functional diversity²⁷³ (**Figure 23**). PPARs gained significant interest in CLL research because of their implication in lipid metabolism, believed to be the main metabolic specificity in CLL²⁷⁴:
 - PPAR α : is highly expressed in energetic tissues such as the liver, the adipose tissues, and the muscles. It acts as a lipid sensor and can bind many polyunsaturated FAs (PUFAs) as well as phytanic acid, a branched chain FA present in dietary products. It then modulates DNA transcription of genes involved in FAO, and fatty FAs uptake²⁷⁵. PPAR α is overexpressed in circulating CLL but not in healthy B cells²⁷⁴ and ligand stimulation upregulates mRNA levels of CPT1A and CACT. Its pharmacological inhibition induces death even in the presence of a microenvironment coculture protection, as well as a reduced proliferation rate *in vitro* and *in vivo*²⁷⁶.
 - PPAR β/δ : is ubiquitously expressed but is particularly present in lipid-dependent tissues. It is activated by unsaturated fatty acids and is involved in regulating lipid and glucose metabolism²⁷⁷. PPAR β/δ exhibits high expression in CLL compared to peripheral blood mononuclear cells (PBMC) as well as other hematological malignancies. Its expression is

similar independently of the IGHV mutational status or the presence of del11q and del17p as well as in circulating or lymph nodes CLL²⁷⁸.

- PPAR γ : is expressed in various tissues such as adipose tissues, muscles, intestines, and immune cells. They can bind unsaturated FAs as well as lipid derivatives called eicosanoids. They regulate glucose homeostasis and lipid metabolism by increasing FAO²⁷⁹. In CLL, The few researches about PPAR γ are controversial. While Spaner et al. demonstrated no expression of PPAR γ in CLL cells²⁷⁴, another study presented positive results that show an induced apoptotic response upon PPAR γ pharmacological inhibition²⁸⁰. PPARs give additional scientific evidence of a FAs metabolism in CLL.

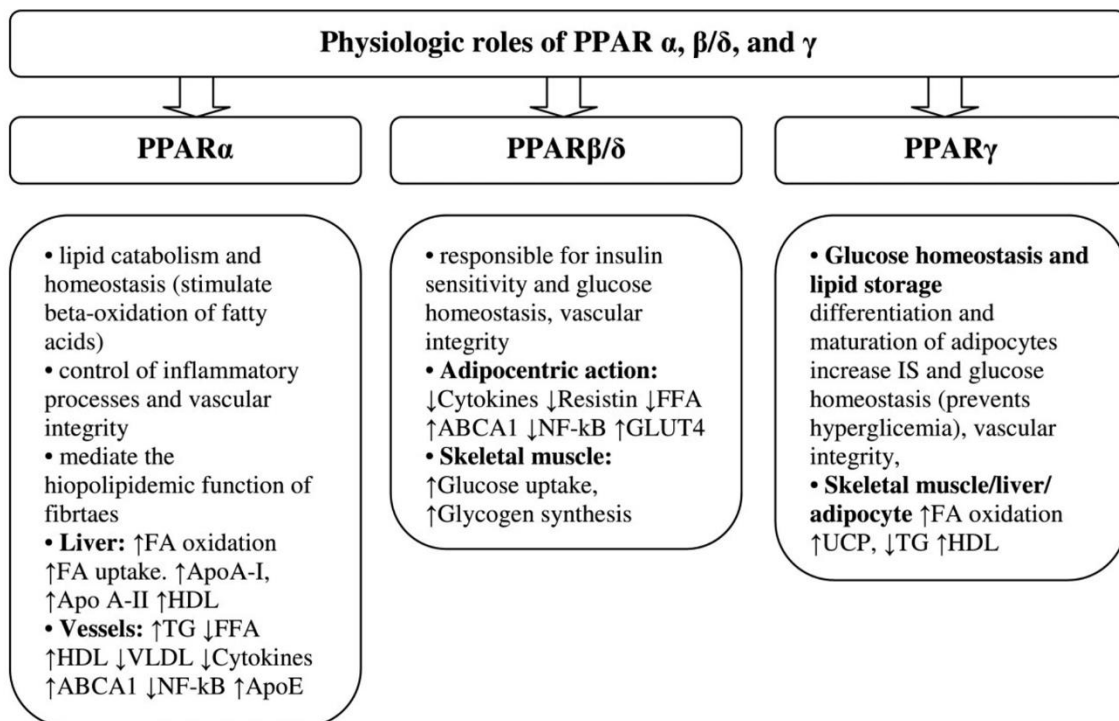


Figure 23: PPARs physiological roles including metabolic regulation. ABCA1: ATP-binding cassette transporter (cholesterol transporter); ApoA-A: Apolipoprotein AI (component of HDL); ApoE: Apolipoprotein E (binds to lipids to form lipoproteins); FA: fatty acid; , FFA: free fatty acid; ; GLUT4: glucose transporter 4; ; HDL: high density lipoprotein; IS: insulin sensitivity; resistin: involved in insulin resistance; TG: triglycerides; UCP: uncoupling protein; VLDL: very-low-density lipoprotein (Reproduced from Grygiel-Górniak, 2014)²⁷⁷.

Chapter III: Peroxisomes

I. Introduction

a. Overview

Peroxisomes are small organelles found in eukaryotic cells. They were discovered in 1954 and called microbodies up until 1966 when the name peroxisome began to be employed due to the capacity of these organelles to degrade H_2O_2 ^{281,282}. Peroxisomes are membrane-bound structures that contain a variety of enzymes essential for different metabolic functions including fatty acid and amino acid metabolisms and reduction of reactive oxygen derivatives. Crucial for human health and development, defects in peroxisomal metabolism or biogenesis are implicated in severe and several peroxisomal disorders²⁸³.

b. Structure and composition

Peroxisomes are usually round-shaped organelles with diameters ranging from 0.1 to 1 μ M. They have a single membrane similar to that of the ER and are composed mainly of phosphatidylcholine (PC) and phosphatidylethanolamine (PE). The peroxisomal membrane contains proteins (PMPs) essential for metabolite transport as well as peroxin (PEX) involved in peroxisome biogenesis. Peroxisomal matrix is very dense and engulfs a large variety of enzymes, substrates, and cofactors. The aggregation of these molecules forms crystalline-like structures called crystalloid cores^{284,285}. Unlike mitochondria, peroxisomes don't have genetic material or ribosomes²⁸¹ and its constituent proteins are encoded by nuclear genes and originate in the cytosol²⁸⁶.

c. De novo biogenesis and division

In eukaryotic cells, new peroxisomes can derive from both the ER and mitochondria (**Figure 24**). Under the effect of peroxins such as PEX3, PEX16, and PEX19, several PMPs are inserted into the ER membrane forming a pre-peroxisomal structure called peroxisome precursor vesicles (ppVs). In parallel, ppVs can be derived from mitochondria under the effect of PEX3 and PEX14. After their detachment, the model proposes the fusion of the ER and mitochondrial ppVs to form a peroxisome. Matrix proteins can then be imported to form a functional peroxisome^{287,288}.

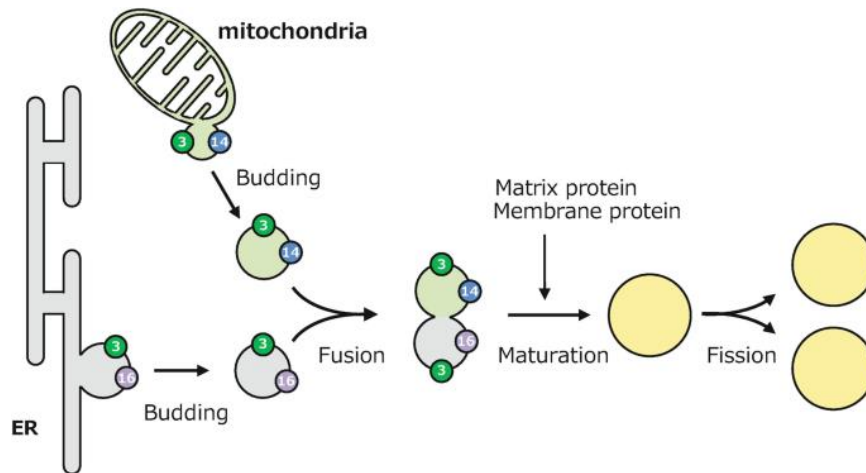


Figure 24: De novo peroxisome biogenesis. Mediated by the PEX family of proteins, ppVs derive from the ER and mitochondria under the effect of several PEXs. After detachment from these organelles, they fuse to form a new peroxisome. Peroxisomal maturation is completed by the import of matrix and membrane proteins (Reproduced from Kawaguchi et al, 2019)²⁸⁹.

Peroxisomes also have the capacity to divide into two peroxisomes daughters (**Figure 25**). This model is known as the growth and division model or the fission model. It includes three steps: elongation, construction, and fission followed by peroxisome translocation with the help of microtubules²⁹⁰.

- Elongation: This step requires PEX11 β that anchors the peroxisome membrane and forms heterodimers under the effect of docosahexaenoic acid (DHA), a product of peroxisomal fatty acid β -oxidation (pFAO).
- Construction: Dynamin-like protein 1 (DLP1) and mitochondrial fission factor (Mff) are recruited to the elongation site forming a functional complex. The complex might include fission 1 (Fis1), less essential for the complex activity.
- Fission: The complex induces Mff-mediated fission giving birth to new peroxisomes.

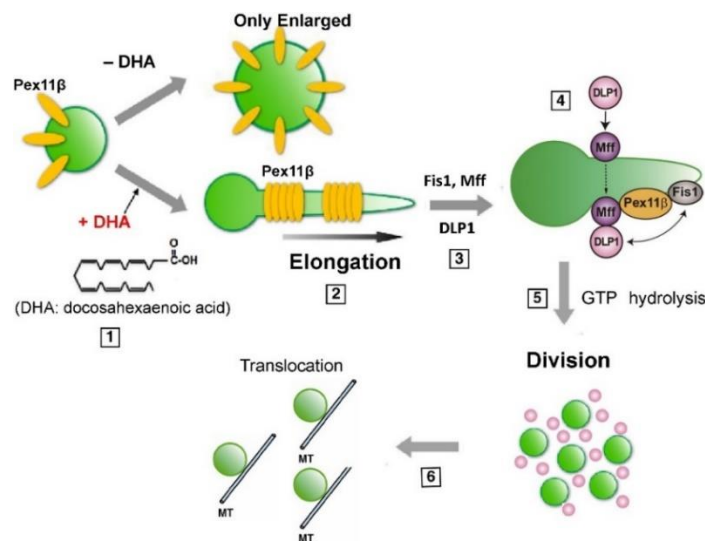


Figure 25: Peroxisome division/fission model (Reproduced from Honsho et al, 2016)²⁹⁰

d. Protein import machinery

As mentioned earlier, peroxisomes cannot generate their own proteins and must import them from the cytosol. In this way, peroxisomes can import fully folded proteins in their oligomeric form. This import pathway requires three steps: 1) Identification of cytosolic cargo proteins by soluble receptors; 2) Translocation of the receptor/cargo complex to the peroxisome membrane; and 3) Internal release of the protein after disassembly of the receptor/cargo complex. In fact, the cytosolic proteins destined to be peroxisomal matrix proteins, contain the characteristic peroxisomal targeting signal (PTS1 and PTS2) in their moiety. PTS1 is a tripeptide sequence located at the C-terminus of peroxisomal proteins that is recognized by the receptor protein PEX5. PTS2 is an aminoacidic sequence located close to the amino-terminus of the peroxisomal matrix proteins and recognized by PEX7²⁹¹. In both cases, the receptor-cargo complex is directed to a docking complex formed on the peroxisomal membrane. This complex is formed of PEX13, PEX14, and PEX17 and facilitates the translocation of the cargo protein within the matrix. The PTS sequence is then cleaved from the protein and the receptor is recycled in the cytosol to perform a new import cycle ²⁹².

II. Peroxisomal functions

a. ROS detoxification

In 1965, Nobel Laureate Christian de Duve proposed the term peroxisome as a name for the previously identified cellular microbodies because of their capacity to generate and degrade hydrogen peroxide. In fact, approximately 30% of the cell H₂O₂ levels come from peroxisomal reactions. Furthermore, these organelles are oxygen-consuming and require oxygen as a co-substrate for many metabolic reactions²⁹³. For this reason, peroxisomes are considered sites for cellular respiratory pathways independent from mitochondrial OXPHOS²⁹⁴.

Many peroxisome enzymes have an oxidase function and catalyze reactions that transfer hydrogen from the substrate to an oxygen molecule generating H₂O₂. This peroxisomal H₂O₂ production comes mainly from pFAO. Less significant, by the effect of xanthine oxidase, peroxisomes can also generate superoxide radicals O₂⁻^{294,295}.

In parallel, peroxisomes contain a group of enzymes to scavenge ROS and prevent their potential damage. Among them, catalase (CAT), the most known and abundant enzyme, is responsible for H₂O₂ breakdown into H₂O and O₂. CAT is directed to the peroxisomal matrix

via PTS1-PEX5 interaction. In some cell types, CAT can have cytosolic and nuclear localization. Peroxiredoxin 5 or PRDX5 is another enzyme that can reduce H_2O_2 into H_2O using the thioredoxins antioxidants as suppliers of reducing equivalents. SOD1, another ROS-regulating enzyme (previously described), can also be localized in peroxisomes²⁹³.

Concerning ROS detoxification, it is important to note that alterations in cellular redox mediated by peroxisomal oxidative stress play a critical role in a large number of human diseases. For instance, type 2 diabetes might be caused by H_2O_2 toxicity towards the insulin-producing β -cells because of a possible increase in pFAO and a lack of CAT in these cells^{293,296}. Studies have also shown that, like mitochondrial dysfunction, peroxisomal ROS imbalance could lead to oxidative stress-related senescence and age-related diseases²⁹⁷.

b. Ether lipids synthesis

Ether lipids are particular glycerophospholipids derived from peroxisomal reactions. They differ from conventional glycerophospholipids by their structure. While conventional glycerophospholipids attach acyl chains to the glycerol backbone by ester bonds (RCOOR'), ether lipids attach an alkyl chain ($\text{C}_n\text{H}_{2n+1}$) to the glycerol backbone by an ether bond ($\text{R-CH}_2\text{-O}$) at the sn-1 position. The sn-2 position of ether phospholipids contains an ester-linked acyl chain. A common form of ether lipids is called plasmalogens or alkenyl-acyl phospholipid, characterized by a vinyl ether bond (R-CH=CH-O) at the sn-1 position²⁹⁸. The difference between the conventional glycerophospholipid and the ether lipids structure is represented in **figure 26** below.

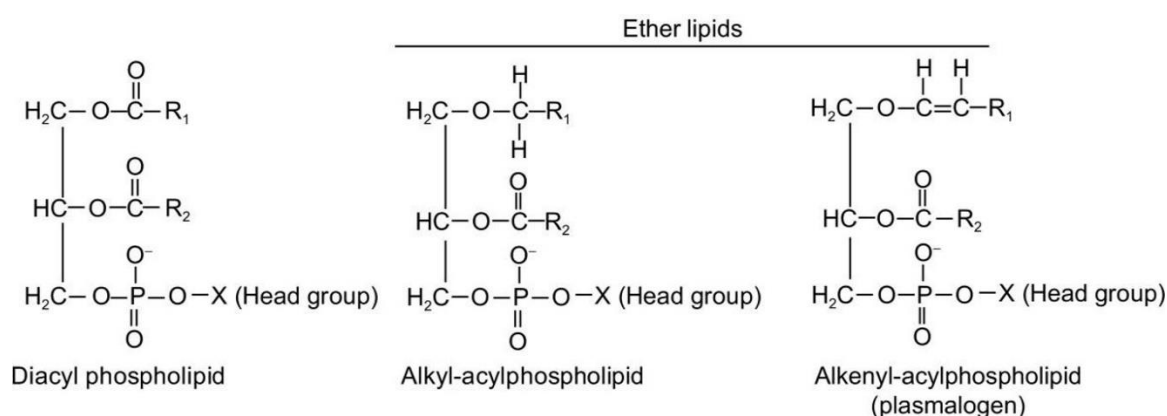


Figure 26: Glycerophospholipid vs. ether lipids structure. Chemical structures of conventional glycerophospholipids (example of a diacyl phospholipid on the left) and ether lipids (middle and right) (Reproduced from Dean et al, 2017)²⁹⁸.

The first part of ether lipids synthesis (**Figure 27**) takes place in the peroxisome via the dihydroxyacetone phosphate (DHAP) pathway. This pathway requires two precursors: DHAP, a glycolysis intermediate, and an acyl-CoA molecule, derived from cytoplasmic FAs. During the first step, an acyl-CoA molecule is transferred to DHAP via a reaction catalyzed by glyceronephosphate O-acyltransferase (GNPAT) to form acyl-DHAP. The alkyl moiety of ether lipids is synthesized by the fatty acyl-CoA reductase (FAR1 or FAR2), a peroxisomal-membrane-associated enzyme. It replaces the acyl group of acyl-DHAP to form alkyl-DHAP, by a reaction catalyzed by alkylglycerone phosphate synthase (AGPS) with the formation of an ether bond. The final peroxisomal step requires an acyl/alkyl DHAP reductase (PexRAP) that transforms alkyl-DHAP to 1-O-Alkyl-G3P (AGP). Ether lipid synthesis is then completed within the ER where the acyl group is added at the sn-2 position forming an alkyl-acylglycerol (AAG) followed by the addition of a cytidine diphosphate-alcohol head group in the sn-3 position forming a mature ether-linked phospholipid²⁹⁸. Plasmalogens are produced in the ER by a series of enzymatic reactions that follow the production of AAG²⁹⁹.

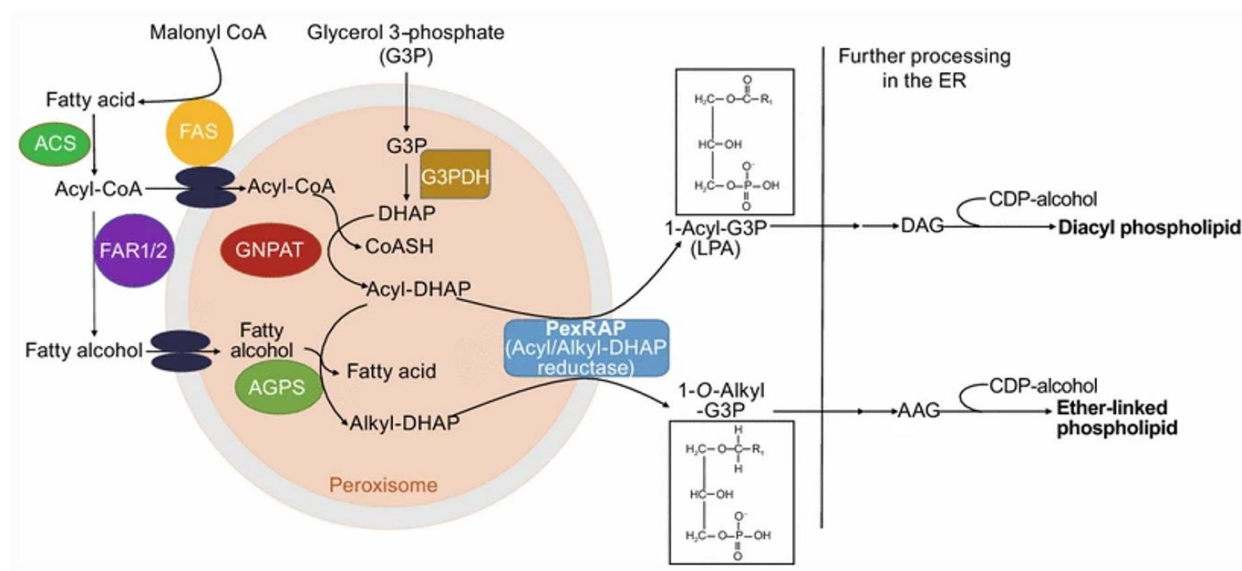


Figure 27: Ether lipids synthesis pathway. DHAP is generated from glycerol-3-phosphate (G3P) by glycerol 3-phosphate dehydrogenase (G3PDH). Fatty acids can be synthesized by the fatty acid synthase (FAS) localized near peroxisomes and further converted into acyl-CoA by acyl-CoA synthetase (ACS). Acyl-CoA is transferred to DHAP by GNPAT or reduced to fatty alcohol by FAR1 or FAR2. AGPS catalyzes the exchange of the acyl group with the alkyl group. PexRAP transfers acyl-DHAP or alkyl-DHAP to the ER for conversion to either conventional glycerophospholipid or ether phospholipid (*Reproduced from Dean et al, 2017*)²⁹⁸.

Ether lipids are major components of biological membranes, especially in the brain and heart tissues³⁰⁰. They allow a closer alignment of PL in the membrane resulting in less fluidity and a more rigid structure. Any alteration in the ether lipid membrane composition can directly affect the cell²⁹⁸. For example, knockout of PexRAP in adult mice leads to neutropenia proving that ether lipids are major components of neutrophils but also essential for their

survival³⁰¹. Congenital deficiency of ether lipid biosynthesis causes rhizomelic chondrodysplasia punctata (RCDP), a rare life-limiting genetic disease that affects bone and cartilage development. Additionally, ether lipids including plasmalogens (**Figure 26, right**), are implicated in the membrane recruitment and function of cellular signaling molecules such as AKT and protein kinase C (PKC). They also serve as ligands for PPAR α and PPAR γ . Plasmalogens are particularly enriched within lipid rafts, which are small membrane domains that act as a platform for cellular processes including key signaling transduction pathways³⁰². They also function as cellular antioxidants because of their unique vinyl ether bond that can be attacked by ROS, preventing the initiation of oxidative attacks on other cellular components²⁹⁸.

c. Bile acid synthesis

Bile acids (BAs) are molecules produced by liver cells (hepatocytes) from cholesterol. BAs are part of the bile, a watery secretion of the liver that also contains enzymes, vitamins, and amino acids stored in the gallbladder^{303,304}. There are two types of bile acids:

- Primary BAs such as cholic acid (CA) and chenodeoxycholic acid (CDCA) are synthesized by a series of enzymatic reactions that occur in different hepatocytic cellular compartments.
- Secondary BAs are formed from primary BAs through intestinal bacterial enzymatic action³⁰⁴.

Primary BAs biosynthesis is essentially performed in the peroxisomes. Cholesterol is first transformed by a series of reactions involving cytosolic, microsomal, and mitochondrial enzymes forming two BAs precursors, the 3 α ,7 α -dihydroxycholestanoic acid (DHCA) and the 3 α ,7 α ,12 α -trihydroxycholestanoic acid (THCA). These C27-bile acid intermediates are then transported within the peroxisome to undergo shortening to C24-bile acid by a β -oxidation reaction. Finally, they are conjugated to taurine or glycine before being excreted from the cell into the bile³⁰⁵.

BAs are essential for proper digestion. After a meal, BAs are released in the small intestines^{303,306} and, due to their amphipathic structure (both hydrophobic and hydrophilic), they form micelles with dietary lipids and fat-soluble vitamins which favors their intestinal absorption. Lastly, they return to the liver to undergo a new cycle³⁰⁷.

III. Peroxisomal fatty acid oxidation

One of the main and essential functions of peroxisomes is fatty acid oxidation. In fact, due to their structure and configuration, some FAs cannot enter the mitochondria to be processed/catabolized, and must first undergo an initial transformation in the peroxisomes. Peroxisomes can break down very-long chain FAs (VLCFAs) and branched chain FAs (BCFAs) by two processes known as FA β -oxidation and FA α -oxidation respectively³⁰⁸. In this section, FAs uptake, degradation, and fate will be discussed in detail.

a. ABCD transporters

The ATP-binding cassette (ABC) is a superfamily of transporters involved in the transport of substrates within the cell and cellular organelles. As its name indicates, the passage of substrates across the membranes requires the use of energy in the form of ATP. Among the members of this superfamily is the ATP-binding cassette subfamily D (ABCD), localized in the peroxisomes of mammalian cells. More precisely, three well-known peroxisomal transporters with distinct substrate preferences are required for the transport of FAs inside the peroxisomes (**Figure 28**). It should be noted that ABCD transporters are only active in dimeric form and that FAs must be in fatty acyl-CoA ester form to enter the organelle³⁰⁹.

- **ABCD1**: also called adrenoleukodystrophy protein (ALDP) mainly transports saturated VLCFAs (C22 to C26), as well as some monounsaturated (C18:1) FAs³¹⁰. A defect in this transporter causes X-linked adrenoleukodystrophy (X-ALD), a neurological inherited disease characterized by the accumulation of VLCFA in the brain, subsequently contributing to the disease symptoms³⁰⁹.
- **ABCD2**: or adrenoleukodystrophy-related protein (ALDR) has a very similar transport function as ABCD1. It has affinity for VLCFAs, monounsaturated FAs (MUFAs) but also PUFAs such as C22:6 and C24:6³¹⁰. Because of this redundant function with ABCD1, many research propose pharmacological induction of ABCD2 to compensate for ABCD1 deficiency as a potential therapeutic target for X-LAD³⁰⁹.
- **ABCD3**: also called peroxisomal membrane protein (PMP70) are specific transporters of BCFAs specially for phytanic acid and pristanic acid³¹⁰. BCFA have one or several methyl groups in their chain. They can be obtained from the diet, especially from milk and milk products in the form of branched-long-chain FAs (BLCFA). Some branched-chain AAs go through fermentation by intestinal bacteria to form branched-short-chain FAs (BSCFA), further elongated into BLCFA. BLCFA are present in human adipose

tissue, the liver, and in the serum in lower concentrations. ABCD3 can also transport long dicarboxylic acids (LDCA)³¹¹, organic compounds originated from the ER ω -oxidation and characterized by the presence of two carboxyl groups (-COOH) in their structure³¹². Furthermore, DHCA and THCA bile acids intermediates enter the peroxisome via ABCD3³¹³.

Interestingly, peroxisomes have the capacity to oxidize medium and long-chain FAs (MCFAs and LCFAs respectively) when mitochondrial FAs uptake is pharmacologically or genetically inhibited. This uptake is mediated by ABCD3³¹⁴. Moreover, a very recent study demonstrated the capacity of ABCD3 to directly transport LCFAs necessary for ether lipid synthesis in HeLa cells³¹⁵.

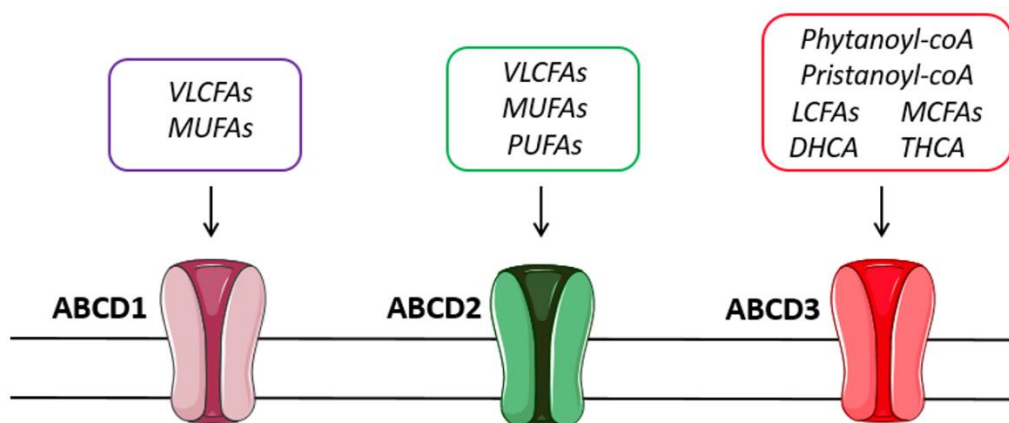


Figure 28: Peroxisomal ABCD transporters

In the transport/processing of FAs in the peroxisomes, the relationship between peroxisomes and lipid droplets (LDs), which has recently begun to be studied in eukaryotic cells, should be taken into account. Indeed, recent data support the idea that contact with LDs facilitates FAs trafficking in peroxisomes. In HeLa cells, Chang et al. suggested a LD-peroxisome contact mediated by M1 spastin (found on LD) and ABCD1. This contact recruits ESCRT-III protein essential for FAs trafficking³¹⁶. Another study demonstrated the role of the phosphatidylinositol PI-4,5-P2 generated by PI5P4K in mediating the transfer of VLCFAs from LDs to peroxisomes³¹⁷.

b. Peroxisomal α -oxidation

Phytanic acid is a 3-methyl FA that requires its transformation into 2-methyl FAs to be able to enter the peroxisomal FA β -oxidation cycle. This transformation process is known as the peroxisomal α -oxidation. Phytanic acid is transported in the plasma in the form of TG. Inside the cell, multiple phytanoyl-CoA synthetases were identified in the cytosol, ER, and

mitochondria, and are responsible for activating this FA into phytanoyl-CoA. Once in the peroxisome, phytanoyl-CoA is transformed into pristanic acid in three successive metabolic reactions. The first reaction requires oxygen to generate succinate, a TCA cycle intermediate, which is then transported to the mitochondria to join the TCA cycle via the peroxisomal porin PXMP2. The second reaction generates formic acid and pristanal, the latter is transformed into pristanic acid by a third reaction. The third reaction generates NADH, which cannot cross the peroxisomal membrane and must be reoxidized into NAD^+ using an NADH redox shuttle^{318,319}. Still not well identified in humans, this shuttle might include malate to oxaloacetate or lactate to pyruvate transformations. These conversions require NADH as a substrate therefore regenerating NAD^+ ³²⁰. The third reaction also produces pristanic acid that can be esterified into pristanoyl-CoA and join FA β -oxidation³¹⁹.

c. Peroxisomal β -oxidation

There are two identified peroxisomal FA β -oxidation (pFAO) systems. The first one catabolizes VLCFAs, as well as LDCA³²¹. Similar to mFAO, it includes four successive reactions: dehydrogenation, hydration, dehydrogenation, and thiolysis, each round of reaction generating an acetyl-CoA. Different enzymes catalyze these reactions. **Figure 29** represents the differences between the pathways in the peroxisomal and mitochondrial compartments. In both organelles, the reactions are catalyzed by enzymes that have the same functionality except the first chemical reaction that is catalyzed by two different enzymes: an oxidase in peroxisomes and a dehydrogenase in mitochondria²⁴⁵. Because peroxisomes lack a respiratory chain, acyl-CoA oxidase (ACOX), the enzyme that catalyzes the first rate-limiting reaction transfers electrons directly to oxygen to produce H_2O_2 . In the peroxisomal β -oxidation system, ACOX1 is the key executioner³²². Because little research is done on pFAO, it is still unclear how many cycles of β -oxidation can occur³²³. In 1992, Vanhove et al. demonstrated the capacity of ACOX1 to oxidize hexanoyl-CoA, an acyl-CoA with six carbons in its chain³²⁴. Another study demonstrated the capacity of octanoic acid (C8 FA) to undergo two peroxisomal β -oxidation cycles in rat liver³²⁵. This suggests that FAs carbon reduction can go as far as 4C fatty acyl-CoA known as butyryl-CoA³²³.

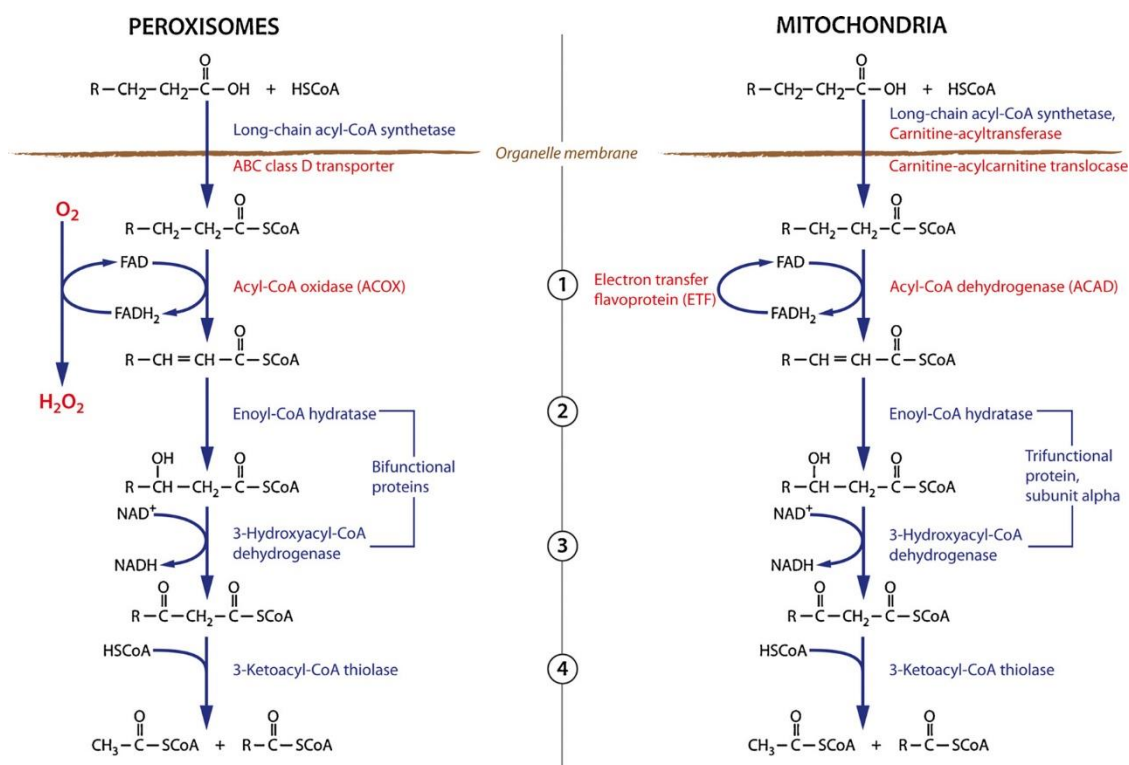


Figure 29: Peroxisomes and mitochondria FA β -oxidation pathways comparison (Reproduced from Schrader et al, 2015)²⁴⁵.

The second peroxisomal β -oxidation system has different substrate specificity. It is responsible for catabolizing branched-chain FAs such as pristanic acid derived from phytanic acid, as well as the bile intermediates DHCA and THCA described previously. This system includes the ACOX2 isoform for the first reaction³²¹. Pristanic acid undergoes three β -oxidation cycles that generate in total one acetyl-CoA, two propionyl-CoA (a C3 acyl-CoA), and 4,8 dimethyl-C9-CoA³²⁶. As for DHCA and THCA, they undergo one β -oxidation cycle that removes three carbons from their chain forming the bile acids CoA esters (CA-CoA and CDCA-CoA) that are further conjugated to taurine and glycine before bile excretion as previously described in chapter II³²⁷.

Just like mitochondria, peroxisomes can oxidize MUFA and PUFA. However, the substrates are different between the two compartments, with a preference for very long chains in peroxisomes³²⁷. Additionally, peroxisomal β -oxidation is required as a final step in some PUFA biosynthesis. For example, after different elongation reactions in the ER, one β -oxidation cycle is required to form DHA³²⁸, an important component of the cell membrane³²⁹ and an immunomodulator with anti-inflammatory properties³³⁰. The peroxisomal ACOX1 participates in this β -oxidation cycle³²⁸.

Finally, it is important to remark that PPAR α contributes to pFAO regulation. VLCFA and BCFA might be inducers of PPAR α dependent gene transcription, while ACOX1,

bifunctional proteins, and thiolase enzymes are entirely dependent on the PPAR α transcriptional regulation³²¹.

IV. Peroxisome-mitochondria connection

Peroxisomes do not have the metabolic capacity to utilize pFAO end products, which must therefore be exported from the organelle for further metabolizing. Primarily, these end products are transported to the mitochondria for processing and for feeding the mitochondrial ETC (**Figure 30**). Once again, carnitine plays a crucial role in this transport, but this time two other types of acyltransferase are involved³¹⁹. They catalyze the reversible transfer of acyl groups from acyl-CoA to carnitine, to generate acylcarnitines³³¹.

- Carnitine acetyltransferase (CRAT): This enzyme is not only present in peroxisomes, but also in the nucleus and mitochondrial matrix³³¹. It generates acetyl-carnitine from the acetyl-CoA produced by each cycle of peroxisomal β -oxidation. It is also responsible for generating propionylcarnitine from the propionyl-CoA produced from α -oxidation. Overall it has a preference for short-chain acylcarnitine (up to C5)³³².
- Carnitine octanoyltransferase (CROT): This enzyme is exclusively present in peroxisomes. It is highly active toward C4 to C12 acyl-CoA substrates³³¹. Very recently, Sanford et al. demonstrated the upregulation of CROT following cellular metabolic stress under the direct effect of *TP53*³³³.

Once synthesized, these products can leave peroxisomes to join mitochondria via PXMP2, a non-selective membrane channel protein that allows the diffusion of small molecules³²⁷. Then, bypassing CPT1, these peroxisome-derived small molecules are transferred across the inner mitochondrial membrane via CACT, the second mitochondrial carnitine shuttle enzyme^{334,335}. In the mitochondrial matrix, the peroxisomal acylcarnitines could be converted back to acyl-CoA before joining the mFAO cycle. Depending on their chain length, this reaction can be catalyzed by two enzymes: Medium chain length acylcarnitines (C8 to C12) are handled by CPT2³³⁶, while the medium chain length C6-carnitines, as well as the short chain length acylcarnitines (C2 and C4) are converted by mitochondrial CRAT³³⁷.

In the definitive steps, acetyl-CoA is released from acetyl-carnitine to join the TCA cycle, while the acyl-CoA released from acylcarnitine undergo the needed rounds of mFAO to be fully broken, producing acetyl-CoA and the reducing equivalents that feed the TCA cycle and the ETC, respectively³³⁴.

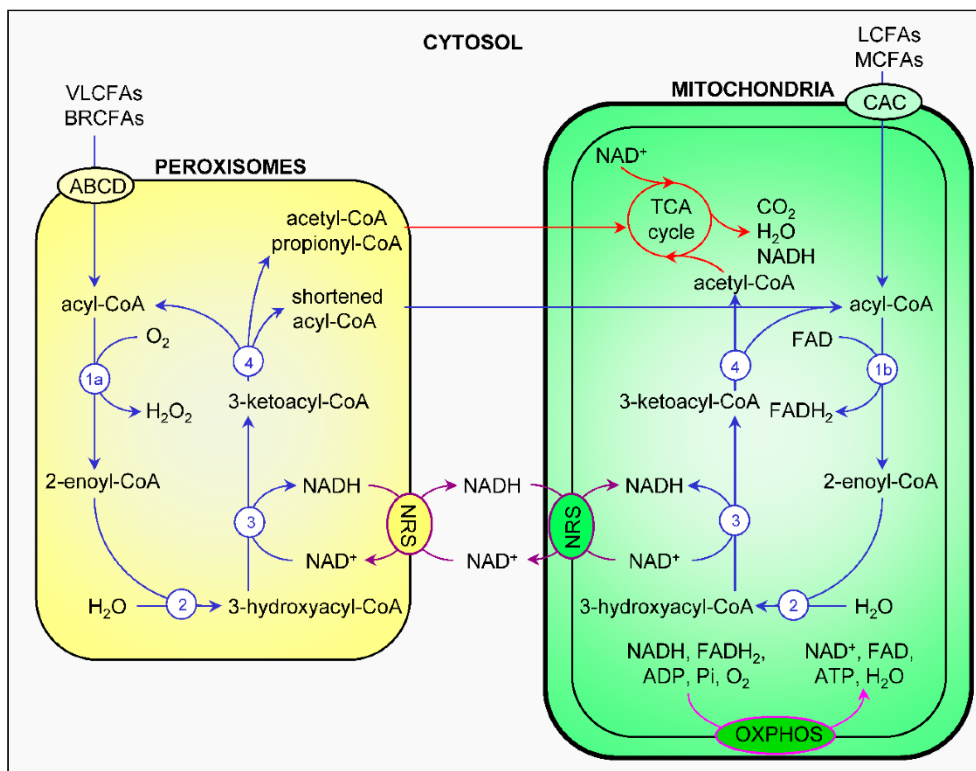


Figure 30: Peroxisome-mitochondria connection. Peroxisomal fatty acid oxidation products including shortened acyl-coA are transferred into mitochondria to join the mFAO and contribute in fuelling the TCA cycle. The reducing equivalents produced are used by the OXPHOS. NADH redox shuttles (NRS) serve to regenerate NAD⁺ from NADH (Reproduced from Fransen et al, 2017)³³⁸.

V. Peroxisome and cancer

The involvement of peroxisomes in cancer phenomena is neither well recognized nor taken into account in the same way as mitochondria. However, in recent years, studies of peroxisome deregulation in the tumor cells have attracted considerable interest due to their involvement in cancer metabolism. Controversially, some findings suggest a reduction in peroxisome abundance and activity, while others suggest an increase in peroxisome metabolic activities promoting tumor growth³³⁹.

Thus, on the one hand, some studies show a decrease in the level or activity of certain pFAO enzymes. For example, ACOX1 activity is reduced in breast and colon carcinoma³⁴⁰, and mRNA and protein levels of the peroxisomal bifunctional protein HSD17B4 and thiolase enzymes are decreased in non-small cell lung carcinoma³⁴¹. CAT protein levels and activity are also reduced in colon carcinoma^{342,340}.

On the other hand, in contradiction to the above studies, a wide panoply of manuscripts describe that FAO enzymes are upregulated in the tumor cells. For instance, ACOX1 is upregulated in HER2-positive breast cancer and correlated to a low OS³⁴³. The pFAO

bifunctional protein HSD17B4 is overexpressed in prostate cancer and can potentially predict a poor disease outcome³⁴⁴. AMACR, an enzyme involved in BCAAs α -oxidation is also overexpressed in prostate cancer³⁴⁵ and its pharmacological inhibition suppresses tumor growth in an *in vivo* aggressive prostate cancer mouse model³⁴⁶. Additionally, melanoma persister cells are highly dependable on pFAO. These residual cancer cells are diminished after pharmacological inhibition of upregulated ACOX1³⁴⁷. CRAT and CROT, rheostats of FAs shuttle between peroxisomes and the mitochondria, are highly expressed in bloodstream metastatic melanoma cells³⁴⁸. Remarkably, under nutrient depletion conditions, CROT is overexpressed under the direct effect of *TP53* and promotes cell survival and proliferation in a breast cancer cell line³³³. Moreover, lymphoma cell proliferation may be promoted by ACOX1, being upregulated and associated with resistance to doxorubicin-based drugs. As expected, ACOX1 downregulation resensitizes the lymphoma cells to doxorubicin treatment³⁴⁹. AGPS, an enzyme implicated in ether lipid biosynthesis, is highly expressed in multiple aggressive cancer cell lines compared to less aggressive, but also in primary breast cancer cells compared to normal breast tissue³⁵⁰. AGPS inhibitor 1a demonstrated efficacy against cell survival and migration in different cancer cell lines³⁵¹. Finally, an association between peroxisome proliferation and cancer treatment appears to be associated with some clinical approaches. This way, an increase in peroxisome proliferation is associated with the deacetylase inhibitor Vorinostat used to kill resistant forms of lymphoma³⁵².

After all, mounting evidence suggests a potential role of peroxisomal metabolism in cancer cell survival. Thus, anti-peroxisomal metabolism strategies could be intriguing in cancer treatment, and the assessment of the peroxisomal dysregulation could pave the way to efficient and original anticancer strategies³³⁹.

Objectives

Objectives

The aim of my PhD project is to identify a metabolic specificity common in CLL. Because of the essential role of ATP in providing energy for the cell function and therefore survival, I was particularly interested in identifying a metabolic pathway central in fuelling ATP generation in CLL.

The objectives were the following:

- Identify the main source of ATP generation in CLL: mitochondria or aerobic glycolysis?
- Determine a metabolic pathway specific for CLL cells and central in fuelling the ATP production within the cell.
- Verify the specificity of this metabolic pathway in a large panel of CLL primary cells with different cytogenetic abnormalities.
- Identify the effect of targeting this pathway on CLL cells survival.
- Check if this pathway is still relevant when mimicking the interactions with the microenvironment.
- Identify the effect of combining an inhibitor of this pathway with drugs used in CLL therapy.

Results

ACOX1-mediated peroxisomal fatty acid oxidation contributes to metabolic reprogramming and survival in chronic lymphocytic leukemia

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ABSTRACT

Chronic lymphocytic leukemia (CLL) is still an incurable disease, with many patients developing resistance to conventional and targeted therapies. To better understand the physiology of CLL and facilitate the development of innovative treatment options, we examined specific metabolic features in the tumor CLL B-lymphocytes. We observed metabolic reprogramming, characterized by a high level of mitochondrial oxidative phosphorylation activity, a low glycolytic rate, and the presence of C2- to C6-carnitine end-products revealing an unexpected, essential role for peroxisomal fatty acid beta-oxidation (pFAO). Accordingly, downmodulation of ACOX1 (a rate-limiting pFAO enzyme overexpressed in CLL cells) was enough to shift the CLL cells' metabolism from lipids to a carbon- and amino-acid-based phenotype. Complete blockade of ACOX1 resulted in lipid droplet accumulation and caspase-dependent death in CLL cells, including those from individuals with poor cytogenetic and clinical prognostic factors. In a therapeutic translational approach, ACOX1 inhibition spared non-tumor blood cells from CLL patients but led to the death of circulating, BCR-stimulated CLL B-lymphocytes and CLL B-cells receiving pro-survival stromal signals. Furthermore, a combination of ACOX1 and BTK inhibitors had a synergistic killing effect. Overall, our results highlight a less-studied but essential metabolic pathway in CLL and pave the way towards the development of new, metabolism-based treatment options.

Keywords: ACOX1, CLL, fatty acid beta-oxidation, metabolic reprogramming, mitochondria, peroxisomes, therapy.

INTRODUCTION

Chronic lymphocytic leukemia (CLL, the most common adult leukemia in Western countries) is characterized by the accumulation of monoclonal B-cells (CD19+, CD5+, and CD23+) in the blood, bone marrow, lymph nodes, and spleen [1, 2]. Despite significant progress in targeted therapies, such as Bruton tyrosine kinase (BTK) or Bcl-2 inhibitors [3-5], CLL remains incurable. Thus, a better understanding of CLL cell physiology appears to be critical to the development of new therapeutic options.

The anabolic and catabolic processes that shape metabolism are at the heart of cellular physiology. In cancer cells, specific metabolic features (metabolic “reprogramming” or “rewiring”) have a major role in cell growth and progression and so become potential therapeutic targets [6]. Beyond the Warburg effect (whereby tumor cells “ferment” glucose outside the mitochondria), the metabolic specificities of CLL B-cells are heterogeneous (like the disease), with alterations in glucose, amino-acid and lipid metabolisms [7, 8] (Fig. 1). Glycolysis helps to fuel mitochondrial oxidative phosphorylation (OXPHOS) and is more active in unmutated IGHV CLL cells (associated with a poor prognosis) [9, 10]. Amino-acids like glutamine and arginine are centrally positioned in the CLL cell’s carbon and nitrogen metabolic pathways [11, 12]. Ions like Ca^{2+} and $\text{Fe}^{2+/3+}$ influence the CLL cell’s signal transduction mechanisms [13, 14]. Furthermore, B-cell receptor (BCR) signaling might induce metabolic modifications in CLL [8, 15, 16]. Much evidence of the involvement of lipid metabolism in CLL survival and growth has been reported, [17-19] and aberrant expression of lipoprotein lipase (LPL) and other key proteins involved in fatty acid beta-oxidation (FAO) are hallmarks of CLL [20-26]. Thus, lipid metabolism appears to be one of the most important metabolic pathways involved in the lifespan of CLL cells.

Lipid metabolism entails the oxidation of fatty acids (FAs), which either generates energy or leads to the synthesis of new lipids [27]. This oxidation occurs variously in the mitochondria (beta-oxidation), peroxisome (alpha- and beta-oxidation), and endoplasmic reticulum (omega-oxidation) [27]. Among them, mitochondrial FAO (mFAO) and peroxisomal FAO (pFAO) constitute the main energy generation pathways [28]. The specific differences between mFAO and pFAO are related to their respective FA substrates and the initial FA processing steps. Whereas mitochondria directly handle long-chain FAs like palmitic or oleic acids, peroxisomes accept a more diverse spectrum, including dicarboxylic acids and very long-, long-, medium- and branched-chain FAs [29-32]. All these fatty acids enter the cell via specific transporters (such as FAT/CD36) and are converted to acyl-CoA before entering the

organelles for processing [29]. Mitochondrial fatty acyl-CoA uptake requires the carnitine shuttle consisting of carnitine acyltransferase 1 (CPT1), carnitine-acylcarnitine translocase (CACT), and carnitine acyltransferase 2 (CPT2), whereas uptake occurs directly via ABCD transporters in peroxisomes. In terms of further processing, like mFAO, pFAO produces an acetyl-CoA for each cycle of FA shortening, but the process is not complete and the chain-shortened acyl-CoAs produced move to the mitochondria for further oxidation. This transport requires the peroxisomal carnitine octanoyltransferase and carnitine acetyltransferase (CROT and CRAT, respectively), which combine acyl-CoA with carnitine to form fatty acylcarnitines. They can then enter the mitochondria via CACT and be converted back to acyl-CoA by the inner mitochondrial membrane protein CPT2 or by CRAT, also located in the mitochondrial matrix [28]. Next, acyl-CoA joins the FAO cycle in the mitochondrial matrix, resulting in the production of one acetyl-CoA for each cycle. Lastly, acetyl-CoA joins the mitochondrial tricarboxylic acid (TCA) cycle, and the NADH and FADH₂ produced by both FAO and the TCA cycle are used by the mitochondrial electron transport chain to generate ATP [27, 29] (Fig. 1).

In CLL, most of the proposed FAO-targeting metabolic approaches to eliminate CLL cells are based on mFAO inhibition (e.g., LPL, CPT1 blockade) [22, 25, 33]. The role of pFAO in CLL is often underestimated or overlooked. However, our detailed analysis of the FAO metabolic landscape of primary CLL cells revealed a key role for pFAO and Acyl-CoA oxidase 1 (ACOX1), a specific and rate-limiting pFAO enzyme [30] that we found to be overexpressed in CLL and that has been implicated in human disorders and cancer development [34, 35].

RESULTS

Compared with healthy B-cells, tumor CLL B-cells exhibit elevated levels of mitochondrial respiration, a low glucose flux, and low glycolytic activity.

To improve our knowledge of the metabolic physiology of CLL, we profiled primary CD5+ tumor B-cells. Our initial results corroborated previous reports on the key role of mitochondrial OXPHOS in the metabolism of CLL cells [10]. Indeed, the tumor CLL cells have much the same mass of mitochondria as healthy B-cells but show higher rates of basal mitochondrial respiration, ATP production, and mitochondrial transmembrane potential ($\Delta\Psi_m$, a marker of electron transport activity) (Figs. 2a-c). Based on these results, we focused our metabolic analyses on main pathways fueling mitochondrial OXPHOS.

We first assessed glycolysis, which (in addition to other functions not related to energy production) fuels mitochondrial OXPHOS in CLL cells [8-10]. To evaluate this in detail, we performed metabolomic analyses in a panel of healthy and CLL B-cells (clinical and genetic characteristics described in Supplementary Table 1). Compared with CLL cells, healthy B-lymphocytes show elevated glycolytic activity and significantly higher levels of glucose-6-P, pyruvate, and lactate intermediates (Fig. 2d). This was confirmed by two complementary approaches: (i) the measurement of glucose uptake, which appeared to be significantly higher in healthy B-cells (Fig. 2e), and (ii) Seahorse glycolysis stress test, which showed that glycolytic activity was three times greater in healthy B-cells than in CLL B-lymphocytes (with about 49.5% of mitochondrial glycolytic activity in healthy B-cells and only 16% in CLL cells) (Figs. 2f, g). The large glycolytic reserve of CLL cells is noteworthy and indicates that they are able to perform glycolysis - even though they do not actively utilize this metabolic pathway in the basal state. These results show that although glycolysis is important in CLL physiology, it is not a primary supplier of the cells' mitochondrial OXPHOS [9, 10].

Peroxisomal FAO (pFAO): an unexpected cornerstone of CLL metabolism.

An in-depth, comparative metabolomics analysis of healthy B-cells and CLL cells revealed elevated levels of carnitine and betaine in the tumor B-lymphocytes (Fig. 3a). Betaine has various cellular functions but it is known to regulate mitochondrial and pFAO enzymes [36]. Furthermore, the amounts of CPT1-derived C14- and C16-acylcarnitines and C2- to C6-acylcarnitines (which can be generated by pFAO) were also significantly higher in CLL cells (Fig. 3b) [28, 37, 38]. Among these, hexanoylcarnitine (C6) is almost exclusively generated by pFAO. Thus, it appeared that the FAO metabolism of CLL might include the often-analyzed CPT1-mediated pathway [22, 25], as well as the less studied pFAO.

To check this hypothesis, we first reanalyzed a published RNAseq dataset comparing healthy B-cells (B-cells from peripheral blood -NBC-PB-) and a large cohort of CLL B-cells [39]. A gene set enrichment analysis (GSEA) showed that 11 metabolic sets were enriched in CLL genes vs. healthy B-cells (Fig. 3c and Supplementary Fig. 1). The most significantly enriched metabolic pathway in CLL cells was FAO, which includes CPT1-mediated FAO and pFAO (Fig 3c and Supplementary Figs. 1 and 2). A GSEA of peroxisome-related genes (including biogenesis, pFAO and other peroxisomal functions) also showed a significant enrichment of this set in CLL cells compared with healthy B-cells - highlighting the importance of this organelle in CLL physiology (Figure 3d and Supplementary Fig. 3). Secondly, a qPCR analysis of CLL cells from our cohort of patients corroborated the overexpression of genes involved in CPT1-mediated FAO and pFAO. As shown in Fig. 3e, genes overexpressed in CLL B-lymphocytes (compared with healthy B-cells) included CPT1, CPT2 and key pFAO genes, such as the peroxisomal transporter ABCD2, the carnitine O-octanoyltransferase (CROT) and the rate-limiting pFAO enzyme acyl-CoA-oxidase 1 (ACOX1) [28, 29]. Interestingly, the pharmacological inhibition of FAO by perhexiline [25] (which blocks both CPT1-mediated FAO and pFAO by preventing the entry of peroxisomal-derived medium chain acylcarnitines into mitochondria via CPT2) induced high levels of cytotoxicity, even at low concentrations (Fig. 3f). Taken as a whole, our data indicate that the metabolic reprogramming characterizing CLL involves not only the extensively studied CPT1-mediated FAO but also pFAO.

To assess the importance of pFAO metabolism in CLL, we focused on ACOX1: a pFAO-limiting enzyme whose overexpression in CLL was confirmed by qPCR, immunoblot and confocal microscopy approaches (Figs. 3e, g, and h). We initially evaluated the consequences of lentiviral knockdown of ACOX1 on CLL metabolism. We implemented this approach on the well-characterized OSU-CLL cell line [40], which showed much the same perhexiline response (Supplementary Fig. 4a) and levels of C2- to C6-acylcarnitines as primary CLL cells (Supplementary Fig. 4b). Metabolomic profiling and immunoblotting confirmed that the reduction in ACOX1 expression (shACOX1 cells; Fig. 4a) was sufficient to shift the metabolism of the CLL cells towards a more carbon-based, amino-acid phenotype. This included the expected decrease in levels of pFAO-generated C2- to C6-acylcarnitines (Fig. 4a) and higher levels of glucose, pyruvate, lactate, glutamine, and α -ketoglutarate (Fig. 4b). Thus, pFAO appeared to be a metabolic cornerstone that influenced the CLL cell's entire metabolism.

Inhibition of ACOX1 reduces ATP levels and selectively kills CLL cells, independently of the patient's clinical or genetic prognostic features.

If pFAO has a central role in CLL metabolism, could then pFAO blockade be used as a treatment option in CLL? To evaluate this possibility, we tested the response of the CLL cells to the specific pharmacological blockade of ACOX1 with 10,12-tricosadiynoic acid (TRCDA) [41] (Fig. 4c). As expected, treatment of primary CLL cells with TRCDA quickly eliminated the 30% of the cell's H₂O₂ produced by the enzyme [30, 42-44] (Fig. 4d). The TRCDA treatment also prevented the generation of C2- to C6-carnitine derivatives in the CLL cells (Fig. 4e), confirming that ACOX1 and pFAO had been modulated by this inhibitor. The inhibition of pFAO was also associated with a time-dependent accumulation of lipid droplets (LDs) in the CLL cells (Figs. 4f, g) - suggesting a link between peroxisomes and LDs [43] - and a significant linear, decrease in the amount of cellular ATP (Fig. 5a). As expected, the ATP levels in healthy B-cells (which are more dependent on a glucose metabolism) were unaffected by TRCDA treatment (Fig. 5a). Interestingly, the loss of ATP induced by TRCDA in CLL cells was also observed in tumor B-lymphocytes from Binet B/C stage patients, patients in relapse after an immunochemotherapy or a targeted ibrutinib treatment, or B-lymphocytes with genetic markers of poor prognostic, including unmutated IGHV, 11q and 17p deletions (del(11q) and del(17p), respectively), a complex karyotype (CK), or an hyper-complex karyotype (HCK) (Fig. 5a and Supplementary Table 1). It therefore appears that a significant proportion of the ATP generated by CLL cells depends on pFAO, and that ATP generation by pFAO is a general metabolic characteristic of CLL B-lymphocytes (independently of clinical or genetic prognostic factors). It is noteworthy that ACOX1/pFAO blockade also led to a similar loss of ATP in the OSU-CLL cell line (Supplementary Fig. 4c). In shACOX1 cells, the metabolic reprogramming towards a carbon- and amino-acid-based phenotype described above (Fig. 4b) appeared to contribute to better maintenance of ATP levels after pFAO inhibition (Supplementary Fig. 4c).

As a potential option for treating CLL, TRCDA led not only to ATP loss in CLL cells but enabled programmed cell death (PCD) (Fig. 5b). Importantly, TRCDA treatment spared both healthy B-lymphocytes and patients' non-tumor blood cells involved in cell death clearance (e.g., T-lymphocytes and natural killer (NK) cells) (Fig. 5b). This PCD process was significantly minimized by ACOX1 downregulation (Supplementary Fig. 4d). Moreover, and as expected from the ATP measurement presented in Fig. 5a, TRCDA-treated CLL cells underwent PCD irrespective of the IGVH mutational status (Fig. 5c) or the presence of other cytogenetic alterations associated with drug resistance (including del(17p), del(11q), and CK/HCK) (Fig. 5d). Thus, the inhibition of pFAO/ACOX1 selectively induced killing of the leukemic CD5⁺ clone and spared the CLL patient's non-tumor immune cells.

It has been widely described that in the lymph nodes, CLL cells reprogram their metabolism by BCR-triggering or by receiving survival or pro-proliferative signals from the microenvironment. Under these conditions, the CLL cells become more glycolytic and less sensitive to the currently approved anti-CLL treatments [8, 45, 46]. Thus, we analyzed the TRCDA sensitivity of CLL cells that had been BCR-activated by soluble anti-IgM, co-cultured with stromal cells or stimulated by CD40L and CpG. Our results indicated that the inhibition of ACOX1/pFAO by TRCDA remained an effective means of inducing death in CLL after BCR triggering, regardless of the presence of factors for a favorable prognosis (mutated IGHV) or unfavorable prognosis (unmutated IGHV) (Fig. 5e, middle and Supplementary Fig. 5a). It should be noted that, under our experimental conditions, BCR stimulation by soluble α -IgM is not pro-apoptotic per se (Supplementary Fig. 5b). BCR activation was confirmed by measuring ERK phosphorylation (Fig. 5e, left). Indeed, the metabolomic profiling of BCR-activated CLL cells revealed that although a metabolic shift could occur, the levels of peroxisomal C2- to C6-carnitine derivatives remained similar to those in unstimulated CLL B-cells (Fig. 5e, right). The pFAO pathway was therefore still active after BCR triggering. Similar results were obtained in CLL cells receiving (or not) survival signals from the stromal cells, with no significant differences in the PCD induction by TRCDA (Fig. 5f, middle). Under these conditions, we observed a trend towards higher levels of the C2- to C6-acylcarnitines in CLL cells co-cultured with the stromal cell line (Fig. 5f, right). The protective effect of stromal cells was confirmed by assessing the spontaneous cell death of primary CLL cells after 24 hours of *in vitro* culture; spontaneous cell death was significantly diminished when CLL cells were co-cultured with the stromal cell line. (Fig. 5f, left). Finally, TRCDA is also an effective means of inducing death in CLL cells stimulated by the combination of CD40L and CpG (mimicking a pro-proliferative environment) (Supplementary Fig. 6). Taken together, our results strongly suggest that pFAO remains essential in BCR-stimulated CLL cells and in CLL cells receiving pro-survival signals or pro-proliferative stimulation.

Inhibition of ACOX1/pFAO induces caspase-3-dependent apoptosis in CLL B-cells.

To gain insights into the mechanism of TRCDA-induced cytotoxicity in CLL, we analyzed the cell death pathway enabled by this ACOX1 inhibitor. Cytofluorometric assessments indicated that ACOX1/pFAO inhibition caused a significant loss of $\Delta\Psi_m$, which was accompanied by the overgeneration of mitochondrial reactive oxygen species (ROS) 24 hours post-treatment (Figs. 6a, b). These results indicated that the PCD process activated by TRCDA inhibition in CLL involves mitochondria.

TRCDA-induced PCD in CLL was precluded by pretreatment of the CLL cells with the broad-spectrum caspase inhibitor Q-VD-OPh (Fig. 6c). A complementary approach indicated that this killing was indeed mediated by caspase-3 (Fig. 6d). These results were fully corroborated in the OSU-CLL cell line model used in our study (Supplementary Figs. 4e-g) or in other CLL cell lines models widely used in CLL *in vitro* and *in vivo* assessments, including the PGA1, MEC1 and HG3 cell lines [47-49] (Supplementary Fig. 7). Taken as a whole, these results indicated that the complete inhibition of ACOX1/pFAO metabolism induces mitochondrial-mediated caspase-3-dependent PCD in CLL cells.

A combination of BTK inhibitors with ACOX1/pFAO blockade has synergistic cytotoxic effects in CLL.

Our results suggested that the ACOX1/pFAO inhibition might be a promising way of specifically eliminating CLL B-cells, irrespective of the presence or absence of key genetic alterations associated with drug resistance. To further analyze the clinical relevance of pFAO inhibition in CLL, we determined whether ACOX1/pFAO blockade induced apoptosis more effectively when combined with chemotherapeutic agents or targeted therapies currently used to treat CLL, such as BTK inhibitors (BTKi) or Bcl-2 inhibitors. Specifically, CLL cells were co-treated with TRCDA and fludarabine, venetoclax, ibrutinib or acalabrutinib at doses that induced low levels of apoptosis when each drug was used alone (Supplementary Figs. 8a-d). Although fludarabine and TRCDA showed an additional apoptogenic effect in CLL cells after 24 hours of treatment (irrespective of karyotype complexity) (Fig. 7a), the combination of venetoclax and TRCDA did not show any additional or synergistic cytotoxic effects (Fig. 7b). This might be related to the metabolic changes induced by venetoclax [50] or to the recently described venetoclax blockade of the complex I of the mitochondrial electron transport chain, which may allow a concomitant/competitive effect of TRCDA and venetoclax on the same mitochondrial PCD pathway. It is noteworthy that fludarabine-resistant cells with del(17p) were sensitive to the combination of fludarabine and TRCDA. In contrast to co-treatment with fludarabine or venetoclax, co-treatment with the BTKi ibrutinib or acalabrutinib produced a significant synergistic apoptogenic effect in CLL cells, including those with cytogenetic alterations associated with drug resistance (i.e. del(17p), a CK, or an HCK) (Fig. 7c). Importantly, the synergistic effect of TRCDA and BTKi was maintained in CLL cells activated by BCR triggering - a signaling pathway involving the BTK protein (Fig. 7d). Overall, our results indicate that the inhibition of ACOX1/pFAO metabolism (whether alone or in combination with BTKi treatment) might be an effective means of eliminating CLL cells.

DISCUSSION

Energy metabolism rewiring or reprogramming is now a well-established feature of cancer [7]. Otto Warburg pioneered this field by observing that even in the presence of oxygen, cancer cells could reprogram their energy production and adopt a mitochondrial OXPHOS-independent glycolytic program. However, as reported for other leukemias [51-53], CLL cells do not show a Warburg effect but display more intense mitochondrial OXPHOS, elevated levels of ROS, and a greater antioxidant capacity than healthy B-lymphocytes [9, 54]. Our multiparameter approach not only confirmed the greater levels of mitochondrial activity in CLL cells but also highlighted their lower glycolytic capacity, relative to healthy B-lymphocytes. This included CLL cells with genetic alterations associated with drug resistance (e.g., del(11q), del(17p), a CK, and an HCK), which strongly suggests that there are common metabolic features beyond glycolysis in this highly heterogeneous disease.

Along with amino acids (such as glutamine) and glucose, FAs are extremely important substrates for mitochondrial OXPHOS. The contribution of FAs and CPT1-mediated FAO to CLL cell survival, growth, and reprogrammed metabolism has been extensively described [17-19]. LPL expression increases in poor-prognosis, unmutated IGHV CLL [20, 55], CPT1 is upregulated in CLL [22, 25], and the low-density lipoprotein regulates cytokine-signaling and CLL proliferation [26]. Moreover, inhibition of FA synthesis or CPT1 blockade induces cytotoxicity in primary CLL cells [22, 33, 56]. These results suggest that targeting CPT1-mediated FAO could be a good metabolic approach to combat CLL. However, this approach might pose a problem in terms of specificity because many non-malignant cells also depend on this metabolic pathway [57, 58]. Other limitations include the *in vivo* liver toxicity produced by CPT1 inhibitors [59].

Compared to healthy B-lymphocytes, our study revealed a significant overexpression of peroxisomal genes involved in pFAO (such as ACOX1, ABCD2, and CROT), the presence of increased levels of betaine (a compound involved in the increased expression of pFAO enzymes [36] and a precursor of carnitine biosynthesis [60]) and higher levels of short-chain acylcarnitines in CLL malignant cells, confirming that pFAO also contributes to CLL metabolism. This is consistent with a previous study reporting that serum levels of short-chain (C2 and C6) acylcarnitines were significantly higher in CLL patients than in healthy donors [61]. Thus, it appears that both mFAO and pFAO contribute to CLL metabolism. However, pFAO seems to play a critical role in CLL cell survival. For example, the treatment of the CLL cells with the specific CPT1/mFAO inhibitor etomoxir does not cause cell death when used at

doses that do not induce off-target effects [62, 63]. In contrast, the inhibition of pFAO alone (this work) or the simultaneous inhibition of CPT1 and carnitine shuttle enzymes involved in pFAO with ST1326 or perhexiline successfully induces apoptosis in the CLL cells [25, 56, 64, 65].

Little is known about the metabolic roles of peroxisomes and the pFAO pathway in tumor cells. It was recently reported that the metabolism of melanoma “persistent” cells depends on pFAO [35]. ACOX1, a matrix-restricted peroxisomal enzyme that catalyzes the first step in pFAO, has been linked to peroxisomal human disorders [34, 35] and was shown to be upregulated in HER-2 positive breast cancer, making a significant correlation to poor overall survival [66]. In a study of lymphoma cells, it was found that ACOX1 regulated cell proliferation and that overexpression of this enzyme reduced doxorubicin responsiveness [34]. Furthermore, treatment with the histone deacetylase inhibitor vorinostat led to an increase in the number of peroxisomes in lymphoma cells [67].

To determine the involvement of pFAO in CLL metabolic reprogramming, we used a two-pronged approach by (i) decreasing ACOX1 expression in CLL cells (i.e. to a level similar to that observed in healthy B-cells) and (ii) pharmacologically abolishing ACOX1’s enzymatic function. The results of the first approach confirmed that the downmodulation of ACOX1 led to reorganization of the CLL metabolism by favoring a carbon (glucose) and amino acid (glutamine) phenotype, which appeared to be able to maintain the mitochondrial OXPHOS supply. This metabolism reprogramming appeared to generate enough energy for the CLL cell to survive. In contrast, the complete inhibition of ACOX1/pFAO led a more drastic effect, as evidenced by impairments in cell functions and the subsequent triggering of a cell death program. This was observed in CLL cells independently of their IGHV mutational status and the presence of genetic alterations associated with drug resistance. The pFAO-mediated processing of FAs appears therefore as a cornerstone in CLL metabolism.

Moving from a basic research perspective to a more translational perspective, the metabolic relevance of pFAO appears to be specific for CLL tumor cells. Inhibition of ACOX1/pFAO did not appear to be relevant for healthy B-cells or for immune cells involved in cell death clearance, such as T-cells and NK-cells. Furthermore, given that drug resistance remains an issue in CLL (due to the need for long-term treatments), we tested the effect of combining ACOX1 inhibition with current treatments for CLL, such as fludarabine, venetoclax, ibrutinib, and acalabrutinib. We observed an additive effect with fludarabine/ACOX1 inhibition and, more interestingly, a synergistic effect between ACOX1

and BTK inhibition on circulating and BCR-stimulated CLL cells. Therefore, coupling of ACOX1 inhibition and one of these drugs might be of value in relapsed CLL. In this way, it will be interesting to test, in a more specific study, whether the combination ACOX1/BTK inhibitors overcome the resistance of the CLL cells with del(8p) [68, 69] or with genetic alterations associated to ibrutinib resistance, such as the mutations in the BTK or phospholipase C gamma 2 proteins [70]. An intriguing finding is the lack of cooperation between TRCDA and venetoclax. Recent studies show that venetoclax suppresses mitochondrial respiration by a direct inhibition of the complex I of the mitochondrial electron transport chain [71]. It is therefore possible that venetoclax-induced mitochondrial dysfunction may render irrelevant the mitochondrial defects caused by the pFAO blockade.

Despite the original results reported here, our study has limitations mainly associated to the uncovering of a less considered/studied metabolic pathway. First, the moderate efficiency of the ACOX1 specific inhibitor TRCDA [41, 72]. Other molecules, such as the thiorizadine, which has been also used as ACOX1 inhibitor, lack of specificity [73, 74]. As a consequence, the induction of apoptosis by TRCDA in the primary CLL cells was only achieved at a micromolar concentration *in vitro*, far from the standard requirements in drug development (nanomolar level). Based on our original results that highlight the therapeutic opportunity offered by the pFAO targeting, we may anticipate that a new set of inhibitors will be generated with a better efficacy and/or bioavailability. Second, the absence of a mouse *in vivo* approach that corroborates the results obtained in our panel of 50 CLL primary cells. Of note, it is generally agreed that current CLL animal models do not fully mimic the situation in humans. Moreover, before to start an animal approach it seems necessary to analyze whether the metabolism of the malignant B-cells of usually used mouse CLL models (e.g., E μ -TCL1) is similar or not to those the CLL cells from patients. This has not been done for pFAO and needs to be evaluated in a more specific work. Thus, at this time, we cannot rule out the possibility that the pFAO inhibitors might behave differently in CLL mouse models and CLL patients. In performing experiments in a panel of primary CLL cells from patients representing the heterogeneity of the disease and in proposing assays in CLL circulating (quiescent) and BCR-activated cells or in CLL cells receiving pro-survival and pro-proliferative signals, we have tried to get closer to the reality characterizing this human disease. In any case, complementary preclinical and toxicological studies will be necessary before the therapeutic effect of pFAO inhibitors can be fully proposed for a CLL treatment in humans.

Our work presents several strengths. First, it incorporates a first translational approach initiated by the discovering of an essential and understudied metabolic CLL pathway and followed by the analysis of the energetic consequences of pFAO inhibition in CLL. Second, from a mechanistic point of view, our work reveals that it is possible to activate, by a metabolic inhibitor, a cell death pathway in the tumor cells that is not down-modulated by pro-survival or pro-proliferative signals. This mode of PCD specifically targets the malignant cells, sparing other key immune subsets. Third, in reporting ACOX1 as a molecular determinant regulating pFAO we uncover a potential link between ACOX1 mRNA expression and CLL. Finally, in combining pFAO and BTK inhibitors, we describe the potentiality of a combinatory treatment implicating newly developed CLL targeted therapies.

Resistance to immunochemotherapies and targeted therapies remains a major problem in CLL. In cases of secondary resistance, CLL progression is often rapid and aggressive, subsequent treatments are generally less effective, and the cancer may progress through a Richter transformation with a very poor prognosis [75]. Moreover, patients frequently develop one or more resistant clones that escape today's treatments. This is why targeting a common physiological feature of CLL is an attractive strategy and might circumvent the development of further resistance. Hence, the often-neglected pFAO pathway appears to be an interesting possibility.

METHODS

Characterization of primary cells

The procedures in our manuscript complied with the Declaration Helsinki and were approved by the institution review board at Groupe Hospitalier Pitié-Salpêtrière (CPPIDF6, May 21st, 2014, Paris, France) and by the French Ministry of Higher Education, Research and Innovation (CODECOH DC-2020-3855). After obtaining written consent, we collected peripheral blood samples from 50 CLL patients diagnosed by the application of classical morphological and immunophenotypic criteria [76] (age ranged from 42 to 98, with a ratio of 27 men/23 women). The Binet stage, IGHV mutational status, and cytogenetic data are summarized in Supplementary Table 1. The complexity of the karyotype relates to the number of chromosomal abnormalities: non-complex karyotype (non-CK: 0-2 abnormalities), complex karyotype (CK: 3-4 abnormalities) and hyper-complex karyotype (HCK: 5 or more abnormalities). Deletions (17p13 (affecting *TP53*), 11q22 (affecting *ATM*) and 13q14) and trisomy 12 were detected by fluorescence *in situ* hybridization (FISH), as previously described [77]. Healthy donor blood was obtained from the Etablissement Français du Sang (EFS, Paris, France).

Peripheral blood mononuclear cells (PBMC) were purified by Ficoll-Hypaque gradient centrifugation. Healthy B-cells were positively selected with an anti-CD19 monoclonal antibody coupled to magnetic microbeads (Miltenyi Biotech). This technique was also applied to CLL samples presenting low levels of lymphocytosis. There were no obvious metabolic or cell-death-related differences between selected and non-selected CLL cells. To identify T-cells and NK cells in the CLL patients' blood, we used anti-human CD3-FITC and CD56-PE-Vio770 antibodies, respectively (Miltenyi Biotech).

Cell culture and drug treatments

Primary B-lymphocytes and the OSU-CLL, PGA1, MEC1, and HG3 cell lines [40, 47-49] were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (complete medium).

FAO was inhibited by treating the cells for 24 h with the ACOX1/pFAO inhibitor 10,12-tricosadiynoic acid (TRCDA, 5 to 95 μ M) or the CPT1 and CPT2 inhibitor perhexiline (1 to 20 μ M). In combinatorial drug tests, CLL cells were treated for 24 h alone or in conjunction with TRCDA (50 μ M), fludarabine (fluda, 5 μ M), ibrutinib (ibru, 5 μ M), acalabrutinib (acala, 5 μ M), or venetoclax (VTX, 1 nM) (Selleckchem). Only primary CLL cells with a spontaneous mortality rate below 25% after 24 h in culture were used in the *in vitro* assays.

For BCR stimulation, primary cells were treated for 24 h with 20 µg/mL of soluble anti-IgM (Jackson ImmunoResearch) prior to TRCDA treatment for 24 h, as specified in Fig. 5.

The stromal cell line M210-B4 was cultured in complete medium and seeded for 24 h before adding the primary CLL cells in a stromal cell/CLL B-cell ratio of 1:100. Next, CLL cells were treated at the indicated concentration of TRCDA for 24 h.

Experiments involving a pro-proliferative environment were performed by stimulating the primary CLL cells for 24 h with the combination of CD40L (MedChemExpress; 25 ng/ml) + CpG (InvivoGen; 50 ng/ml) prior to TRCDA treatment for 24 h.

To evaluate the involvement of caspases in TRCDA-induced cell death, the broad-spectrum caspase inhibitor quinoline-Val-Asp-difluorophenoxymethylketone (Q-VD-OPh; 10 µM) was added 30 min before the treatment with TRCDA.

Seahorse metabolic assays

Primary B-lymphocytes were plated in a 96-well Seahorse plate (Agilent Technologies) at a density of 6×10^5 cells per well (for the mitochondrial stress test) or 8×10^5 cells per well (for the glycolysis stress test), pre-treated for 20 min with Corning® Cell-Tak™ (Fisher Scientific), and cultured for 1 h in NaHCO₃-free DMEM (for the mitochondrial stress test) or glucose/glutamine/NaHCO₃-free medium (for the glycolysis stress assay). The assays were performed with a Seahorse XFe96 Analyzer, according to the manufacturer's instructions (Agilent Technologies). For the mitochondrial stress test, the oxygen consumption rate (OCR) was measured at baseline (basal respiration) and after sequential injections of oligomycin (1 µM), fluorocarbonyl cyanide phenylhydrazone (FCCP; 1 µM), and rotenone/antimycin A (1 µM). The oligomycin-sensitive OCR is the rate required for ATP production. The OCR after addition of the uncoupler FCCP corresponds to the maximal mitochondrial respiratory capacity of the cells (maximal respiration). For the glycolysis stress assay, the extracellular acidification rate (ECAR) was analyzed after the sequential injection of glucose (10 mM), oligomycin (1 µM), and 2-deoxy-d-glucose (2-DG; 10 mM). The glycolytic capacity corresponds to the maximal rate of conversion of glucose to pyruvate or lactate that can be achieved by a given cell type and is also a measure of maximal ATP production through glycolysis. The raw data were normalized according to the samples' protein content.

Liquid chromatography–mass spectrometry (LC-MS) metabolomic analyses

Metabolite levels were measured using LC-MS. To prepare the samples, primary B-lymphocytes and OSU-CLL cells were washed three times with PBS, and metabolites were

extracted with a mixture of 50% methanol, 30% acetonitrile and 20% water (1 ml per 1×10^6 cells). After centrifugation, supernatants were separated by LC with a SeQuant ZIC-pHilic column (Millipore), an aqueous mobile phase of 20 mM ammonium carbonate + 0.1% ammonium hydroxide, a solid mobile phase (acetonitrile), a linear gradient from 80% organic to 80% aqueous over 15 minutes, a column temperature of 48°C, and a flow rate of 200 μ l/min. A Thermo Q-Exactive Plus mass spectrometer (Thermo Fisher) was used to detect metabolites across a mass range of 75–1,000 m/z and at a resolution of 35,000 (200 m/z), with electrospray ionization and in polarity switching mode. Lock masses were used to ensure mass accuracy below 5 ppm. Data were acquired with Thermo Xcalibur software (Thermo Fisher). The metabolites' peak areas were determined using Thermo TraceFinder software (Thermo Fisher), on the basis of the exact mass of each singly charged ion and the known retention time on the HPLC column. Statistical analyses were performed using Metaboanalyst 5.0 software [78]. Metabolite peak areas were normalized using the sum normalization method and then autoscaled. The algorithm for heatmap clustering was based on measurement of the Euclidean distance for similarity and the Ward method for clustering. The results are presented as the fold difference between healthy and CLL B-cells or between wild-type (WT) OSU-CLL cells and ACOX1-downregulated OSU-CLL cells (shACOX1).

Cell and mitochondrial assays

Cell death in the total population (10,000 cells) was assessed using annexin-V-APC (0.1 μ g/ml; BD Biosciences) and propidium iodide double labeling in a FACSCanto II (BD Biosciences). Data were analyzed using FlowJo software and (after accounting for spontaneous death) expressed as the percentage of induced cell death.

Caspase-3 activation was analyzed using the CaspGlow Fluorescein Active Caspase-3 Staining Kit, according to the manufacturer's instructions (Thermo Fisher).

For measurements of mitochondrial ROS, mitochondrial mass, and the mitochondrial membrane potential ($\Delta\Psi$ m), cells were incubated respectively with MitoSOX (Thermo Fisher; 5 μ M), Mitotracker Green (Thermo Fisher; 1 μ M), and tetramethyl rhodamine ethyl ester (TMRE; 2.5 μ M), before assessment in a FACSCanto II in the total population (10,000 cells). The data were analyzed using FlowJo software and expressed as the mean fluorescence intensity (MFI) or as a ratio between treated and untreated (control) cells.

Glucose uptake assay

Cells were seeded in a 96-well plate (1×10^6 cell/well) for 24 h in complete medium and then starved for 6 h in glucose-free medium supplemented with 2% fetal bovine serum. Glucose uptake was measured in an Infinite M100 PRO plate reader (Tecan) with the 2-Deoxyglucose Glucose Uptake Assay Kit (Abcam).

RNAseq processing and gene set enrichment analysis (GSEA)

RNAseq matrices from healthy and CLL B-cells were obtained from <http://resources.idibaps.org/paper/the-reference-epigenome-and-regulatory-chromatin-landscape-of-chronic-lymphocytic-leukemia> [39]. More precisely, matrices from "Normalized signal intensities of CLL reference epigenome & B-cells for RNAseq" and "Normalized signal intensities of CLL extended dataset & B-cells for RNAseq" were downloaded and merged, with the deletion of duplicated CLL samples. Both matrices were in fpkm with gencode 22 annotations. After merging, the data were transformed in $\log_2(\text{fpkm}+0.1)$.

For the GSEA analysis, 32 metabolic pathways or the peroxisomal gene sets annotated in the source data section were extracted from the $\log_2(\text{fpkm}+0.1)$ matrix using the molecular signature database (MSigDB, <https://www.gsea-msigdb.org/gsea>). GSEA was performed using the Java GSEA desktop application (version 4.3.2). The analysis involved a gene-set permutation algorithm (1,000 permutations) to calculate an enrichment score and a p-value for each gene set. Gene sets with a false discovery rate $<0.25\%$ and an adjusted p-value <0.05 were considered to be relevant. The GSEA desktop application was used to generate a heatmap of the gene expression levels within the selected, enriched gene set across the various samples.

Immunoblots

Cells were lysed in M-PER buffer (Pierce Biotechnology) supplemented with protease and phosphatase inhibitor cocktail. Proteins were assayed using the Qubit Protein Broad Range Assay kit (Thermo Fisher). Firstly, 10-30 μg of total protein were loaded onto a Mini Protean TGX gel 12% (Bio-Rad) and transferred onto a nitrocellulose membrane. The membranes were blocked and incubated with antibody in TBS 0.1% Tween 20 plus 5% non-fat dry milk or 5% BSA. Primary antibodies against ACOX1 (Abcam), β -actin, pan-ERK (BD Biosciences), and phospho-p44/42 MAPK (ERK1/2; T202/Y204; Cell Signaling) were used. Immunoreactive proteins were detected using horse-radish-peroxidase-conjugated secondary antibodies and visualized with Clarity or max ECL (Bio-Rad). Immunoblot images were acquired in the Chemidoc Imaging System and analyzed using Image Lab software (Bio-Rad).

H₂O₂ assay

H₂O₂ levels were measured using the ROS-Glo H₂O₂ Assay (Promega). Briefly, cells were seeded in a 96-well plate, and the H₂O₂ substrate solution was added alone or in the presence of TRCDA (50 μM) for the indicated time. Next, ROS-Glo detection solution was added. After 20 min, luminescence was measured on an Infinite M100 PRO plate reader.

ATP assay

A cell Titer-Glo Luminescent Assay (Promega) was used to measure ATP levels on an Infinite M100 PRO plate reader. The results were expressed as a percentage relative to untreated (control) cells.

Lipid droplet assay

Lipid droplets were quantified using the Lipid Droplet Fluorescence Assay Kit (Cayman), according to the manufacturer's instructions. Briefly, CLL cells were incubated for 24 h with TRCDA (50 μM) in a 96-well plate, washed, and fixed for 10 min with formaldehyde before the addition of Nile Red solution (10 min). Next, fluorescence in the total population (10,000 cells) was measured with flow cytometry on a FACSCanto II. Data were analyzed using FlowJo software and expressed as a fold difference relative to untreated (control) cells.

Immunofluorescence

Primary B-lymphocytes seeded on coverslips were fixed in 4% paraformaldehyde and incubated with the nuclear dye Hoechst 33342 and an anti-ACOX1 antibody (Proteintech), detected with an anti-rabbit IgG conjugated with Alexa[®] 594 Fluor (Thermo Fisher Scientific), and mounted with Fluoromount (Southern Biotech) according to standard procedures. The slides were imaged with a LSM 710 Zeiss confocal microscope and analyzed using Image J.

Vectors and lentiviral transduction

To downregulate ACOX1, we utilized the vector pLV[shRNA]-Puro-U6 (Vectorbuilder) cloned with the target sequence TTGCAGTGGTCTTCCAAATAT. Virus was produced in HEK-293T cells after CaCl₂-mediated transient transfection of the lentiviral construct and the packaging/envelope plasmids pMD2.G and psPAX-2 (Addgene 12259 and 12260, respectively). Forty-eight hours after transfection, the lentiviral supernatants were harvested, clarified by filtration, concentrated by centrifugation (in a Takara LentiXConcentrator), and then frozen prior to transduction of 5x10⁶ OSU-CLL cells. Next, the cells were cultured in complete medium for 48 hours before the application of puromycin selection (250 ng/ml). The

culture was continued until the control (non-transduced) OSU-CLL cells died (~10 d). Lastly, the selected shACOX1 OSU-CLL cells were expanded and analyzed.

Quantitative RT-PCR

Total RNA was extracted from cells using a Nucleospin® RNA II kit (Macherey-Nagel). cDNA was prepared using Superscript® IV reverse transcriptase (Life Technologies). Quantitative RT-PCR was performed using the TaqMan® Gene Expression Assays (Life Technologies) for *ACOX1* (Hs00244515_m1), *ABCD2* (Hs00193054_m1), *CROT* (Hs00221733_m1), *CPT1A* (Hs00912671_m1), and *CPT2* (Hs00988962_m1). PCR reactions were performed in triplicate, using TaqMan® Fast Advanced Master Mix (Life Technologies). The products were amplified at 60°C for 40 cycles in a ViiA7 Real-Time PCR System (Life Technologies). Data were evaluated using the comparative threshold cycle method. The data were normalized against the expression of the housekeeping gene *ABL1* (Hs01104728_m1).

Electron microscopy

CLL cells treated (or not) with 50 µM TRCDA were fixed at room temperature (1 h) in 100 mM sodium cacodylate (pH 7.3), 1.5% paraformaldehyde, and 1.5% glutaraldehyde and then fixed at 4°C (1 h) in the same buffer supplemented with 1% OsO₄ and 1% potassium ferrocyanide. After washing, cells were incubated in 1% aqueous uranyl acetate for 1 h at room temperature. After a further brief rinse in water, cells were dehydrated in a series of increasingly ethanolic solutions (30%, 50%, 70%, 95% and then 3 x 100%, for 15 min each), a 1/1 (v/v) ethanol/propylene oxide mixture (10 min) and then pure propylene oxide (2 x 5 min) before final embedding in Epon. Ultrathin sections (80 nm) were prepared, stained with lead citrate, and photographed on a Jeol 1400 Flash transmission electron microscope operating at 120 keV and equipped with a RIO CMOS camera (Ametek SAS).

Statistics

The data were quoted as the mean ± SEM, the mean ± SD, or the median (range). All statistical analyses were performed using GraphPad Prism. Mann-Whitney, Wilcoxon signed rank, or two-tailed Student's *t*-tests were applied in comparisons of two datasets. For comparisons of three or more datasets, a one-way analysis of variance (ANOVA) was followed by Dunnett's *post hoc* test. The levels of statistical significance were defined as **p*<0.05, ** <0.01, ****p*<0.001, and *****p*<0.0001.

Chemicals

All chemicals and reagents were purchased from Merck, except when noted otherwise.

Data availability

Data are available within the manuscript, figures, or supplementary information. Further correspondence and material requests should be addressed to the corresponding author. Source data are provided with this paper.

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AUTHOR CONTRIBUTIONS

M.T. conceived and performed CLL experimental work, analyzed the data, and helped to write the manuscript. M.A. and L.D. conceived and performed CLL experimental work and analyzed the data. I.N. performed metabolomic studies, analyzed the data, and helped in metabolomic profile presentation. K.D. performed CLL/stromal cell coculture approaches and analyzed the data. C.I. performed electronic microscopy and analyzed the results. A.B. performed immunofluorescence and analyzed the results. D.R.-W. and K.M. provided CLL samples and critical advice. H. M.-B. and B.B. provided critical advice on the work and the manuscript. B.S. conceived and performed electronic microscopy work, and analyzed the data. E.C. performed cytogenetic analysis, completed the mutational status and karyotype of the CLL patients, and provided critical advice throughout the study. F.N.-K. provided CLL samples, performed cytogenetic analysis, and provided critical advice throughout the study. D.G. conceived and supervised experimental work, analyzed the data, provided critical advice throughout the study, and helped to write the manuscript. S.A.S. conceived and supervised all aspects of the project, designed experiments, interpreted the data, and wrote the manuscript. All authors reviewed and approved the manuscript.

ETHICS DECLARATIONS

Competing interests

The authors declare no competing interests.

Ethical approval

Patient samples were collected after informed consent according to the Declaration of Helsinki.

FIGURE LEGENDS

Figure 1. Schematic representation of the three major metabolic pathways involved in OXPHOS energy production in CLL. Glucose is catabolized by a series of reactions called glycolysis, in which pyruvate is the end product. Pyruvate can be converted to lactate (Warburg effect) or it can enter the mitochondria to be transformed to acetyl-CoA, an intermediate of the TCA cycle. Once in the mitochondria, glutamine can be converted to glutamate, which in turn is converted to α -KG, also a TCA cycle intermediate. Fatty acids (FAs) enter the peroxisomes and mitochondria via the ABCD and CPT1 transporters, respectively. In the peroxisome, FAs are degraded to short-chain carnitines, which enter the mitochondrial matrix via CPT2 or CRAT for further oxidation. The product of β -oxidation (acetyl-CoA) enters the tricarboxylic acid (TCA) cycle. This cycle produces NADH and FADH₂, which are used by the electron transport chain (ETC) to produce ATP. ABCD, ATP binding cassette subfamily D member; ACAA1, acetyl-CoA acyltransferase 1; ACOX, acyl-CoA oxidase 1; ACS, acyl coenzyme A synthetase; ATP, adenosine triphosphate; α -KG, alpha-ketoglutarate; CACT, carnitine/acylcarnitine translocase CPT, carnitine palmitoyltransferase; CRAT, carnitine O-acetyltransferase; CROT, carnitine O-octanoyltransferase; D-BP, D-bifunctional protein; FADH₂, flavin adenine dinucleotide; NADH, nicotinamide adenine dinucleotide; SCP-X, sterol carrier protein 2.

Figure 2. Primary CLL B-cells show higher levels of mitochondrial respiration and lower levels of glycolytic activity than healthy B-cells. (a) Flow cytometry measurement of the mitochondrial mass, using Mitotracker Green labeling of healthy (n=5) and CLL (n=15) B-cells and expressed as MFI. (b) *Left*, representative mitochondrial stress tests in healthy and CLL cells, performed in a Seahorse XFe96 analyzer and showing the OCR. Indices of mitochondrial function were determined by the sequential application of pharmacological inhibitors, oligomycin, FCCP, and a combination of rotenone and antimycin A. *Right*, basal OCR, ATP production, and maximal respiration in healthy (n=3) and CLL (n=6) cells in independent Seahorse experiments. (c) The loss of mitochondrial transmembrane potential ($\Delta\Psi_m$) in healthy B-cells (n=5 samples) and CLL B-cells (n=13 samples) was measured using flow cytometry. (d) *Upper panel*, a heat map showing the relative area under the peak of nine key glycolytic derivatives measured with mass spectrometry in healthy B-cells (n=6) and CLL (n=14) B-cells. Each column corresponds to a sample. *Lower panels*, fold changes in levels of glucose-6-phosphate, pyruvate, and lactate metabolites. For group comparisons, the values of

the healthy B-cells were averaged and set to 1. Fold changes in the CLL cells were then calculated relative to the mean value in the healthy group. **(e)** Glucose uptake was measured with 2-DG in healthy cells (n=3 independent experiments) and CLL cells (n=6 independent experiments). **(f)** A glycolysis stress test in healthy and CLL cells was carried out on a Seahorse XF analyzer by the sequential application of glucose or the pharmacological inhibitors of OXPHOS (oligomycin) and glycolysis (2-DG); this enables us to differentiate between glycolysis, glycolytic capacity, glycolytic reserve, and non-glycolytic acidification. Representative curves are shown. ECAR in healthy B-cells (left) and CLL cells (right) is shown as percentage of the three components (glycolysis, glycolytic reserve, and non-glycolytic acidification), setting the maximum observed ECAR to 100%. **(g)** Glycolysis levels in ECAR were calculated from experiments on independent samples of healthy B-cells (n=3) and CLL cells (n=7). For the results in the Figure, statistical significance was calculated using the Mann-Whitney test in (a), (b), (c), (e) and (g) and Student's *t* test in (d). The bars correspond to the mean \pm SEM. (b) Basal respiration $p=0.0476$, ATP production $p=0.0476$. (c) $\Delta\Psi_m$ $p=0.0028$. (d) Glucose-6-P $p=0.027$, pyruvate $p=0.0015$, lactate $p=0.0248$. (e) $p=0.0238$. (g) $p=0.0167$.

Figure 3. pFAO contributes to metabolic rewiring in CLL. **(a)** *Left*, a heat map showing seven metabolites whose levels were significantly elevated (as measured with mass spectrometry) in CLL cells (n=14) versus healthy B-cells (n=6). The results are expressed as the relative area under the peak, and each column corresponds to a different sample. The color of the cell indicates the relative content of the metabolite (from blue to red). *Right*, a box plot of fold changes in levels of betaine and carnitine metabolites, showing the median (range) values. For group comparisons, the values of the healthy B-cells were averaged and set to 1. Fold changes were then calculated relative to the mean value in the healthy group. **(b)** Fold changes in C2-carn, C4-carn, C6-carn (*left panels*), C14-carn, and C16-carn (*right panels*) metabolites in the CLL and healthy B-lymphocytes described in **(a)**, as measured with mass spectrometry. Group comparisons were done as previously described. **(c)** *Left*, a GSEA analysis of a published RNAseq dataset [39], showing the metabolic gene sets in CLL B-cells (n=78) vs. healthy B-cells (n=3), in order of the normalized enrichment score (NES). Bars indicate a significant enrichment at a nominal p-value. *Right*, a GSEA plot of the published RNAseq dataset [39], depicting the FAO set of genes in CLL cells vs. healthy B-lymphocytes and showing the profile of the running enrichment score (ES) and the positions of the gene set members on the ranked list. **(d)** a GSEA plot of the peroxisomal set of genes obtained from the

same RNAseq dataset as in (c), showing the profile of the running ES and the positions of gene set members on the rank-ordered list. (e) mRNA levels of ACOX1 (healthy=4, CLL=11), ABCD2 (healthy=4, CLL=8), CROT (healthy=3, CLL=9), CPT1A (healthy=4, CLL=7) and CPT2 (healthy=4, CLL=9) in our cohort of patients were assessed using quantitative PCR. The results were normalized against ABL1 mRNA expression and fold changes were calculated relative to the mean values of healthy (control) samples. The values on the y-axis are plotted on a logarithmic scale. The bars correspond to the mean \pm SD. (f) Cell death was determined by annexin-V/PI co-labeling of primary CLL cells treated with the indicated concentrations of perhexiline. The percentage of annexin-V positive cells was quantified (n=5). (g) ACOX1 in healthy and CLL B-lymphocytes was detected by immunoblotting. Equal loading was confirmed by β -actin detection. The optical density (OD) ratio represents the differences in protein expression in CLL cells, relative to the mean values for healthy B-cells (set to 1). (h) Representative confocal images of healthy and CLL cells labeled with an antibody against ACOX1 (red) and with Hoechst reagent (blue, nucleus), showing the enhanced expression of the protein in the CLL cells. White bar: 10 μ m. Statistical significance was calculated using Student's *t* test for (a) and (b), the Mann-Whitney test for (e), and the one-sample *t*-test for (g). The bars correspond to the mean \pm SEM, unless indicated otherwise. (a) Betaine $p < 0.0001$, carnitine $p = 0.003$. (b) C2-carn $p = 0.0102$, C4-carn $p = 0.0157$, C6-carn $p = 0.0332$, C14-carn $p = 0.04$, C16-carn $p = 0.0484$. (e) ACOX1 $p = 0.0022$, ABCD2 $p = 0.002$, CROT $p = 0.041$, CPT1A $p = 0.006$, CPT2 $p = 0.021$. (g) $p = 0.04$.

Figure 4. Modulation of ACOX1 and pFAO induces metabolic reprogramming and lipid droplet accumulation in CLL cells. (a) *Left*, ACOX1 visualized by immunoblotting in WT control OSU-CLL cells and shACOX1 OSU-CLL cells (shACOX1). Equal loading was confirmed by β -actin probing. The optical density (OD) ratio corresponds to the differences in protein expression. *Right*, plots showing the normalized, autoscaled area under the peak for C2-, C4-, and C6-carnitines in OSU-CLL WT cells and shACOX1 cells, as measured with mass spectrometry. Box plots represent the median (range) values. Each point represents a triplicate. The loss of C2 to C6-carnitines in shACOX1 cells is also depicted in a histogram as the fold change versus WT cells. (b) Key metabolites in glycolysis (glucose, pyruvate, and lactate) and in the glutamine metabolic pathway (glutamine and α -ketoglutarate, α KG) in shACOX1 and OSU-CLL WT cells were measured with mass spectrometry and are presented as the fold change. (c) Schematic representation of the key steps of pFAO and CPT1-mediated

FAO (mFAO) described in Figure 1, including here the targeting of ACOX1 by 10,12-tricosadiynoic acid (TRCDA). **(d)** H₂O₂ was measured in primary CLL cells 5 to 30 min after treatment with TRCDA (50 μM). The amount of H₂O₂ was expressed as a percentage of the value for untreated (control) cells (set to 100%) (n=5). **(e)** ACOX1 inhibition by TRCDA reduced the levels of pFAO carnitine derivatives in primary CLL cells. *Left*, C2-, C4-, and C6-carnitines measured with mass spectrometry in CLL cells treated with TRCDA at the indicated concentrations. Curves represent the percentage relative to untreated (control) cells (100%). *Right*, fold changes in levels of C2-, C4-, and C6-carnitines in untreated (control) or TRCDA-treated (50 μM) CLL cells, measured with mass spectrometry (n=6). The bars correspond to the mean ± SEM. **(f)** *Left*, representative cytometric panels of lipid droplet (LD) quantification by Nile red fluorescence in untreated (Ctrl) and TRCDA-treated CLL cells (50 μM). *Right*, the MFI recorded in the flow cytometer was represented in the histogram as the fold change in TRCDA-treated cells relative to untreated (Ctrl) cells (n=6). **(g)** *Left*, representative electron micrographs of untreated (control) and TRCDA-treated CLL B-cells (50 μM; 12 and 24 h post-treatment). White squares and inserts indicate a lipid droplet. Scale bars are 1 μm for cell images and 200 nm for inserts. *Right*, the percentage of cells containing LDs was assessed in five patients (100 cells counted per patient) and represented in a histogram. Box plots represent the median (range) values. For the results in the Figure, statistical significance was calculated using Student's *t* test in (a), (b), (e), and (f) and a one-way ANOVA in (d) and (g). The bars correspond to the mean ± SEM. (a) C2-carn $p=0.0008$ and C4-carn $p<0.0001$. (b) glucose $p=0.0012$, pyruvate $p=0.00027$, lactate $p=0.0105$, glutamine $p<0.0001$, αKG $p=0.00775$. (d) 5 min $p<0.0001$, 10 min $p=0.0099$, 15 min $p=0.0003$, 30 min $p=0.0022$. (e) *Left*, C2-carn $p<0.0001$, C4-carn $p<0.0001$, C6-carn 10 μM $p=0.0013$, C6-carn 25 μM $p=0.00028$, C6-carn 50μM $p<0.0001$. *Right*, C2-carn $p=0.0003$, C4-carn $p=0.0004$, C6-carn $p=0.0045$. (f) $p=0.0457$. (g) 12 h $p=0.0127$, 24 h $p=0.0005$.

Figure 5. ACOX1/pFAO inhibition induces ATP loss and selectively targets circulating, BCR-stimulated CLL B-cells, and CLL B-cells receiving pro-survival stromal signals. **(a)** *Left*, ATP levels recorded in healthy (n=5) and primary CLL cells (classified as IGHV-mutated (IGHV-M) (n=15) and IGHV-unmutated (IGHV-UM) (n=13)) incubated for 24 h with TRCDA (50 μM and 75 μM) and in untreated (control) cells. The ATP results are expressed as a percentage relative to the value in control cells (set to 100%). *Right*, Similar ATP assays were performed on primary CLL cells classified as having a non-complex karyotype (non-CK; n=22) or a complex/hyper complex karyotype (CK/HCK; n=11). The ATP results are expressed as a

percentage relative to the value in control cells (set to 100%). **(b)** *Left*, cell death was measured through annexin-V/PI co-labeling of healthy B-cells (n=3) and primary CLL cells (n=3) incubated for 24 h with TRCDA at the indicated concentration. The percentages of annexin-V positive cells were recorded and plotted. *Right*, cell death was measured through annexin-V labeling of T-lymphocytes (T-cells) and natural killer (NK) cells from CLL patients (n=5) and healthy (n=2) incubated for 24 h with TRCDA (50 μ M and 75 μ M). The percentages of annexin-V positive cells were recorded and expressed as a histogram. **(c)** Cell death was assessed by annexin-V/PI co-labeling of primary CLL cells (classified as IGHV-M (n=15) and IGHV-UM (n=12)) incubated for 24 h with TRCDA (50 μ M and 75 μ M). The percentages of annexin-V positive cells were recorded and plotted. Box plots represent the median (range) values. **(d)** Cell death was measured through annexin-V/PI co-labeling of primary CLL cells (classified as non-del(17p)/del(11q) (n=21), del(17p)/del(11q) (del (17p), yellow circles; n=5; del(11q), red triangles; n=4), non-CK (n=19) and CK/HCK (n=9)) treated with 50 μ M or 75 μ M of TRCDA for 24 h. The percentages of annexin-V positive cells were recorded and expressed as a histogram. **(e)** *Left*, CLL B-cells were stimulated for 24 h with 20 μ g/mL soluble anti-IgM, and BCR-activation was verified by immunoblotting phosphorylated ERK in two representative IGHV-mutated (IGHV-M) and IGHV-unmutated (IGHV-UM) samples. Equal loading was confirmed by measurement of total ERK. *Middle*, unstimulated (CLL) and BCR-stimulated CLL cells (CLL + α -IgM) (n=9) were incubated for 24 h with TRCDA and cell death was measured through annexin-V/PI co-labeling. Induced cell death was calculated as above and expressed as a violin plot. *Right*, unstimulated CLL cells (CLL) and BCR-stimulated CLL cells (CLL + α -IgM) were analyzed using mass spectrometry, and levels of C2-, C4-, and C6-carnitines were represented as the fold change for CLL + α -IgM versus CLL (n=5). **(f)** CLL cells were co-cultured (CLL-strom) or not (CLL) with the stromal cell line M210-B4 for 24 h and then treated for 24 h with TRCDA. *Left panel*, the protective effect of stromal/CLL culture was checked by measuring spontaneous cell death by annexin-V/PI co-labeling, as above (n=5). *Middle*, cell death was assessed by annexin-V/PI co-labeling of CLL cells co-cultured (CLL-strom) or not (CLL) with stromal cells and treated with TRCDA (n=5). The percentages of annexin-V positive cells were recorded and expressed as a violin plot. *Right*, CLL cells co-cultured (CLL-strom) or not (CLL) with stromal cells were analyzed using mass spectrometry, and levels of C2-, C4-, and C6-carnitines were represented in the histogram as the fold change for CLL-strom versus CLL (n=5). For the results in the Figure, statistical significance was calculated using Student's *t* test in (a, right panel), (b), (c), (d), (e), and (f), a one-way ANOVA

in (a, left panel), and the Wilcoxon signed rank in (f; middle violin plot). The bars correspond to the mean \pm SEM, except in Fig. b, right, where the mean \pm SD is given. (a) $p < 0.0001$. (b) 50 μM $p = 0.01$, 75 μM $p < 0.0001$, 85 μM $p = 0.0015$. (c) 50 μM $p = 0.0215$, 75 μM $p = 0.0206$. (f) *Left*, $p = 0.0049$. *Right*, C2-carn $p = 0.0126$, C4-carn $p = 0.028$, C6-carn $p = 0.034$.

Figure 6. ACOX1/pFAO inhibition induced mitochondrion-mediated caspase-3-dependent apoptosis in primary CLL B-cells. (a) The loss of $\Delta\Psi\text{m}$ was measured with TMRE staining in CLL B-cells not treated (0 μM) or treated for 24 h with TRCDA (50 μM or 75 μM). The MFI recorded in nine independent experiments is presented as the ratio between treated and untreated cells (untreated = 1). (b) Mitochondrial ROS levels were assayed using flow cytometry and the Mitosox dye in CLL B-cells not treated (0 μM) or treated for 24 h with TRCDA (50 μM or 75 μM). The MFI recorded in five independent experiments is plotted as the ratio between treated and untreated cells (untreated = 1). (c) *Left*, cell viability was determined by annexin-V/PI co-labeling of CLL cells that were pre-incubated (or not) with Q-VD-OPh (10 μM , 30 min) and then treated with TRCDA (50 μM or 75 μM). Annexin-V positive cells were quantified and expressed as a percentage ($n = 3$). *Right*, representative flow cytometry plots show the percentage of annexin-V positive cells. (d) Caspase-3 activation was analyzed by flow cytometry with a CaspGLOW fluorescein active caspase-3 staining kit in CLL B-cells not treated (0 μM) or treated for 24 h with TRCDA (50 μM or 75 μM). *Left*, the mean fluorescence intensity (MFI) assessed in four independent experiments is depicted as the ratio between treated and untreated cells (untreated = 1). *Right*, representative flow cytometry histograms of untreated (control) and TRCDA-treated cells, showing the percentage of caspase-3 positive cells. For the results in the Figure, statistical significance was calculated using Student's t test in (c) and a one-way ANOVA in (a), (b), and (d). The bars correspond to the mean \pm SEM. (a) 50 μM $p = 0.0006$, 75 μM $p < 0.0001$ (b) 50 μM $p = 0.0217$, 75 μM $p = 0.0077$. (c) 50 μM : $p = 0.0035$, 75 μM $p = 0.0066$. (d) 75 μM $p = 0.0264$.

Figure 7. A combination of ACOX1/pFAO and BTK inhibitors has a synergistic killing effect on circulating and BCR-stimulated primary CLL B-cells. (a) Primary cells from patients classified as having a non-complex karyotype (non-CK) or complex karyotype (CK) were selected, and cell death was measured through annexin-V/PI co-labeling of cells incubated for 24 h with TRCDA (50 μM) or fludarabine (Fluda; 5 μM) alone or in combination. *Left*, annexin-V positive cells were recorded and plotted. *Right*, heatmap visualization of the

induced cell death recorded under each condition (represented in columns). Each row represents a different CLL sample. The color of the cell indicates the percentage of cell death (from white to dark red). **(b)** Cell death was measured after incubating the CLL cells for 24 h with TRCDA (50 μ M) or venetoclax (VTX; 1 nM) alone or in combination. The percentages of annexin-V positive cells are represented as a box plot (*left*) or a heatmap (*right*), as in (a). **(c)** Primary cells from patients classified as having a non-CK, a CK or an HCK were selected, and cell death was assessed after 24 h of treatment with TRCDA (50 μ M), ibrutinib (Ibru; 5 μ M) or acalabrutinib (acala, 5 μ M) alone or in combination. The results are represented as a box plot (*left*) or a heatmap (*right*), as above. **(d)** *Left*, the CLL B-cells selected in (c) were stimulated for 24 h with 20 μ g/mL soluble α -IgM and then treated for 24 h with TRCDA (50 μ M) or ibrutinib (Ibru, 5 μ M) alone or in combination. Cell death was measured through annexin-V/PI co-labeling. The percentages of annexin-V positive cells were recorded and represented as a box plot (*left*) or a heatmap (*right*), as above. For the results in the Figure, statistical significance was calculated using Student's *t* test. Box plots show the median (range) values for (a) TRCDA vs. TRCDA+Fluda: $p=0.0144$, Fluda vs. TRCDA+Fluda: $p=0.0123$. (b) TRCDA vs. TRCDA+VTX: $p=0.037$. (c) TRCDA vs. TRCDA+Ibru: $p=0.0011$, Ibru vs. TRCDA+Ibru: $p=0.0023$, TRCDA vs. TRCDA+acala: $p=0.0042$, acala vs. TRCDA+acala: $p=0.0002$. (d) TRCDA vs. TRCDA+Ibru: $p=0.0007$, Ibru vs. TRCDA+Ibru: $p=0.002$.

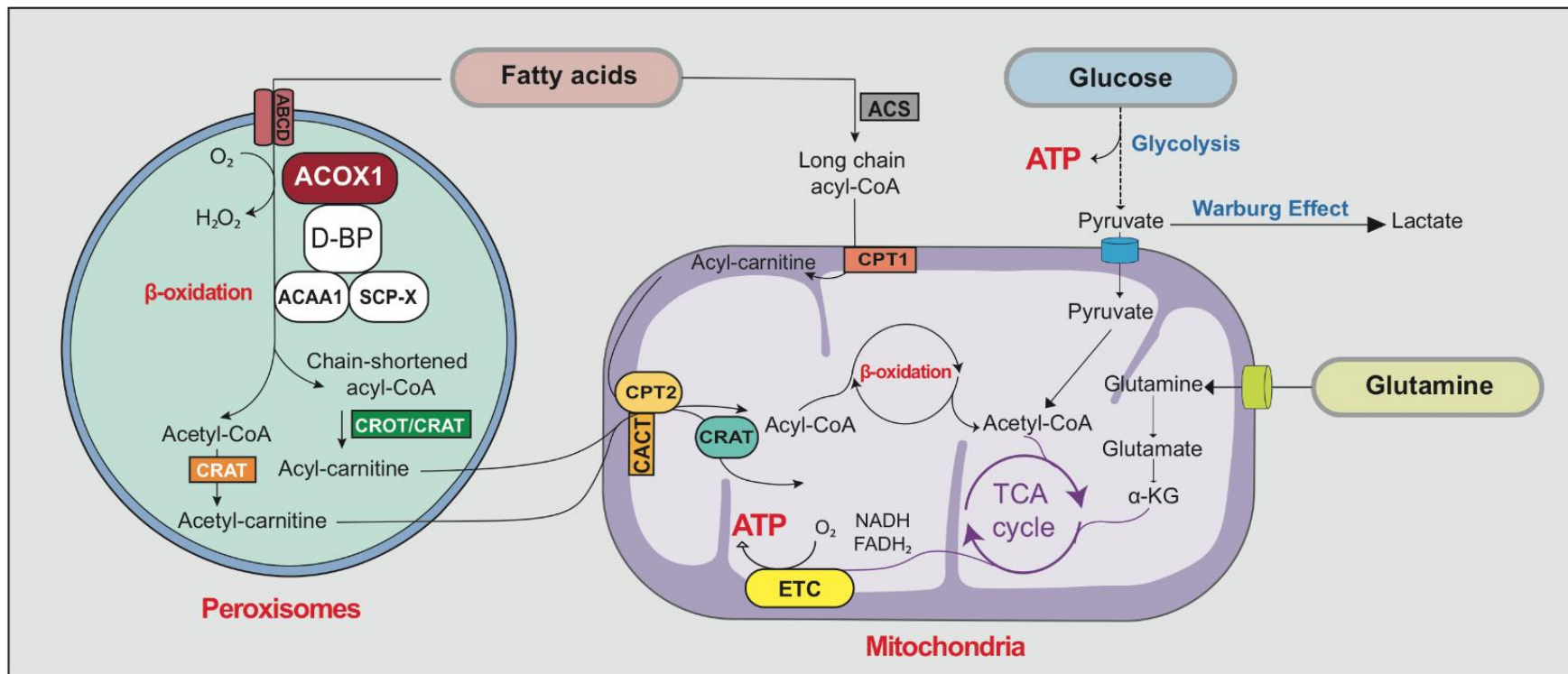


Figure 1

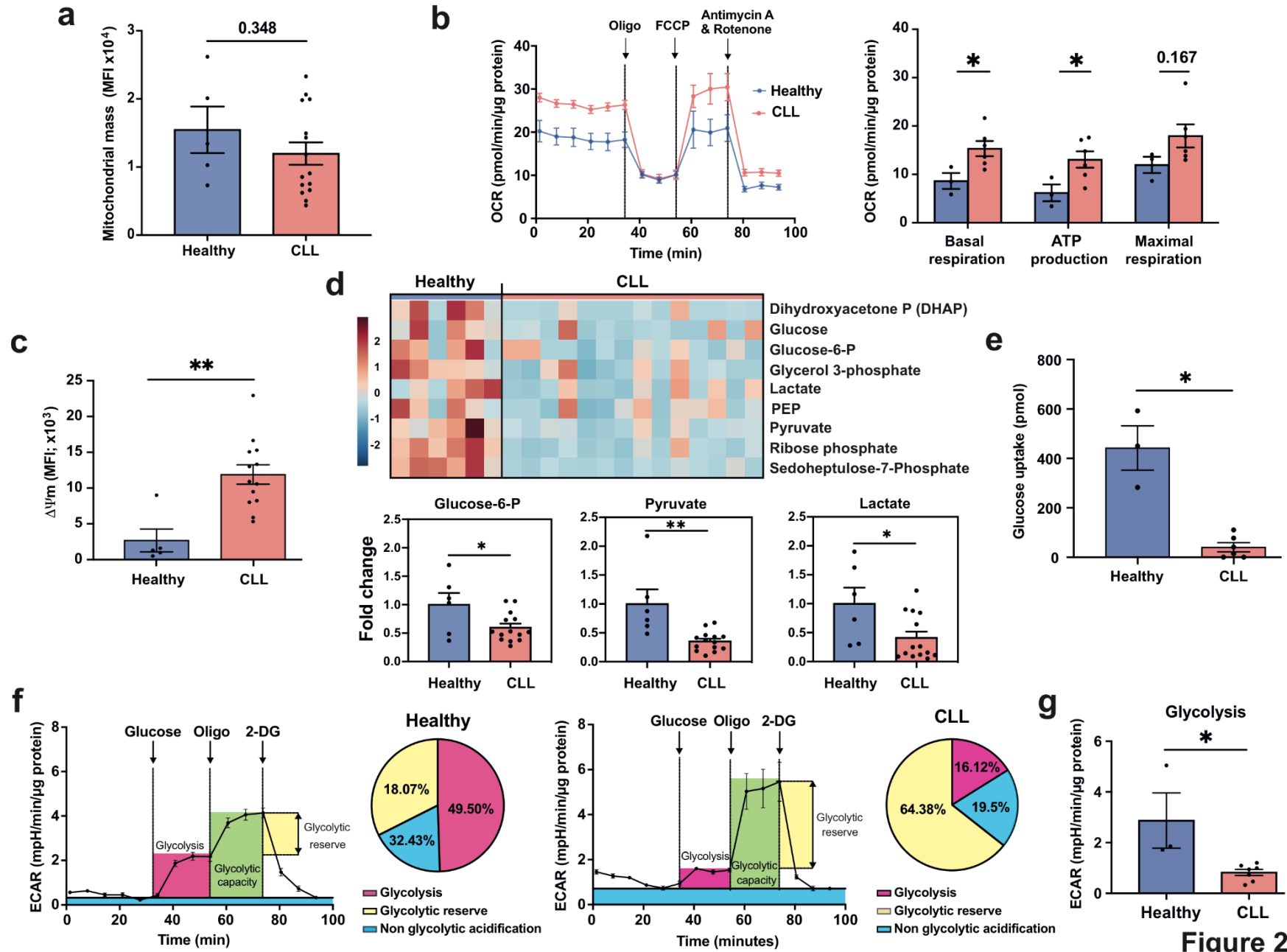


Figure 2

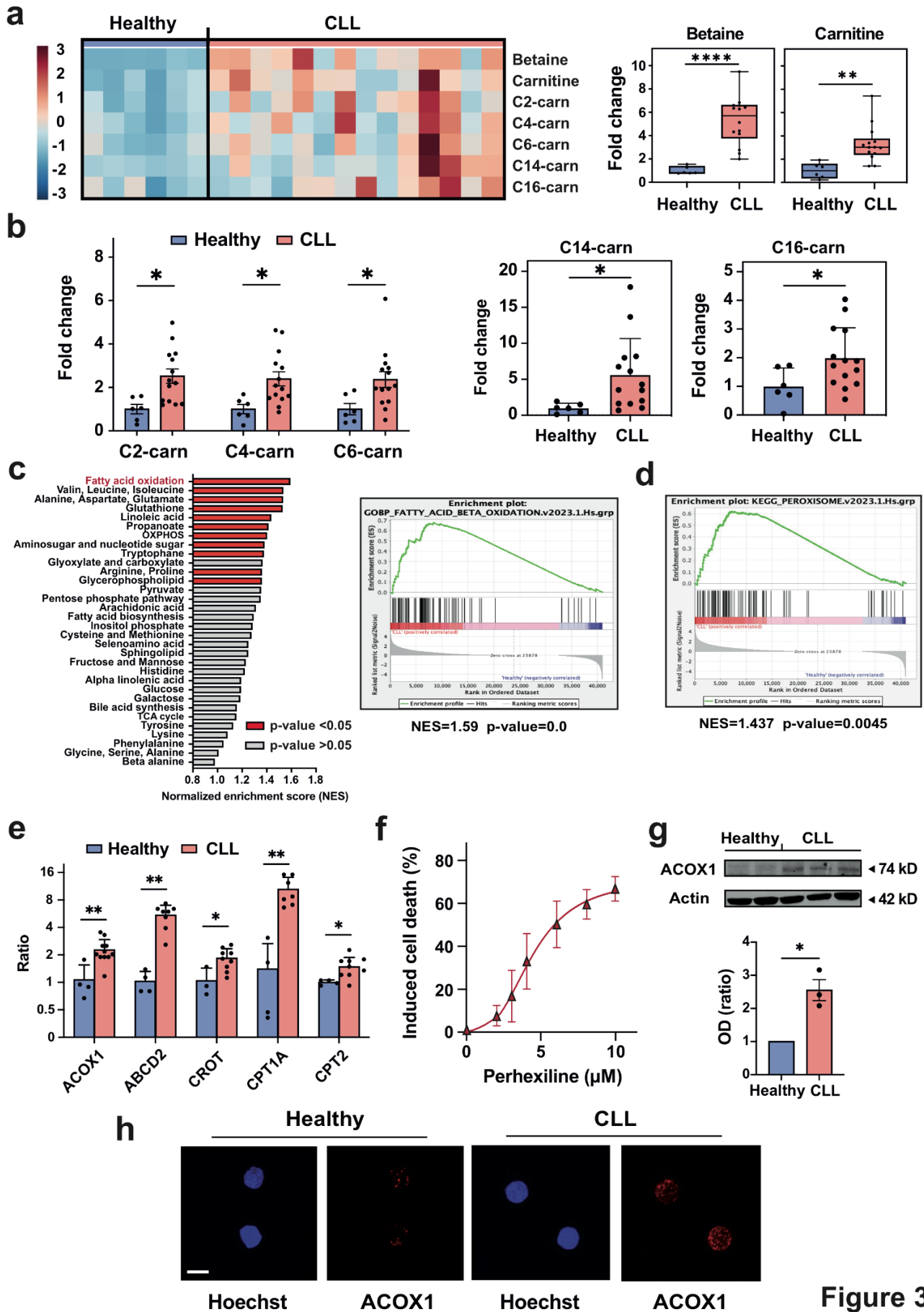


Figure 3

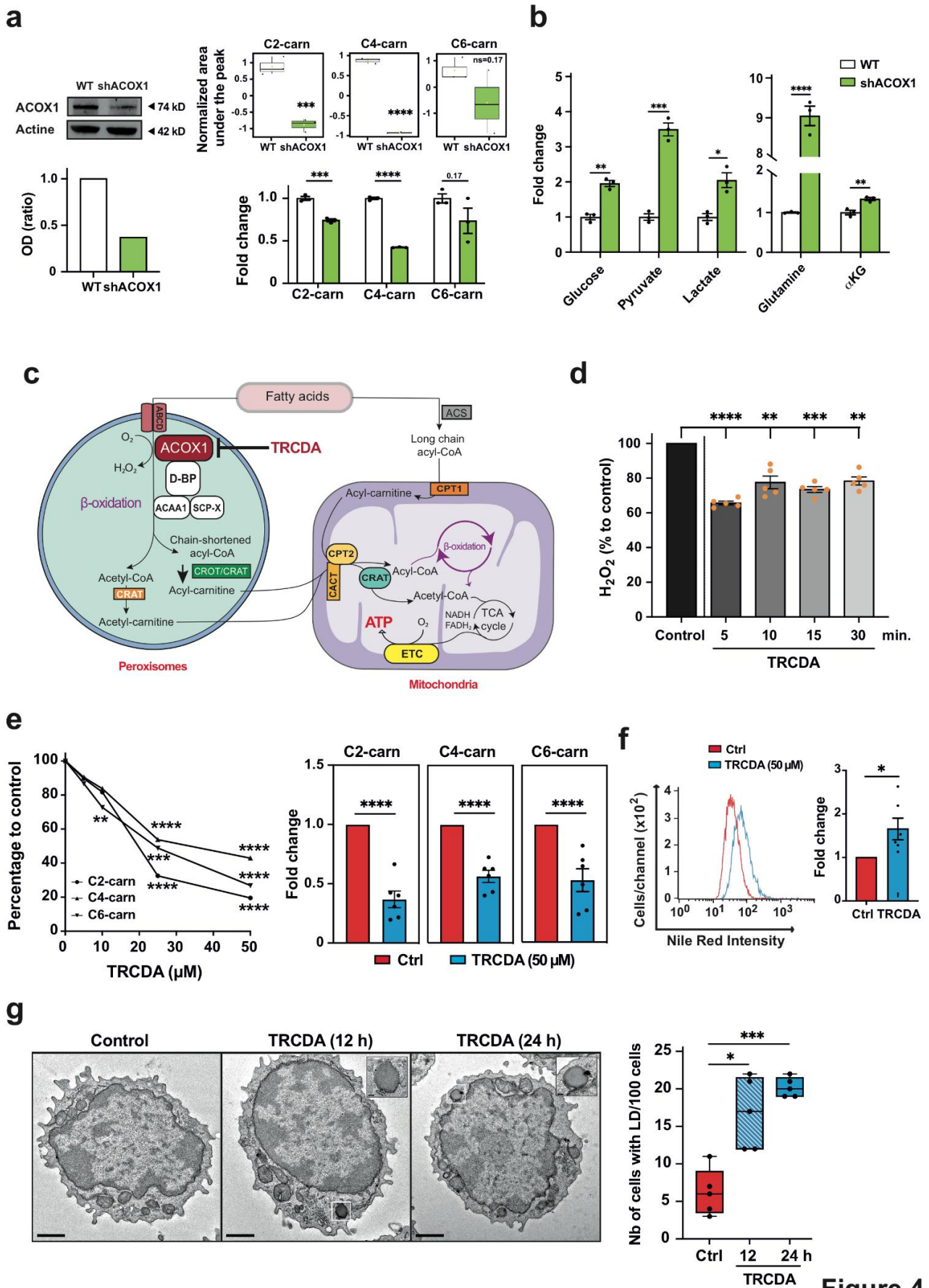


Figure 4

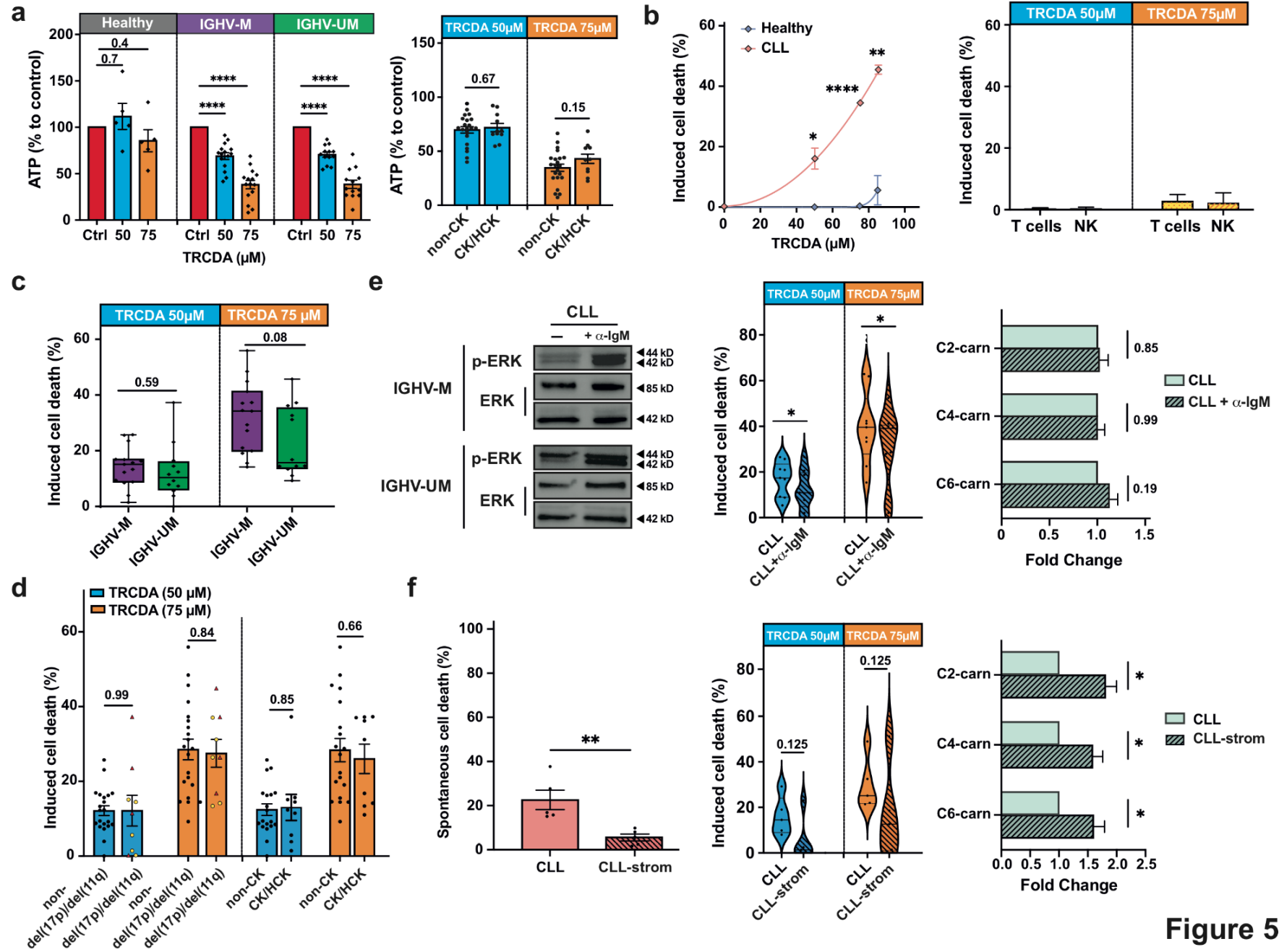


Figure 5

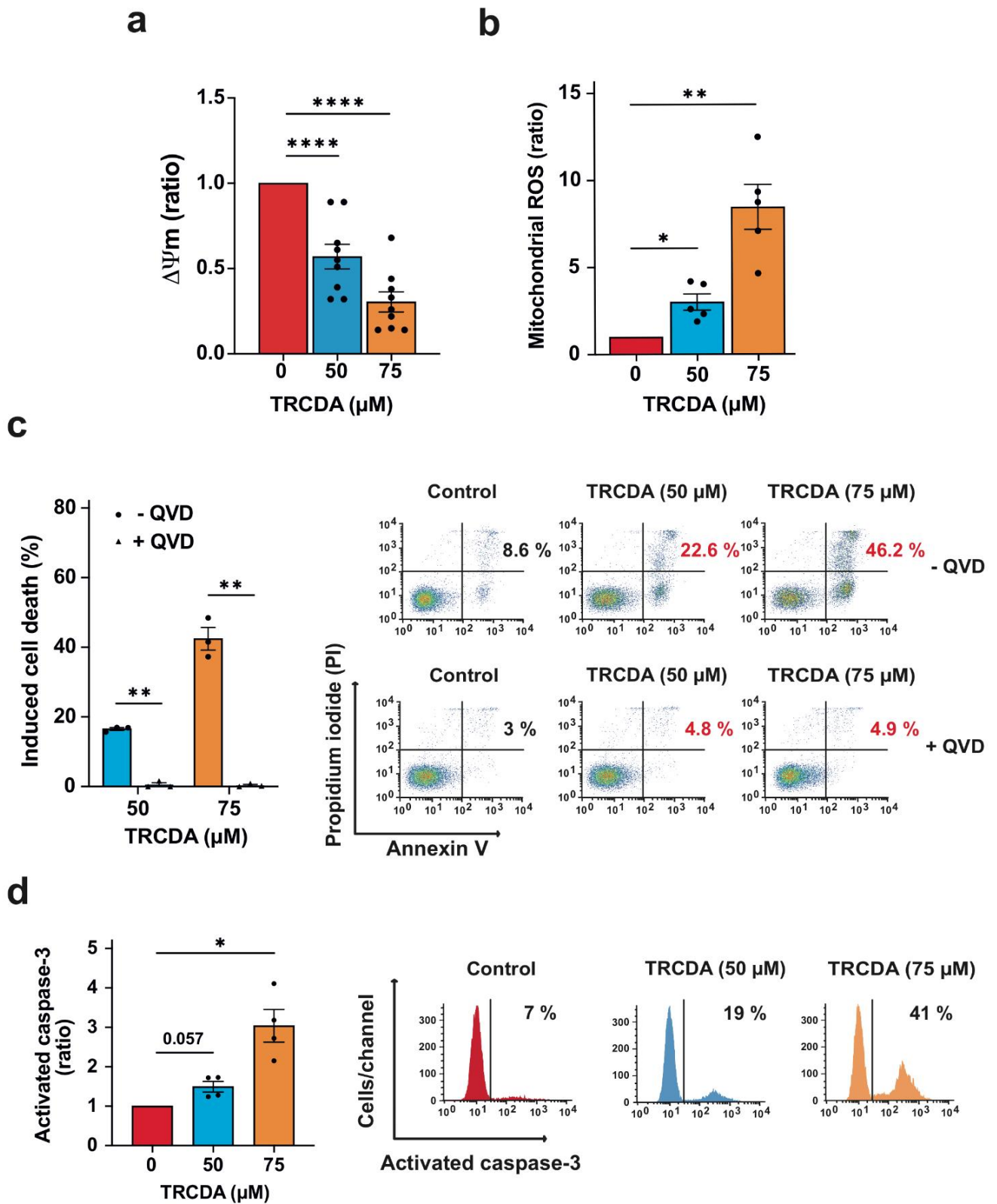


Figure 6

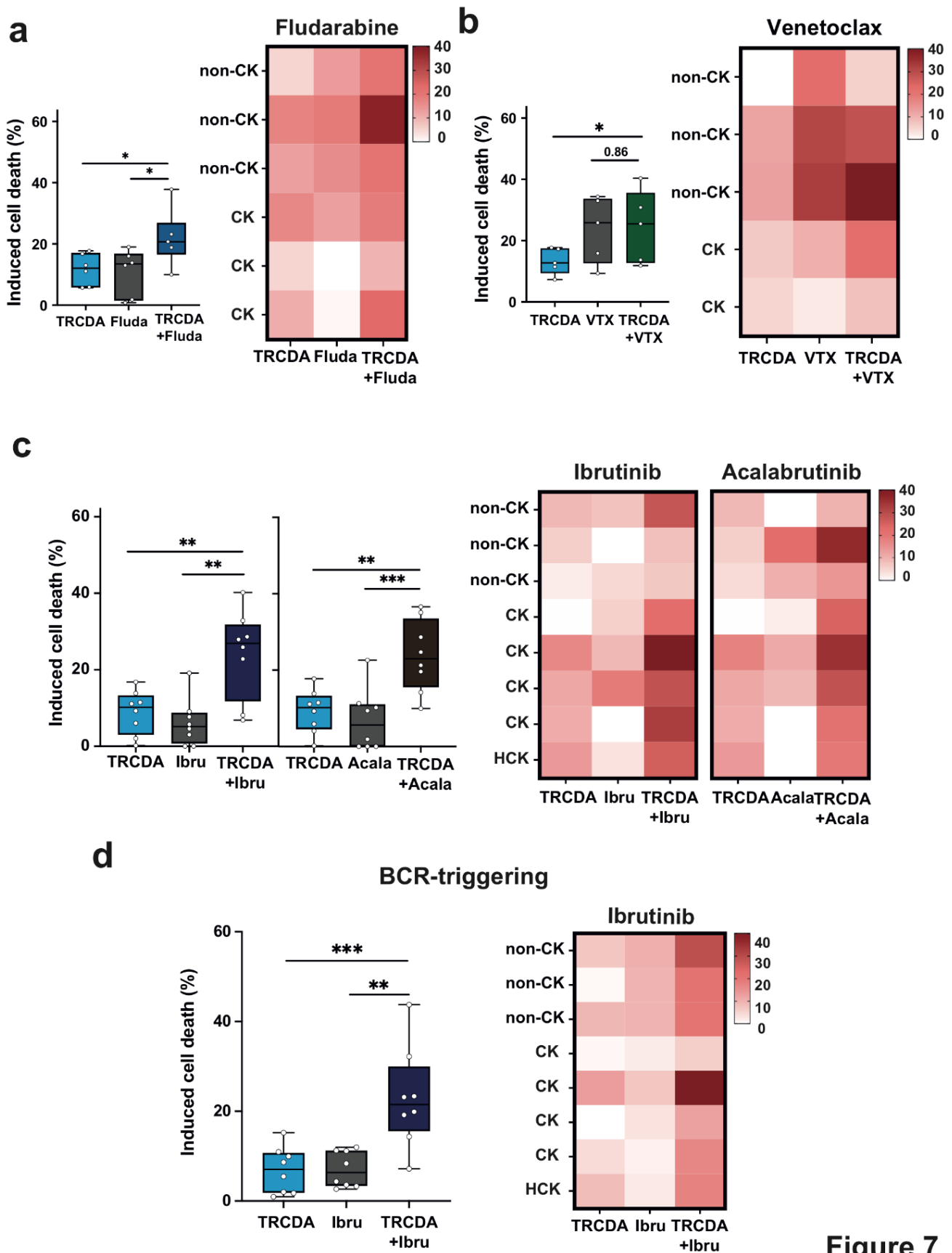


Figure 7

SUPPLEMENTARY INFORMATION

ACOX1-mediated peroxisomal fatty acid oxidation contributes to metabolic reprogramming and survival in chronic lymphocytic leukemia

Tannoury et al.

- SUPPLEMENTARY TABLE 1

- SUPPLEMENTARY FIGURES 1 - 8

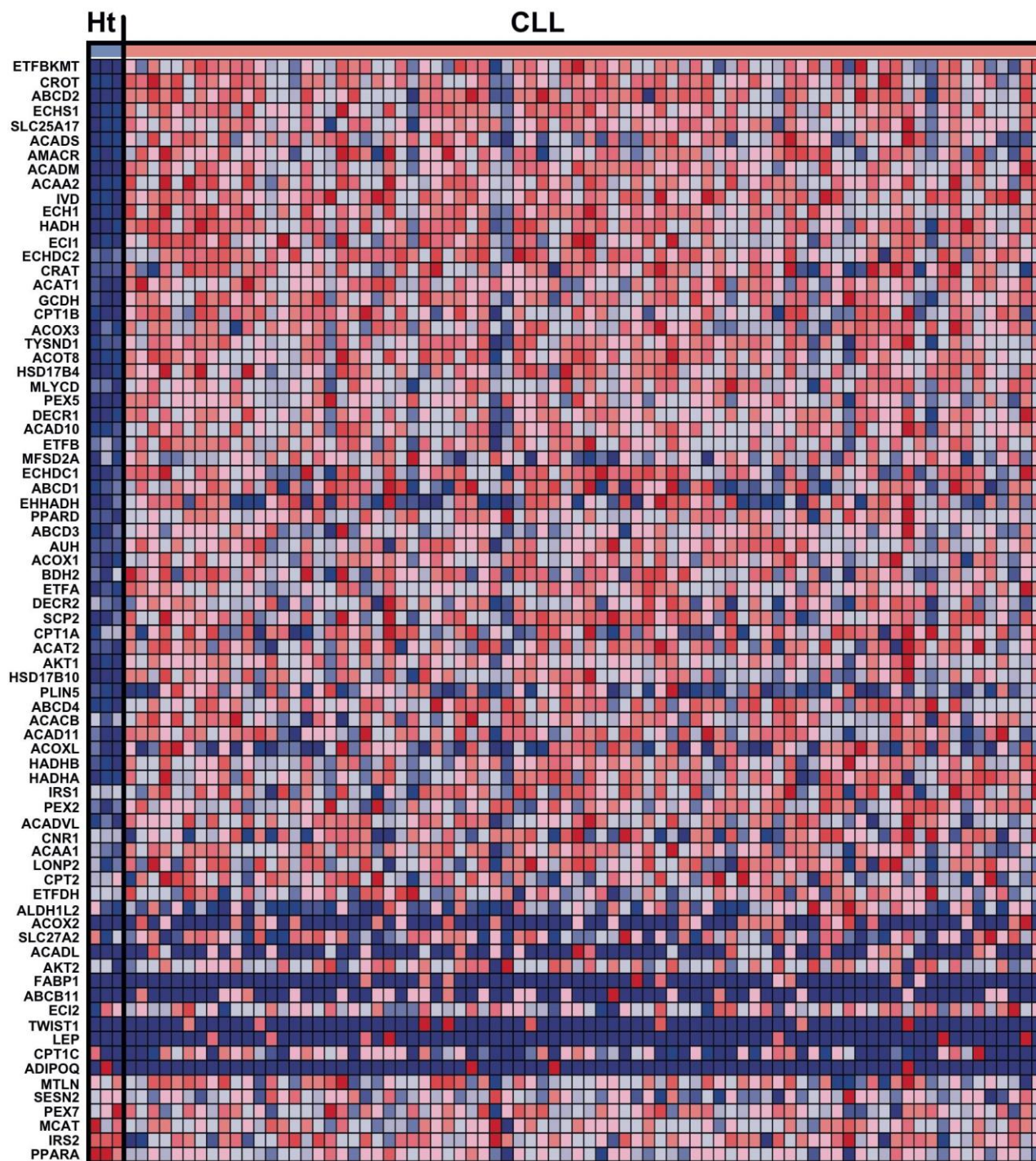
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Patient Number	Stage	IGHV Mutational status	Del(17p)	Del(11q)	Del(13q)	Trisomy 12	Karyotype
Stage A							
CLL_1	A	M	-	-	+	-	non-CK
CLL_2	A	M	-	-	+	+	non-CK
CLL_3	A	M	-	-	-	-	non-CK
CLL_4	A	M	NA	NA	NA	NA	NA
CLL_5	A	M	-	-	-	-	non-CK
CLL_6	A	M	-	+	+	-	non-CK
CLL_7	A	M	-	-	-	+	non-CK
CLL_8	A	M	-	-	+	-	non-CK
CLL_9	A	M	-	-	-	+	non-CK
CLL_10	A	M	-	+	+	-	non-CK
CLL_11	A	M	-	-	+	-	non-CK
CLL_12	A	M	-	-	+	-	non-CK
CLL_13	A	M	-	-	-	-	non-CK
CLL_14	A	M	-	-	+	-	non-CK
CLL_15	A	M	+	-	+	-	HCK
CLL_16	A	UM	+	-	-	-	CK
CLL_17	A	NA	-	-	-	-	non-CK
CLL_18	A	NA	-	-	-	+	non-CK
CLL_19	A	UM	-	-	+	-	non-CK
CLL_20	A	UM	-	-	+	-	non-CK
CLL_21	A	UM	-	-	-	-	non-CK
CLL_22	A	UM	-	-	-	+	non-CK
CLL_23	A	UM	-	+	+	-	HCK
Stage B/C or Relapse							
CLL_24	B	M	-	-	+	-	non-CK
CLL_25	Relapse post-rituximab+chlorambucil	M	-	-	+	-	non-CK
CLL_26	Relapse post-fludarabine+ cyclophosphamide	M	-	-	+	-	NA
CLL_27	C	M	+	-	+	-	non-CK
CLL_28	C	M	-	-	+	-	NA
CLL_29	C	M	-	-	+	-	non-CK
CLL_30	B	M	-	-	+	-	CK
CLL_31	Relapse post-ibrutinib	M	+	-	+	-	HCK
CLL_32	B	UM	-	-	-	-	non-CK
CLL_33	B	UM	-	-	+	-	non-CK
CLL_34	B	UM	-	-	-	-	non-CK
CLL_35	B	UM	-	-	+	-	non-CK
CLL_36	B	UM	-	-	-	+	non-CK
CLL_37	B	UM	-	-	+	-	non-CK
CLL_38	B	UM	-	+	+	-	non-CK
CLL_39	Relapse post rituximab + fludarabine + cyclophosphamide	UM	-	-	+	-	non-CK
CLL_40	B	UM	-	-	+	-	CK
CLL_41	B	M	+	+	-	-	CK
CLL_42	B	UM	-	+	-	+	CK
CLL_43	B	NA	-	-	+	-	CK
CLL_44	Relapse post rituximab + fludarabine + cyclophosphamide	UM	+	-	+	+	HCK
CLL_45	B	UM	-	+	+	-	HCK
CLL_46	B	UM	+	-	-	+	HCK
CLL_47	Relapse post-rituximab+bendamustine	UM	-	+	+	-	HCK
CLL_48	B	UM	+	+	+	-	HCK
CLL_49	B	UM	+	-	+	-	HCK
CLL_50	B	NA	+	-	+	-	HCK

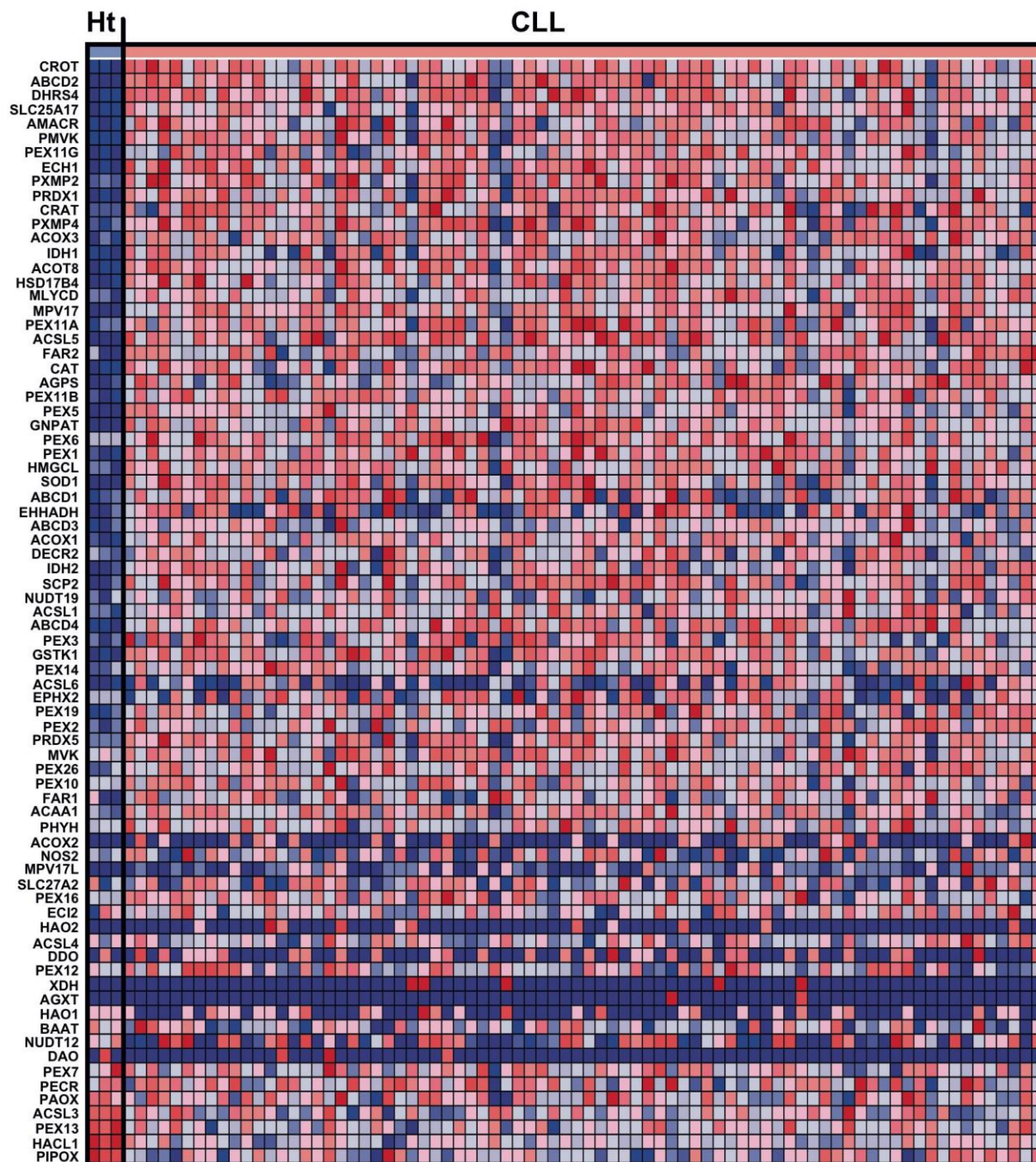
Supplementary Table 1. Features of the CLL patients included in the study. The clinical stage was determined according to Binet's criteria (A, B or C). IGHV mutational status homology: M, mutated (germline homology <98%); UM, unmutated (germline homology ≥98%). Cytogenetic alterations were determined by karyotype/FISH: (-) negative; (+) positive. The complexity of the karyotype depends on the number of chromosomal abnormalities: noncomplex karyotype (non-CK: 0-2 abnormalities), complex karyotype (CK: 3-4 abnormalities) and hyper-complex karyotype (HCK: 5 or more abnormalities). Del, deletion. NA, not available.

Gene set	Size	Enrichment Score	Normalized Enrichment Score	Nominal p-value	FDR q-value
FATTY ACID BETA OXIDATION	75	0.68	1.59	0.000	0.030
VALINE, LEUCINE AND ISOLEUCINE METABOLISM	43	0.70	1.53	0.002	0.052
ALANINE, ASPARTATE, AND GLUTAMATE METABOLISM	31	0.75	1.53	0.001	0.035
GLUTATHIONE METABOLISM	48	0.69	1.53	0.005	0.028
LINOLEIC ACID METABOLISM	28	0.70	1.43	0.021	0.117
PROPANOATE METABOLISM	32	0.68	1.41	0.037	0.133
OXPHOS	131	0.58	1.40	0.003	0.133
AMINOSUGAR AND NUCLEOTIDE SUGAR METABOLISM	43	0.63	1.38	0.038	0.152
TRYPTOPHAN METABOLISM	39	0.64	1.37	0.040	0.142
GLYOXYLATE AND DICARBOXYLATE METABOLISM	15	0.74	1.36	0.057	0.142
ARGININE AND PROLINE METABOLISM	53	0.61	1.36	0.033	0.136
GLYCEROPHOSPHOLIPID METABOLISM	76	0.59	1.36	0.016	0.126
PYRUVATE METABOLISM	39	0.63	1.35	0.060	0.124
PENTOSE PHOSPHATE PATHWAY	26	0.67	1.35	0.055	0.120
ARACHIDONIC ACID METABOLISM	57	0.58	1.31	0.060	0.164
FATTY ACID BIOSYNTHESIS	21	0.66	1.29	0.114	0.182
INOSITOL PHOSPHATE PATHWAY	53	0.57	1.28	0.098	0.186
CYSTEINE AND METHIONINE METABOLISM	33	0.60	1.27	0.126	0.191
SELENOAMINO ACID METABOLISM	25	0.63	1.25	0.133	0.221
SPHINGOLIPID METABOLISM	38	0.58	1.25	0.133	0.210
FRUCTOSE AND MANNOSE METABOLISM	33	0.59	1.23	0.163	0.234
HISTIDINE METABOLISM	28	0.60	1.22	0.187	0.238
ALPHA LINOLENIC ACID METABOLISM	18	0.63	1.19	0.236	0.273
GLUCOSE METABOLISM	91	0.50	1.19	0.151	0.263
GALACTOSE METABOLISM	25	0.59	1.18	0.219	0.262
BILE ACID SYNTHESIS	15	0.63	1.15	0.277	0.299
TCA CYCLE	30	0.56	1.15	0.250	0.295
TYROSINE METABOLISM	41	0.53	1.12	0.279	0.327
LYSINE METABOLISM	43	0.50	1.08	0.364	0.393
PHENYLALANINE METABOLISM	17	0.56	1.04	0.418	0.439
GLYCINE, SERINE AND ALANINE METABOLISM	30	0.49	1.00	0.473	0.495
BETA ALANINE METABOLISM	21	0.50	0.97	0.526	0.530

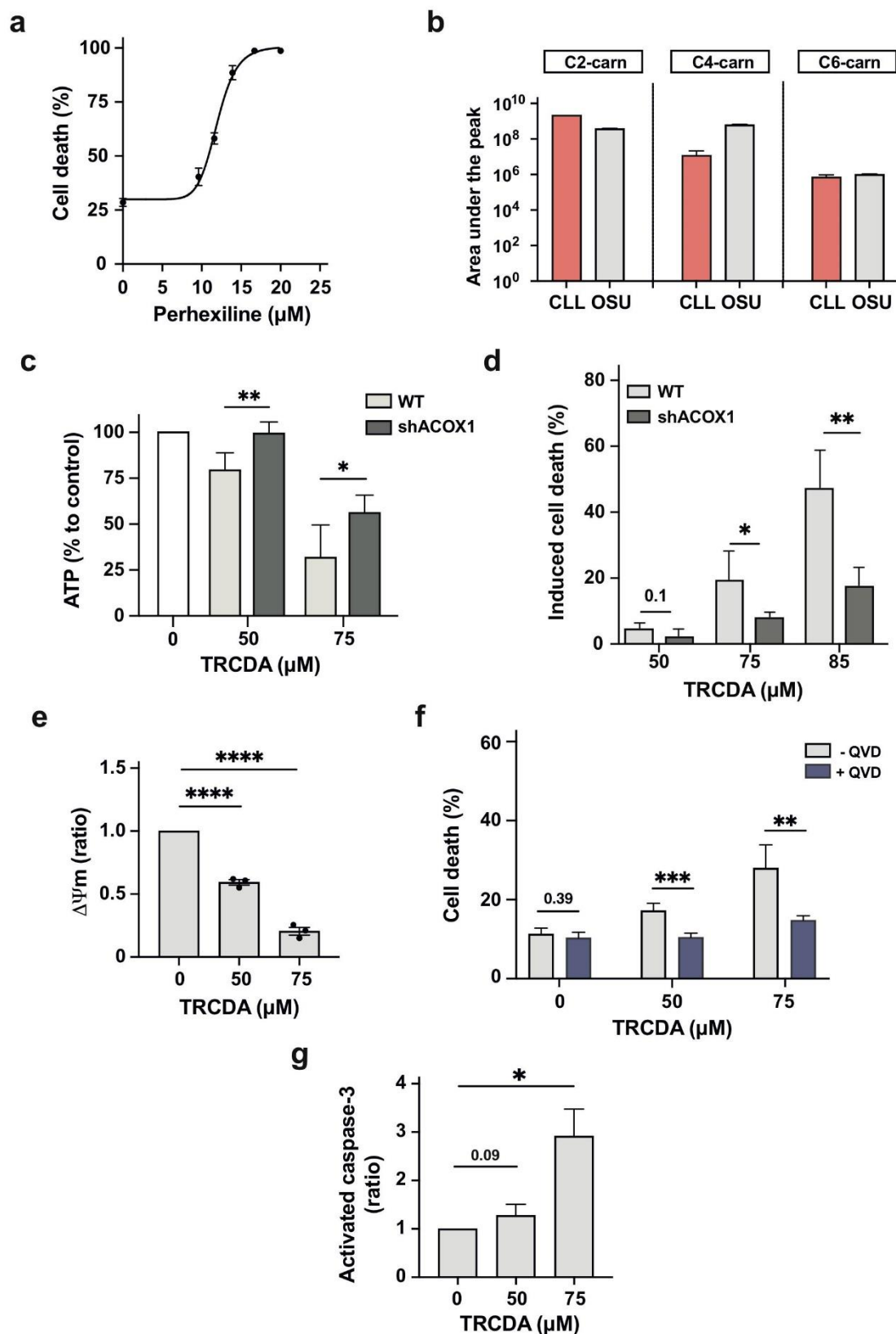
Supplementary Figure 1. The FAO gene pathway is enriched in CLL B-cells, relative to healthy B-cells. Table specifying the results of the GSEA analysis shown in Fig 3c (left), including the metabolic gene sets, the number of genes assigned to each set (size), the enrichment score, the normalized enrichment score, the nominal p-value, and the false discovery rate (FDR) q-value.



Supplementary Figure 2. Genes in the FAO metabolic pathway are enriched in CLL cells, relative to healthy B-cells. A heat map of the FAO genes included in the GSEA analysis described in Fig. 3c (right), showing a comparison of 78 CLL B cells (CLL) vs. 3 healthy B cells (Ht) for the RNAseq data reported by Beekman *et al.* 2018. Nature Medicine 24, 868-880. Gene expression values are represented as colors, ranging from red (highest expression), pink (moderate) and light blue (low) to dark blue (lowest expression).

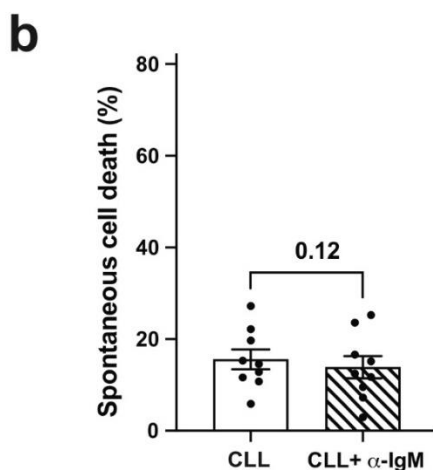
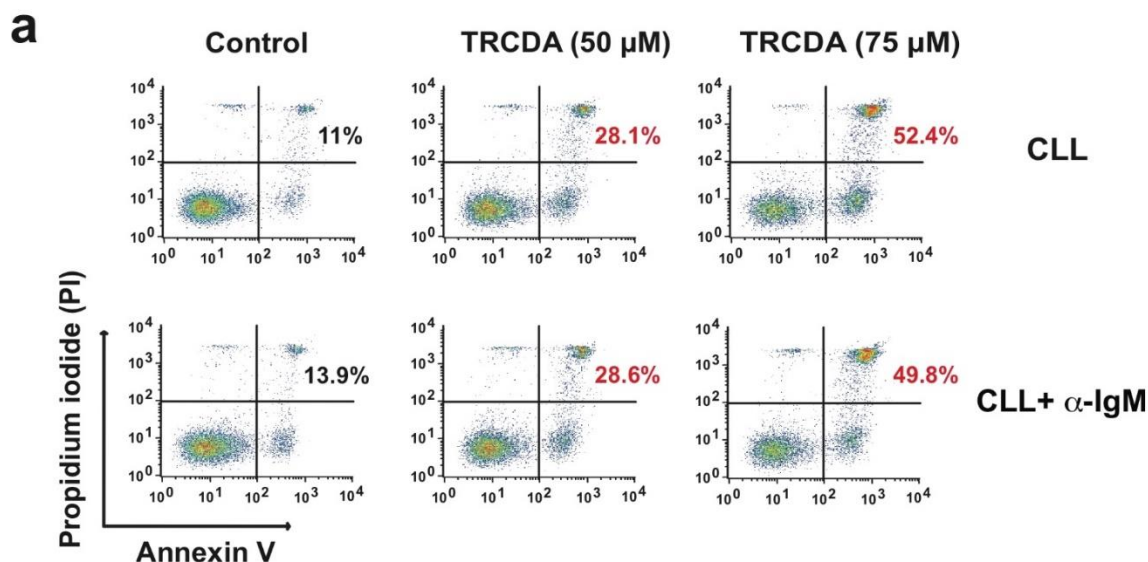


Supplementary Figure 3. Peroxisome-related genes (including genes related to biogenesis, pFAO and other peroxisomal functions) are enriched in CLL cells, relative to healthy B-cells. A heat map of the peroxisome genes included in the GSEA analysis described in Fig. 3d, showing a comparison of 78 CLL B cells (CLL) versus 3 healthy B cells (Ht) for the RNAseq data reported by Beekman *et al.* 2018. Nature Medicine 24, 868-880. Gene expression values are represented as colors, ranging from red (highest expression), pink (moderate) and light blue (low) to dark blue (lowest expression).

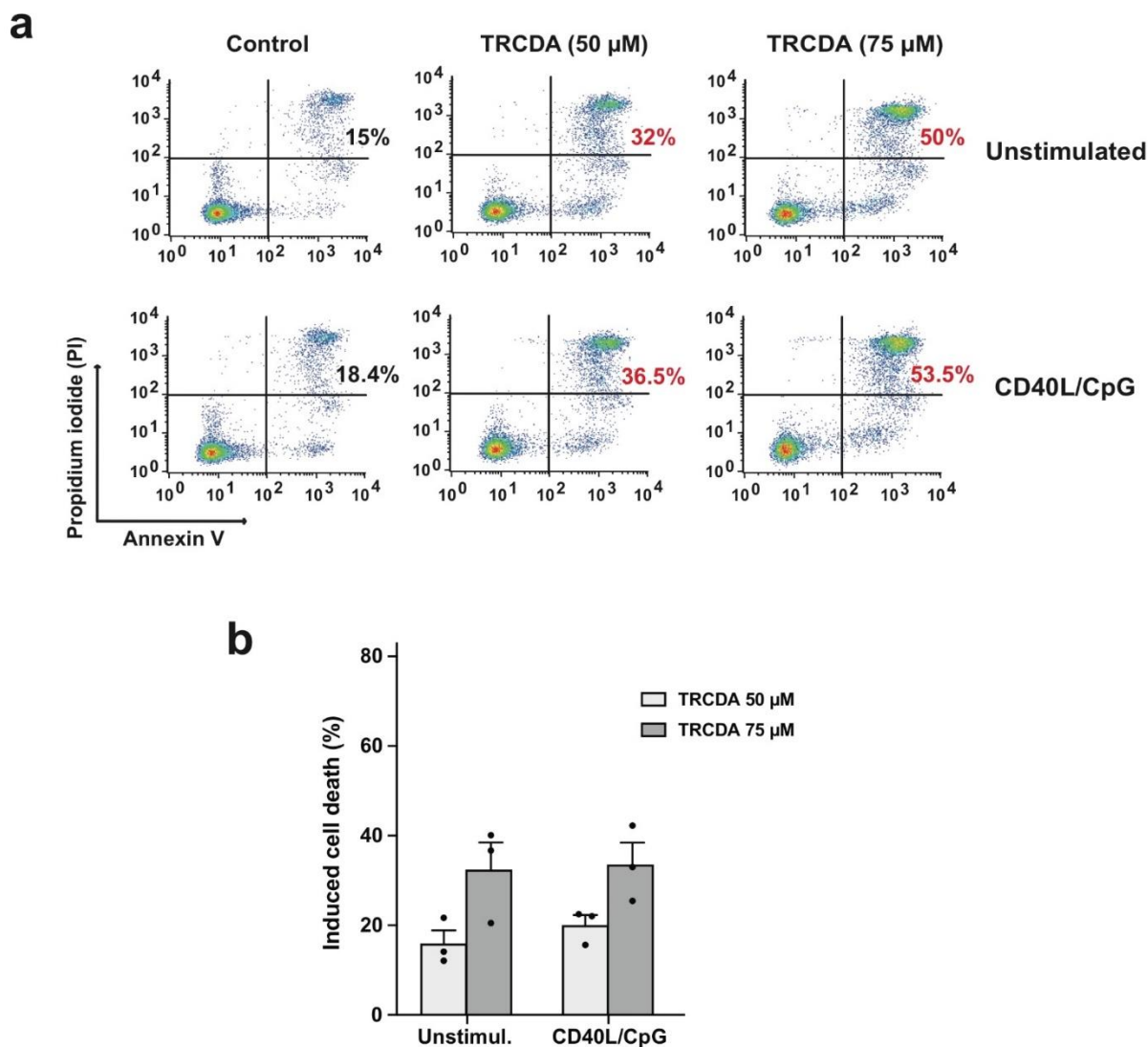


Supplementary Figure 4. pFAO contributes to OSU-CLL metabolism. (a) Cell death was determined through annexin-V/PI co-labeling in the OSU-CLL cell line after treatment with the indicated concentrations of perhexiline. The percentage of annexin-V positive cells was quantified and plotted (n=3). **(b)** C2-, C4-, and C6-carnitines measured using mass

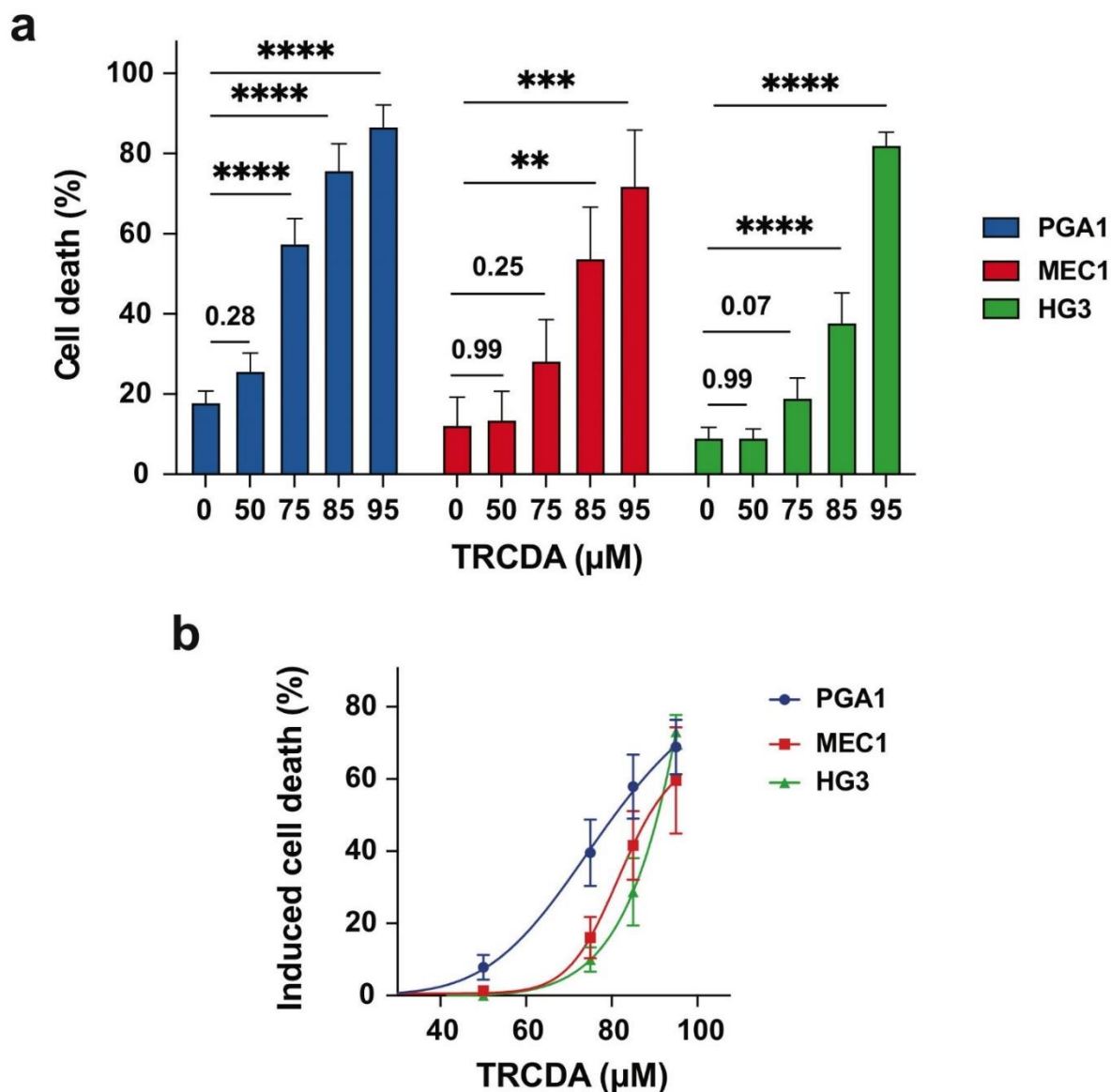
spectrometry in primary CLL cells (CLL) vs. the OSU-CLL cell line (OSU). Metabolite levels are represented as the area under the peak (n=3). **(c)** ATP levels recorded in control (WT) OSU-CLL cells and shACOX1 OSU-CLL cells (shACOX1) treated for 24 h with TRCDA (50 μ M and 75 μ M). The results are expressed as the percentage of ATP relative to nontreated (WT) or shACOX1 cells (set to 100%) (n=4). **(d)** Cell death was measured through annexin-V/PI co-labeling of control (WT) OSU-CLL cells and shACOX1 OSU-CLL cells (shACOX1) treated for 24 h with TRCDA (50 to 85 μ M). The percentages of annexin-V positive cells were recorded and (after accounting for spontaneous death) expressed as the percentage of induced cell death (n=4). **(e)** The loss of $\Delta\Psi_m$ was measured using TMRE staining in the OSU-CLL cell line treated for 24 h with TRCDA (50 μ M or 75 μ M) or not treated (0 μ M). The recorded mean fluorescence intensity is presented as the ratio between treated and nontreated cells (n=3). **(f)** Cell death was determined through annexin-V/PI co-labeling in the OSU-CLL cell line, which was pre-incubated (or not) with QVD (10 μ M, 30 min) and then treated with TRCDA (50 μ M or 75 μ M) or not (0 μ M). Annexin-V-positive cells were quantified and expressed as a percentage (n=4). **(g)** Caspase-3 activation was analyzed by flow cytometry with a CaspGLOW fluorescein active caspase-3 staining kit. The OSU-CLL cell line was treated for 24 h with TRCDA (50 μ M or 75 μ M) or not treated (0 μ M). The mean fluorescence intensity recorded in two independent experiments was plotted as the ratio between treated and nontreated cells (nontreated=1). For the results in the Figure, statistical significance was calculated using a one-way analysis of variance (ANOVA) in (e) and (g) and Student's t-test in (d) and (f). The bars correspond to the mean \pm SD. (c) 50 μ M p=0.0066, 75 μ M p=0.02. (d) 75 μ M p=0.038, 85 μ M p=0.003 (e) p<0.0001. (f) 50 μ M p=0.0008, 75 μ M p=0.0049. (g) 75 μ M p=0.02.



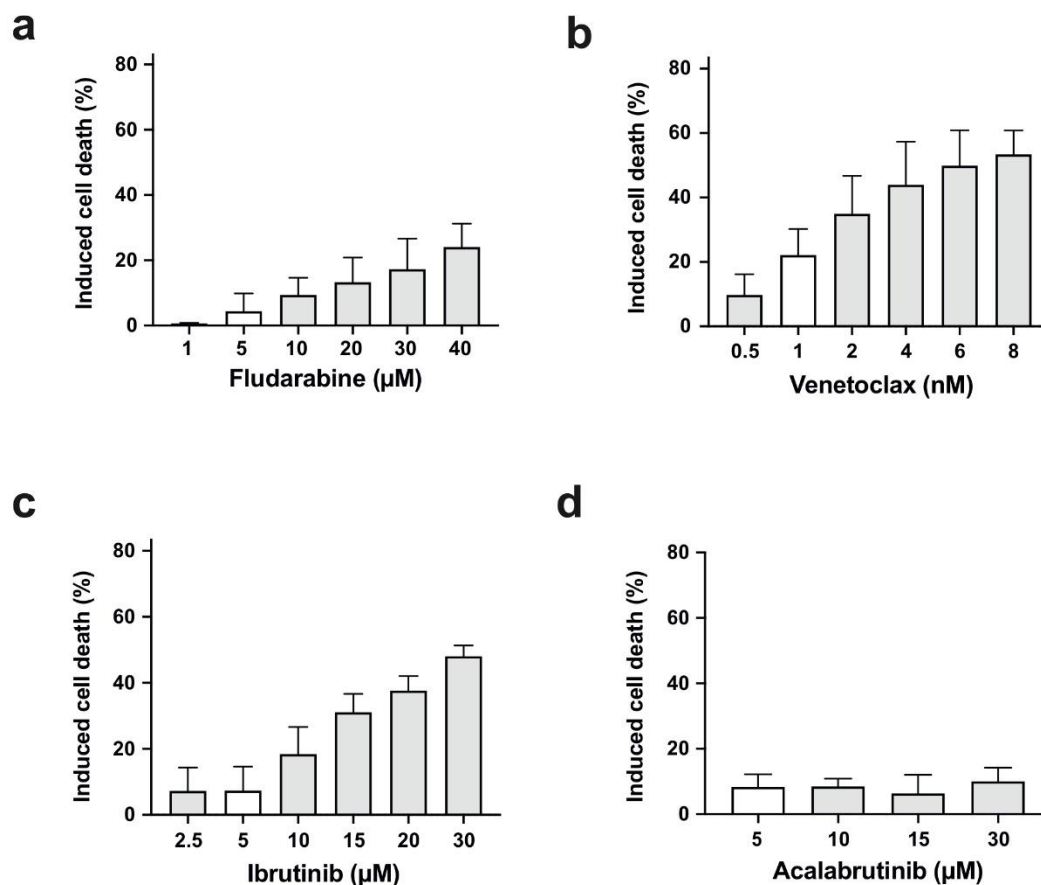
Supplementary Figure 5. TRCDA remains an effective means of inducing death in CLL after BCR triggering by soluble α -IgM. (a) Primary CLL cells ($n=9$) were stimulated or not during 24 h with 20 μ g/mL soluble α -IgM, then incubated for 24 h with TRCDA (50 μ M and 75 μ M) and cell death was measured by annexin-V/PI co-labeling. Representative flow cytometry plots show the percentage of annexin-V positive cells. (b) The percentage of spontaneous cell death (annexin-V-positive cells) in unstimulated control cells (CLL) and soluble α -IgM-stimulated control cells (CLL + α -IgM) from the experiment described in (a) was quantified and plotted as a histogram. Note that, under our experimental conditions, BCR stimulation by soluble α -IgM is not pro-apoptotic per se. Statistical significance was calculated by the student t test. Bars represent mean \pm SEM.



Supplementary Figure 6. TRCDA remains an effective means of induced death in CLL in a pro-proliferative environment. (a) Primary CLL cells ($n=3$) were stimulated or not with 25 ng/mL CD40L and 50 ng/mL CpG for 24 h, then incubated with TRCDA (50 μ M and 75 μ M) for 24 h and cell death was measured by annexin-V/PI co-labeling. Representative flow cytometry plots show the percentage of annexin-V positive cells. (b) After accounting for spontaneous cell death, the percentage of annexin-V-positive cells in TRCDA-treated unstimulated (Unstimul.) and CD40L/CpG stimulated cells from the experiment described in (a) was quantified and graphed as induced cell death. ACOX1/pFAO inhibition by TRCDA remains an effective means of inducing apoptosis after CD40L/CpG pro-proliferative stimulation.



Supplementary Figure 7. Inhibition of ACOX1/pFAO by TRCDA induces cell death in the PGA1, MEC1 and HG3 CLL cell lines. (a) PGA1, MEC1 and HG3 cell lines were treated with TRCDA at the indicated concentrations for 24 h and cell death was measured by annexin-V/PI co-labeling. The percentages of annexin-V positive cells were recorded and graphed as a histogram. (b) After accounting for spontaneous cell death, the percentage of annexin-V-positive cells from the experiment described in (a) was recorded and plotted as induced cell death. Statistical significance was calculated by the one-way analysis of variance (ANOVA). Bars represent mean \pm SD (n=3). PGA1 $p < 0.0001$. MEC1 85 μM $p = 0.0026$, 95 μM $p = 0.0002$. HG3 $p < 0.0001$.



Supplementary Figure 8. *In vitro* cytotoxicity of fludarabine, venetoclax, ibrutinib, and acalabrutinib in primary CLL cells. Cell death (as measured using annexin-V/PI co-labeling) in CLL primary cells treated for 24 h with the indicated concentrations of fludarabine (a), venetoclax (b), ibrutinib (c), or acalabrutinib (d). After accounting for spontaneous apoptosis, the percentage of annexin-V positive cells was quantified and plotted. The bars correspond to the mean \pm SEM (n=2).

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Fig. 3g

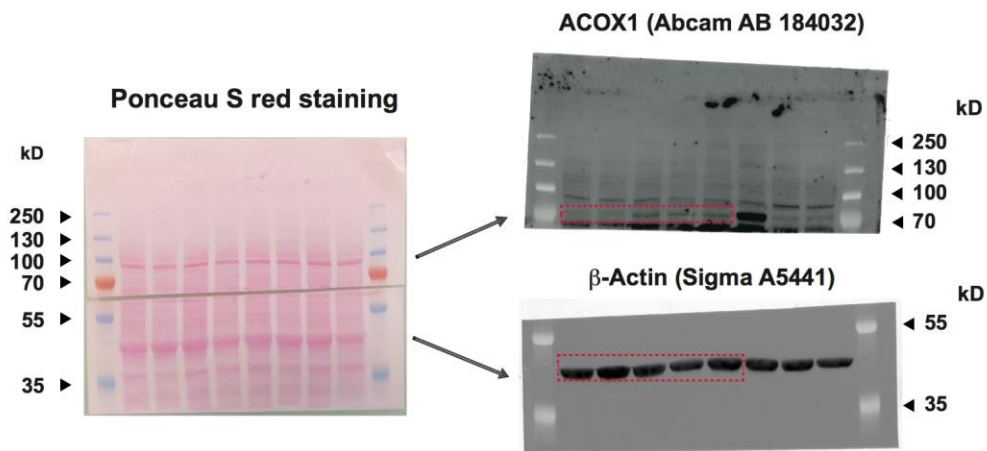


Fig. 4a

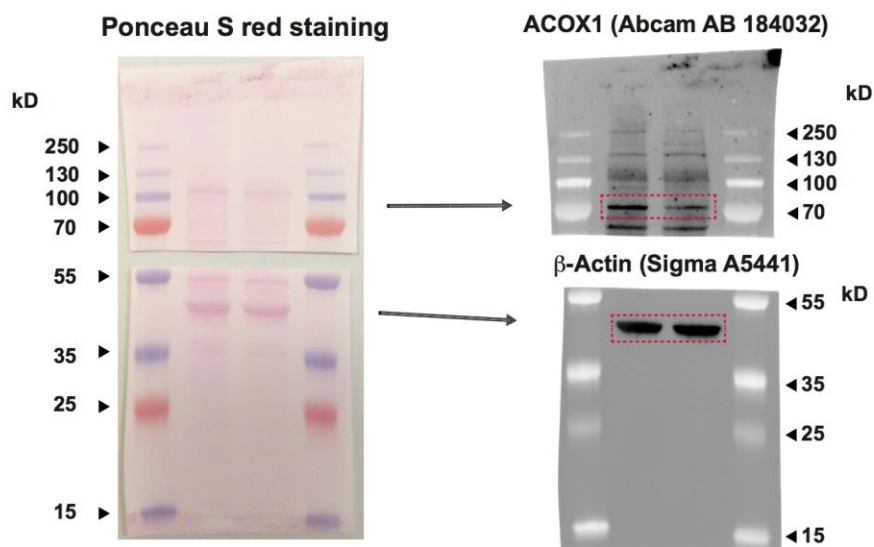
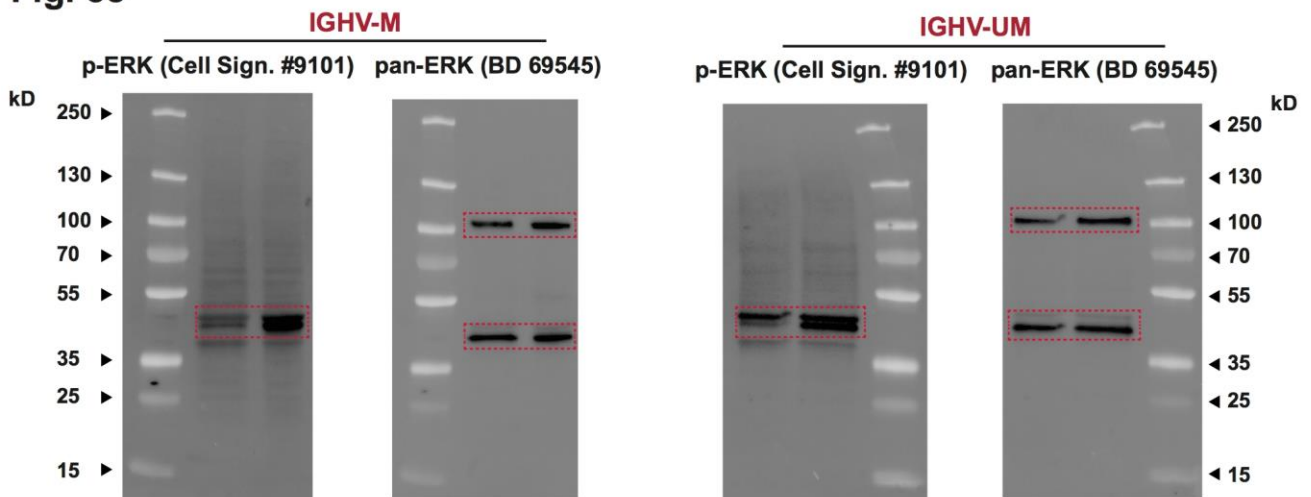


Fig. 5e



Discussion

Discussion

CLL: a heterogeneous incurable disease

CLL, the most common leukemia among adults in Western countries, is a highly heterogeneous disease: i) biologically, it is characterized by genetic variability between patients, which has a direct impact on the course of the disease; ii) clinically, it manifests differently from patient to patient. While some cases are asymptomatic and discovered by chance during routine blood tests, others display an aggressive course and require treatment.

The conventional chemoimmunotherapy FCR remains a first-line treatment option for fit-eligible patients in the absence of TP53 inactivation. However, this type of treatment has its drawbacks: it can be harsh on CLL patients, a disease of the elderly, and therefore affects the patients' general well-being and overall survival. Moreover, relapses after FCR treatment frequently occur, presenting a significant challenge that requires an adjustment to the treatment plan.

Fortunately, scientific and technological advancements (Omics, etc.) have led to a better understanding of the biology of the disease revolutionizing therapeutic approaches against CLL. Targeted therapies were developed offering hope for a more effective and better-tolerated treatment. These new developments propose a more personalized approach based on the patient's genetic profile and general well-being. For instance, BCR inhibitors such as ibrutinib (and derivatives) became a treatment option for CLL patients physically ineligible for chemoimmunotherapy, those with *TP53* aberrations, or FCR-relapsed cases. Venetoclax, a BCL-2 inhibitor, also became another option in case of intolerance to- or relapse after- ibrutinib treatment.

Despite the evident efficacy of these new drugs, resistance mechanisms can occur rendering CLL to date an incurable disease. Understanding the causes of these resistance mechanisms, as well as identifying new targets essential to the development and survival of CLL, may pave the way to better-tailored targeted therapies and improved quality of life for CLL patients.

Exploring cancer cell metabolism

Metabolism is the basis of cellular activity. From generating energy to providing building blocks for cellular structures, this complex network of reactions is essential for maintaining normal cellular function and the health and well-being of the organism as a whole.

Metabolic reprogramming is a hallmark of cancer. As described in the introduction section of this manuscript, tumor cells rewire their metabolism to meet their energy demand for growth, proliferation, and survival. Exploring metabolic rewiring in cancer does not only provide a fundamental understanding of the cell's biochemical characteristics but it also provides new opportunities for novel targeted treatments.

Several drugs targeting metabolic reprogramming demonstrated efficiency in reducing tumor cell growth. Depending on the metabolic rewiring observed in the tumor type, multiple metabolic inhibitors are currently being tested in clinical trials and others are even clinically approved for use³⁵³. For example, in 2018, the FDA approved ivosidenib, an inhibitor of isocitrate dehydrogenase 1 (IDH1) for relapsed or refractory acute myeloid leukemia (AML) presenting an IDH1 gain-of-function mutation. This enzyme mutation causes an abnormal production of 2-Hydroxyglutarate (2-HG), an oncometabolite that disrupts the epigenetic landscape of the cell, which impairs the normal differentiation process, a hallmark of AML³⁵⁴.

Moreover, because the metabolism relies on macronutrients provided by the diet, metabolic-reprogramming-related treatment can include dietary modulation. Nutritional modification based on the tumor cells' demand for nutrients might provide support for cancer management and treatment³⁵³. For example, glucose restriction by a low carbohydrate diet was investigated in many preclinical studies and provided promising evidence of tumor-suppressing effects on several types of cancers such as glioblastoma³⁵⁵.

Regardless of these promising anti-metabolic-reprogramming treatment options, cancer cells can exhibit metabolic plasticity which helps them adapt to this stress. Blocking a metabolic pathway or depriving the cell of an essential substrate could theoretically result in a switch to another metabolic pathway to compensate for and overcome these altered conditions. This is why, combinational approaches could be much more effective. Thus, by taking advantage of the cancer cell metabolic vulnerabilities, metabolic inhibitors can provide a synergistic effect for already used drugs and improve their therapeutic outcome³⁵³.

Metabolic reprogramming in CLL

Over the past few years, CLL cells metabolism has become an interesting field of study among researchers. As detailed previously in the introduction, CLL metabolism seems to be heterogeneous as is the disease itself. CLL cells metabolic preferences may change according to the cytogenetic variations, or the disease progression. To complicate matters, a metabolic switch may occur in response to the infiltration of cells into lymphoid tissue, or even in response to treatment. Metabolic substrate preferences also depend on their use, as both anabolic and catabolic reactions can be fuelled by specific substrates to benefit tumor cells. Therefore, the feasibility of targeting metabolic reprogramming as an approach against CLL becomes more challenging.

To this end, I sought to identify a metabolic signature common to CLL. Specifically, I focused on identifying a metabolic response that would fuel ATP production because of its essential role in providing the energy required for cell function and thus survival. To achieve this, I followed a five-step approach:

1. Identify the main source of ATP generation within CLL cells.
2. Determine if there is a CLL specific metabolic pathway that fuels this ATP production.
3. Verify the generality (or not) of this identified metabolic pathway in a large panel of CLL primary cells with variable cytogenetic abnormalities.
4. Check the relevance of this pathway when mimicking the interactions with the microenvironment.
5. Test the possibility of a combinational metabolic/targeted therapy approach.

By initiating this multistep plan, we came across an underexplored metabolic pathway that involves peroxisomes. Peroxisomes, involved in many cellular metabolic reactions, have received limited exploration and are usually overlooked. So, with a few exceptions, little is known about the role of peroxisomes in cancer cells.

Overall, my thesis work provides clear evidence of the peroxisomal fatty acid β -oxidation (pFAO) implication in CLL metabolic reprogramming and demonstrates the crucial role of this pathway, mediated by ACOX1, in fuelling mitochondrial ATP production. Hence, I give a first step in the use of this metabolic vulnerability as a potential therapeutic target. Finally, this thesis does not only provide evidence on a potential metabolic signature for CLL cells, but it also supports many ideas concerning pFAO in general.

I. Identification of ATP source in CLL

ATP is the energy currency of the cell. It stores and transfers energy upon requirement. Because of its crucial role in diverse cellular metabolic functions, the first step in our study was to identify its main source in CLL cells. When looking for a metabolic signature of CLL B cells, it was rational to compare metabolic differences between tumor B cells and B cells from healthy donors. My results confirmed the reliability of CLL cells on mitochondrial respiration but not on aerobic glycolysis for ATP generation, an observation that was already suggested in the literature¹³⁴. For instance, CLL cells exhibit low glucose uptake compared to healthy B-cells, with a lower level of glucose metabolism intermediates, including pyruvate that can fuel the mitochondrial TCA cycle, and lactate, excluding the potential shift to the characteristic Warburg effect. While glucose metabolism can still be important for other CLL cell functions because of its implication in many other metabolic pathways such as the pentose phosphate pathway²¹⁷, I suggest its exclusion as a main contributor in ATP synthesis. Furthermore, when comparing CLL cells to B cells from healthy donors, I assessed both higher mitochondrial membrane potential and oxygen consumption rate in CLL cells, and that despite a similar number of mitochondria. Altogether, these initial results suggest that these malignant cells use mitochondria to generate ATP and they do so without using aerobic glycolysis.

II. Uncovering a CLL metabolic signature

To unravel a unique metabolic specificity that can serve as fuel for mitochondrial ATP generation, I initiated the search using metabolomics data obtained from primary CLL and healthy B cells. This technique aims to quantify metabolite abundance in a group of samples compared to another. Our cohort of CLL cells included patients with both mutated and unmutated IGHV status, from different Binet clinical stages, and with diverse cytogenetic profiles. This analysis helped us come up with three conclusions:

- i. Glucose metabolism (including classical and aerobic glycolysis) is irrelevant in CLL and is instead present in healthy B cells, corroborating the idea discussed in the previous paragraph.
- ii. The metabolic profile is very heterogeneous among patients confirming what was previously published¹⁵⁷.
- iii. A significant abundance of some metabolites implicated in fatty acid oxidation (FAO) is observed in CLL cells compared with healthy B lymphocytes, independent of CLL genetic specificities.

In fact among the 115 metabolites detected by mass spectrometry, we found a small number of metabolites significantly abundant in CLL compared to healthy B lymphocytes, with no significant difference according to Binet stage, IGHV mutational status, or karyotype complexity (**Figure 31**). Top CLL metabolites included **carnitine**, a key player essential for FAs transport across the mitochondrial membrane, as well as several **long and short-chain acylcarnitines** of different origins, produced by the conjugation of fatty acyl-CoA to carnitines. We therefore hypothesized a dual role for mitochondrial and peroxisomal FAO (mFAO and pFAO, respectively).

Another highly abundant metabolite in CLL, **betaine**, supports our hypothesis. Indeed, betaine, a glycine derivative, is five times more abundant in CLL B cells than in normal B cells. This nutrient, highly present in the human blood plasma, can be synthesized in the liver but it is also very rich in many dietary sources such as whole grain products³⁵⁶. In mammals, betaine supplementation can have a direct impact on promoting FAO. It increases carnitine levels in the muscle³⁵⁷, as well as the expression of LPL, overexpressed in CLL²⁵³, and other fatty acid transporters such as CD36. Interestingly, it increases the expression of key enzymes that regulate both mitochondrial and peroxisomal FAO³⁵⁶, probably by a mechanism involving PPAR α ³⁵⁸.

After exploring metabolite abundance in CLL, gene set enrichment analysis (GSEA) from a public RNAseq database, together with a qPCR approach to our patient cohort, confirmed the overexpression of genes encoding enzymes involved in mitochondrial and peroxisomal FAO in CLL compared to healthy donor B cells. I was particularly interested in studying pFAO, a pathway usually neglected in cancer metabolism studies. The significant overexpression of genes implicated in FAs transport inside peroxisomes, pFAO, and peroxisomal end-products transport to mitochondria gave enough evidence to highlight a possible involvement of peroxisomes in CLL metabolic reprogramming.

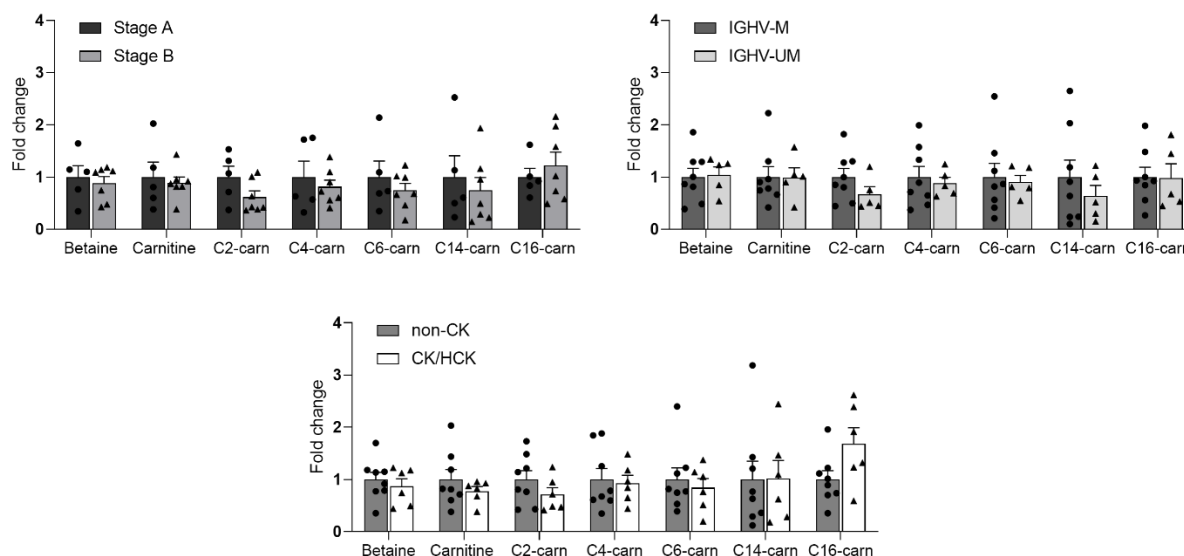


Figure 31: Comparison of metabolites fold changes (FC) according to Binet stages, IGHV mutational status, and the karyotype complexity. FC of metabolites measured by mass spectrometry are plotted as a histogram. The average of the results obtained in stage A, IGHV-M, and non-CK were set as 1. Statistical significance was calculated by the Student t-test, and the results, while not shown in the figure, indicated non-significance ($p > 0.05$) for all comparisons.

While our metabolomics analysis included the end-products of pFAO (short-chain acylcarnitines), it lacked the substrates metabolized by this pathway such as VLCFAs transported by ABCD2, a peroxisomal FAs transporter found overexpressed in our qPCR. Weber et al. demonstrated that among the three VLCFAs that undergo pFAO, (C22:0, C24:0, and C26:0), C26 levels were the higher in healthy CD19+ B lymphocytes³⁵⁹. Therefore, it is interesting to compare the levels of these FAs between CLL and healthy donors B cells. If a higher pFAO activity is characteristic of CLL cells, then lower levels of VLCFAs are expected compared to healthy B lymphocytes since these substrates are not accumulated but metabolized by the cells.

The origin of these FAs in CLL is another question to elucidate. FAs can be both exported from plasma or synthesized by the cell by a series of elongation reactions. For instance, inhibiting LPL, the enzyme that releases FAs from circulating TAG, by orlistat, induces cytotoxicity specifically in CLL cells²⁵⁵. While healthy human blood usually has lower VLCFAs levels than other FAs such as LCFAs, it can be interesting to also compare different FAs levels in the serum of healthy and CLL donors. In this way, we can determine if the origin of these FAs is external sources or generated from endogenous metabolic reactions.

In order to study in detail the pFAO metabolic pathway, I focused on **ACOX1**. I chose this peroxisomal enzyme for several main reasons:

- Both its mRNA and protein expression were upregulated in our cohort of primary CLL cells compared to B cells from healthy donors.
- This enzyme is peroxisome-specific and is rate-limiting in pFAO.
- It is associated with the β -oxidation of FAs transported by ABCD2, the latter is approximately five times more expressed in CLL compared to healthy B cells.
- It is regulated by PPAR α ³²¹, a metabolic regulator found overexpressed in circulating CLL, with a positive correlation to an advanced stage disease²⁷⁴.
- Its expression can be regulated by betaine³⁵⁶, a metabolite found in high abundance in our cohort of patients compared to healthy B lymphocytes.
- It can be pharmacologically inhibited, which can help us determine the importance of this pathway in CLL cells metabolism and survival.

III. *Pharmacological inhibition of ACOX1*

pFAO appears to be a specific metabolic pathway activated in CLL cells. To test this possibility, we sought to inhibit this pathway and then determine the metabolic and survival consequences for the tumor cell. Two available ACOX1 inhibitors exist: thioridazine hydrochloride is an antipsychotic that has an inhibitory effect on ACOX1³⁶⁰ but it has been described to have off-target effects³⁶¹ such as the inhibition of the PI3K/Akt/mTOR pathway³⁶². For this reason, I chose the second ACOX1 inhibitor described: 10,12-tricosadiynoic acid (TRCDA), a highly selective ACOX1 inhibitor with no identified off-target effects to date.

TRCDA is an acetylenic acid, characterized by two triple bonds (C \equiv C) and a carboxylic end (-COOH) in its 23 carbon atoms chain (**Figure 32**). This compound has a selective high affinity towards ACOX1, and no effect on other pFAO enzymes TRCDA inhibition is irreversible and rapid against recombinant ACOX1. Similarly, ACOX1 isolated from rat liver peroxisomes was rapidly and effectively inhibited by TRCDA³⁶³.

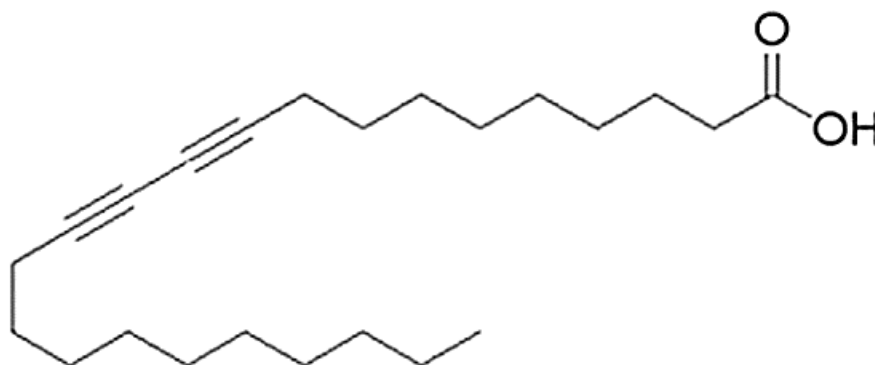


Figure 32: 10,12-tricosadiynoic acid (TRCDA) structure

Testing this inhibitor on primary CLL cells did not only provide us with evidence on the implication of this pathway in CLL, but also evidence in support of not yet well-defined aspects of pFAO:

- TRCDA treatment caused an instant decrease in H₂O₂ levels starting 5min of incubation. As H₂O₂ is a byproduct of pFAO, this result suggests a pFAO activity in CLL primary cells and at the same time proposes the inhibition of ACOX1.
- My data demonstrated that the short-chain acylcarnitines observed in abundance on CLL are of peroxisomal origin. As mentioned in the introduction, the breakdown of FAs by pFAO can go all the way down to generate a four-carbon chain FA. However, in literature, this was supported by evidence concerning the enzymatic activity of ACOX1 but never on the metabolites levels themselves. In our study, the dose-dependent decrease in these short-chained metabolites (C4 and C6 acylcarnitines) provides additional evidence of their generation by this pFAO metabolic pathway.
- The significant decrease in acetyl-carnitine (C2-carn) levels strengthens the hypothesis of a pFAO activity since each β -oxidation cycle generates an acetyl-CoA, transported to mitochondria to join the TCA cycle in the form of C2-carn.
- My results support recent observations demonstrating an interaction between peroxisomes and lipid droplets (LD). We hypothesize that LDs are able to sequester FAs not oxidized by peroxisomes. While other studies demonstrate a possible channeling of FAs from LD to peroxisomes³¹⁷, my work provides evidence that the interaction could occur in the opposite direction and we give two possible explanations:
 - i) Unmetabolized FAs are transferred from the inside of peroxisomes to LD by a direct interaction between the two organelles.
 - ii) The inhibition of pFAO indirectly blocks the entry of FAs in this organelle, and LDs are formed to sequester accumulated FAs in the cytosol to avoid lipotoxicity.

In both cases, the results of my manuscript provide proof of evidence that FAs are oxidized in peroxisomes of primary CLL cells and that the inhibition of this pathway leads to the accumulation of FAs in the form of LDs in the cell. Despite this observation,

a complementary metabolomics approach could further support it. A metabolomics approach including FAs metabolized by pFAO such as VLCFAs, should be done after TRCDA treatment. For instance, we must observe an accumulation of VLCFAs and maybe some LCFAs, which can also be metabolized in peroxisomes. This approach can also provide additional important evidence about which type of FAs is metabolized inside CLL cells peroxisomes.

- TRCDA treatment depleted ATP levels in a dose-dependent manner. This observation supports a peroxisomes-mitochondria connection, in which peroxisomes provide their pFAO end-products to fuel the TCA cycle and the mitochondrial OXPHOS. Consequently, this pathway appears to be part of the fuel for ATP generation in CLL cells. Interestingly, ATP levels remain unchanged when testing the drug on healthy B cells, providing further evidence to our data indicating that the pFAO pathway is only essential in CLL cells, while healthy B-cells most likely depend on glucose as a substrate for ATP generation.

Our data need to be complemented/verified by a metabolic workflow using ¹³C-labeled VLCFA, which will then be traced in CLL cells by mass spectrometry. Peroxisomal short-chain acylcarnitines and ¹³C-labeled TCA cycle intermediates will definitively confirm that part of mitochondrial energy metabolism in CLL cells is powered by peroxisomal sources.

As this metabolic pathway appears to be specific to CLL cells, we raised the following hypothesis: Blocking this "vital" metabolic pathway affects CLL cell survival. This possibility was confirmed by TRCDA dose-dependent induced mortality in the CLL cells. Data published on mFAO inhibition (by inhibiting the carnitine shuttle CPT1) in CLL indirectly support our findings. While ST1326, significantly reduced viability in both quiescent and activated CLL cells²⁶³, another widely used inhibitor, etomoxir, fails to induce similar cytotoxicity when used at doses that do not induce off-target effects (<100μM)^{265,364} raising doubts about the relevance of the cytotoxicity induced in CLL by CPT1/mFAO inhibition. Why does ST1326 induce cytotoxicity in CLL and etomoxir does not? Indeed, ST1326 does not only inhibit CPT1 but also CACT³⁶⁵, a protein that mediates the entry of pFAO end-products into mitochondria. Consequently, the cytotoxicity observed in CLL after ST1326 treatments could be linked to the double blockade of mFAO (via CPT1 inhibition) and pFAO (via CACT blockade). The blockade of CPT1 and CPT2 by perhexiline also confirms the relevance of pFAO as a "vital"

metabolic pathway in CLL and, consequently, as a potential therapeutic target. Indeed, like CACT, CPT2 also contributes to the mitochondrial transfer of pFAO end products into mitochondria. As shown in our article and previously published by others, inhibition of CPT1/CPT2 induces strong cytotoxicity in CLL²⁶².

What is the advantage of specifically inhibiting pFAO (ACOX1) versus inhibiting mFAO (CPT1) or mFAO and pFAO at the same time (CPT1 and CACT or CPT1 and CPT2)? The answer lies in the relevance of mFAO to other cells of the immune system. Indeed, T-cells and NK cells appear to primarily use CPT1-mediated FAO and glycolysis to fuel mitochondrial OXPHOS^{366,367}. Thus, the inhibition of mFAO could certainly provoke alteration in the immune system.

Another point to consider is the overexpression of CPT1 in CLL, which suggests a key role for the protein in this leukemia. However, this prompts the inquiry into the little cytotoxicity caused by etomoxir, while CPT1A expression is found overexpressed as previously published²⁶² and in our cohort of CLL, along those implicated in pFAO. If this is true, why the CPT1-specific inhibition by etomoxir is irrelevant in CLL? A possible explanation that I propose is that pFAO causes a negative retrocontrol on the carnitine shuttle. CPT1 is naturally inhibited by malonyl-coA, produced in the cytoplasm from acetyl-coA³⁶⁸. It is therefore possible that the acetyl-coA produced by pFAO contributes to the synthesis of malonyl-CoA, which in turn inhibits the uptake of long-chain fatty acids into mitochondria. It is, therefore, possible that the acetyl-coA produced from pFAO contributes to the synthesis of malonyl-CoA and therefore controls the mitochondrial long-chain fatty acid uptake. This hypothesis seems to be corroborated by our metabolomic data. We observed a significant accumulation of long-chain C14 and C16 acylcarnitines upon inhibition of pFAO by TRCDA (**Figure 33**).

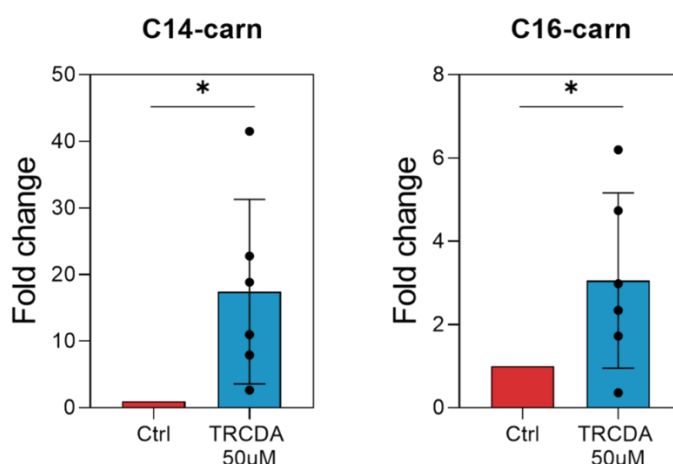


Figure 33: Comparison of C14 and C16-carnitines intermediates in CLL primary cells after 24h of TRCDA 50uM treatment. FC of metabolites measured by mass spectrometry are plotted and the control group was set as 1. Statistical significance was calculated by the student t-test.

Our results also indicate that irrespective of the genetic heterogeneity of CLL B cells, TRCDA induces significant and similar levels of cytotoxicity. This includes CLL cells with del11q, which appears to be dependent on glutamine metabolism. In these cells, however, the involvement of glutamine metabolism in OXPHOS and ATP synthesis has not been demonstrated²¹³. It is therefore possible that glutamine metabolism is involved in other survival functions in del11q CLL cells. In any case, my results allow me to propose that the pFAO pathway is a common metabolic hallmark in CLL, overcoming the heterogeneity characteristic of this disease.

Additionally, the lack of TRCDA effect on B cells from healthy donors, seems to corroborate that pFAO is a metabolic specificity of the tumor CLL cells.

Interestingly enough, TRCDA selective targeting of pFAO also spared key non-tumorous immune cells of the CLL patients (e.g., T- and NK-cells). This gives additional interest in the modulation/inhibition of this metabolic pathway in CLL. Of note the T cells of the CLL patients have poor functionality²⁸. It therefore seems appropriate that any potential treatment for CLL should spare these cells to avoid further impact on immune function. The same applies to NK cells, a key population in the clearance of dying cells.

IV. ACOX1 inhibition vs. downmodulation: questioning the cells' plasticity

One of the most interesting aspects of the discovery of the relevance of pFAO in CLL is that the tumor CLL B cells do not seem to be able to undergo a metabolic reorganization when ACOX1 is inhibited. As a result, these malignant cells trigger a programmed cell death process. Plasticity is the cell's capacity to change its identity in response to extrinsic change and is a hallmark of cancer cells³⁶⁹. For example, upon any stress encountered, cells have the capacity to change their functional characteristic and gain new ones in order to bypass this stress³⁷⁰. The same applies in response to metabolic pressure. For example, a substrate switch enables cancer cells to adapt to challenging metabolic conditions and/or substrate deprivation³⁷¹.

In our case, a compensatory mechanism was observed when we genetically downmodulated ACOX1 in the OSU-CLL cell line, in which we observed a switch to aerobic glycolysis and glutamine metabolism. By increasing their glucose metabolism, the cells are switching back to a "healthy" B-cell metabolism phenotype. Also interesting about this shRNA ACOX1 regulation is the significant reduction in TCA cycle intermediates (**Figure 34**), validating the importance of pFAO in fuelling the mitochondrial metabolism and respiratory chain. The switch to aerobic glycolysis comes to the rescue of the cells where ATP is generated

outside mitochondria, explaining the reduced effect of TRCDA on ATP levels in the CLL shACOX1 modulated cell line.

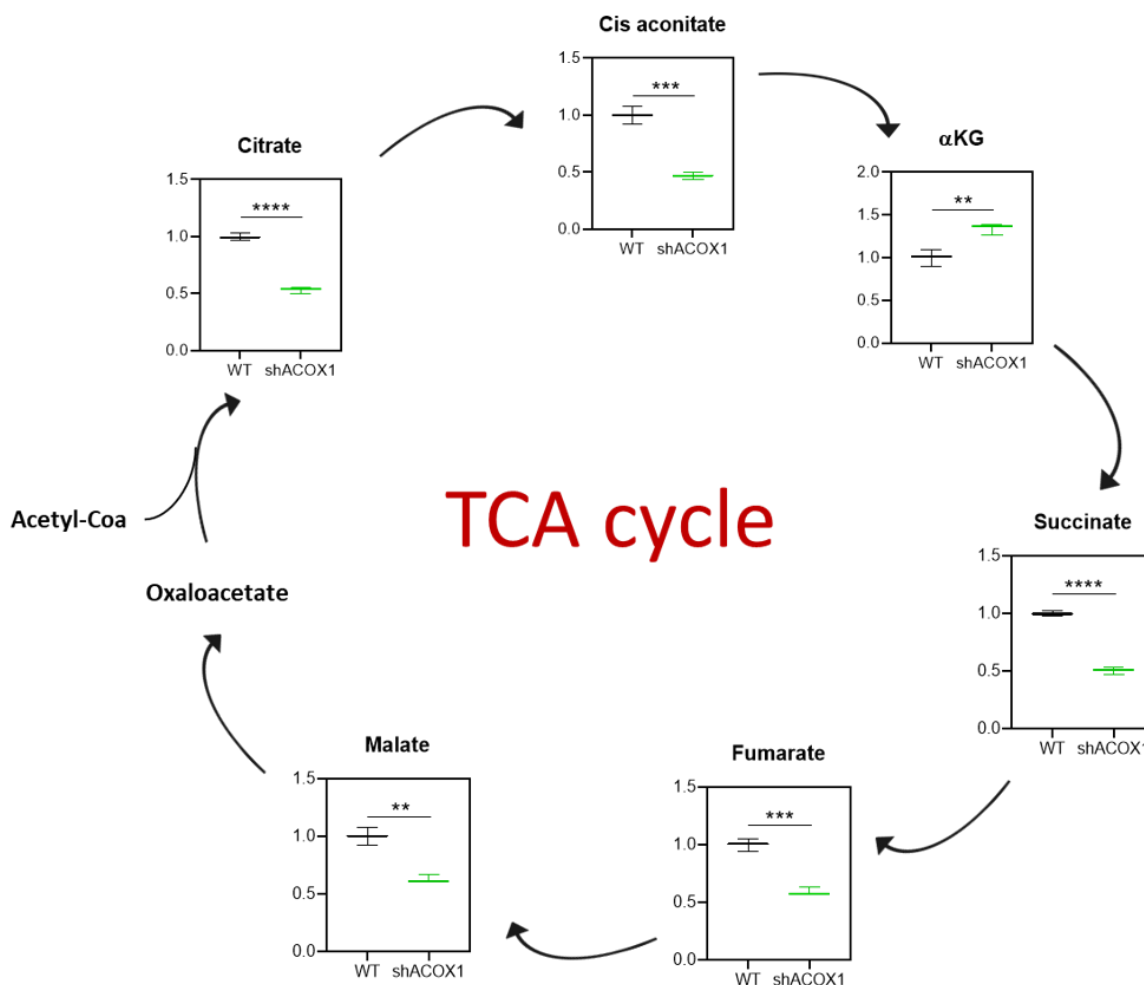


Figure 34: Comparison of TCA cycle intermediates in OSU-CLL shACOX1 compared to OSU-CLL WT. FC of metabolites measured by mass spectrometry are plotted. The average of the results obtained in OSU WT was set as 1. Statistical significance was calculated by the student t-test.

With regard to primary CLL cells, we observed that after treatment with 50 μ M TRCDA (a concentration that induces a low percentage of mortality), these lymphocytes showed increased lactate levels suggesting an attempt to compensate pFAO inhibition (**Figure 35**). However, given that ATP levels in the cells are significantly reduced at this dose, it seems that this attempt is not sufficient to counterbalance the ATP diminution associated with the pFAO blockade. Of note that, mitochondrial disruption and cell death became more pronounced with increasing doses of TRCDA. This suggests the inability of cells to withstand this metabolic alteration affecting pFAO.

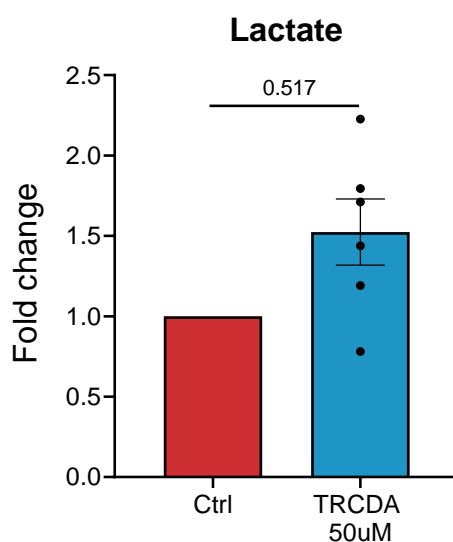


Figure 35: Lactate level variation with TRCDA treatment on CLL primary cells. FC of the metabolite measured by mass spectrometry is plotted. The average of the results obtained in OSU WT was set as 1. Each dot represents a replicate. Statistical significance was calculated by the student t-test.

Taking all our results together, I suggest that the metabolic imbalance induced by TRCDA in the CLL cells directly affects mitochondrial respiration/function. Limited mitochondrial supply after pFAO inhibition results in reduced ATP synthesis, accompanied by a decrease in mitochondrial transmembrane potential and overproduction of ROS. These cellular dysfunctions enable the cell to trigger a caspase-dependent programmed cell death process that leads the cell to death. As described by Zeng et al., TRCDA is a suicide substrate for ACOX1³⁶³. In our study, I propose TRCDA as a suicide substrate for CLL primary cells.

V. *pFAO under microenvironmental stimuli*

Until now, we have been able to provide evidence of the relevance of ACOX1-mediated pFAO in CLL metabolism, and that irrespective of the presence or absence of aggressive cytogenetic alterations, karyotype complexity, or IGHV mutational status. However, this observation is made on circulating/quiescent CLL primary cells. We therefore wanted to check whether the dependence on this metabolic pathway is still applicable when CLL cells are activated.

1. BCR triggering

The first thing we were interested in testing was the effect of BCR stimulation on pFAO. As detailed in the introduction, CLL cell's interaction with some microenvironment surrounding cells can trigger BCR signalling. To provide some evidence on the effect of this

stimulation on pFAO, we checked both the effect of BCR triggering on peroxisomal metabolites levels as well as the effect of TRCDA on CLL viability.

As described in my lead paper, BCR triggering by anti-IgM did not affect peroxisomal acylcarnitine levels and TRCDA was still able to induce programmed cell death. Thus, it seems that BCR stimulation did not modify the ACOX1-mediated pFAO metabolic pathway. Chen et al. have previously demonstrated an increase in mitochondrial respiration in BCR-stimulated cells possibly due to an increase in glucose metabolism¹⁵⁷. My work suggests that, despite a potential metabolic shift that may occur in triggering the BCR, CLL cells continue to use mitochondria to generate ATP and pFAO still plays a major role in feeding mitochondria.

2. CLL- M2 stromal cells coculture

A second approach to assess the role of pFAO in CLL has been the coculture of primary CLL cells with a stromal cell line (M2-10B4)³⁷², designed to produce stimulatory factors and cytokines³⁷³. The aim here is to mimic the microenvironmental interactions that protect the cell and lead to CLL drug resistance. Under these conditions, the viability of primary cells, usually limited in vitro, was improved, corroborating the expected action of this stromal cell line. It's first important to mention the limitations of our approach:

1. Because we used a murine cell line, our results need to be corroborated with a human stromal cell line (work in progress in our lab with the HS-5 cell line).
2. The number of samples analyzed in coculture during my PhD was few in number. This limits the possibility of drawing a general conclusion about the impact of this approach on pFAO metabolism. Testing a wider panel of CLL can provide information about the impact or not, of cytogenetic alterations, IGHV mutational status, and disease aggressiveness, on the effect of the coculture on metabolism.

Taking these limitations into account, our results clearly show a significant increase in short-chain acylcarnitines in CLL cells cocultivated with stromal cells. This increase could signify higher pFAO activity in these conditions. This hypothesis seems supported by a previous study corroborating a higher mitochondrial respiratory activity in CLL cells cocultured with the M2-10B4 stromal cell line¹⁵⁶.

Overall, we speculate that pFAO remains a relevant metabolic pathway in BCR-activated CLL cells or CLL cells receiving prosurvival signals from the stromal cells.

To complete this part of the study, we are currently testing the effect on pFAO of CpG/IL2-induced CLL cell proliferation. Co-cultures with the HS5 human stromal cell line, NK.Tert stromal cells or nurse cells could also be performed to gain a broader view of the impact of the microenvironment on this metabolic pathway

Unraveling a potential combinational therapeutic option

In 1965, Frei et al. introduced the first-ever combinational therapy for children with acute leukemia³⁷⁴. Since then, the interest in combinatory therapeutic approaches has grown as a successful cancer treatment strategy³⁷⁵. Thanks to the advances in cancer biology research, selective targeted therapies can be used in combination to achieve better therapeutic outcomes, as well as a reduction in drug resistance and undesirable toxic effects³⁷⁶. In CLL, targeted therapy combinations such as ibrutinib (BTK inhibitor) and venetoclax (bcl-2 inhibitor) are largely investigated as a therapeutic strategy leading to a complete remission as well as an undetectable minimal residual disease (MRD)³⁷⁷. Many in vitro studies are taking into account ibrutinib in combination with metabolic inhibitors such as 2-DG (glycolysis inhibitor)³⁷⁸ and etomoxir (CPT1 inhibitor)²⁶⁵.

Based on my results, ACOX1-mediated pFAO can be considered a metabolic vulnerability in CLL cells. Therefore, in the latter part of my thesis, I hypothesized that the effect of drugs already used to treat CLL can be enhanced by simultaneously limiting the cell's energy supply by pFAO inhibition. So, I tested the effect of a combination therapy involving TRCDA and some therapeutic drugs currently used in CLL clinical treatment.

Our results showed a synergistic cytotoxic effect when TRCDA was combined with BTK inhibitors (BTKi) such as ibrutinib or acalabrutinib at low in vitro doses. The combination with the conventional therapeutic agent, fludarabine, was simply additive. The interest of this combination of TRCDA/BTKi is that the cytotoxic effect was observed at lower drug concentrations, which may ultimately spare normal cells while showing its toxic effects on malignant CLL cells.

Interestingly, the synergistic cytotoxic effect of the TRCDA/ibrutinib combination remains when cells are BCR-activated with anti-IgM antibody, whereas it becomes additive with acalabrutinib. One possible explanation could be the different cell death effect of ibrutinib and acalabrutinib in vitro. In this way, in our in vitro tests, acalabrutinib alone appears less effective in inducing death in CLL than ibrutinib. Thus, further evaluation with acalabrutinib,

at different doses, is needed to understand the different behavior of these two BTKi in a combinatorial approach with TRCDA.

Finally, it should be noted that the venetoclax/TRCDA combination has not produced positive results. On the contrary, this combination generates antagonistic results (less induction of cell death with the combination of the two drugs than the cytotoxicity induced by each drug alone). Why is this so? It was previously proposed that venetoclax induces a shift toward glutamine metabolism¹⁵⁷. The antagonistic effect observed could therefore be due to the activation of this metabolic pathway (glutaminolysis) as a source for energy production, counteracting the pro-apoptotic effect of TRCDA and contributing to cell survival. Another hypothesis could be that the switch to glutamine metabolism induced by venetoclax increases the synthesis of GSH, which has an antioxidant effect against the ROS generated by TRCDA, reducing their damaging effect and explaining the antagonistic effect observed. In this case, the cytotoxic effect of pFAO inhibition would be caused by a dual effect: inhibition of ATP generation and production of cellular damage by ROS generated by the associated mitochondrial dysfunction. Further analyses involving combined treatment of CLL cells with TRCDA and antioxidants would clarify this question.

Overall, the data provided in this thesis provide, in my opinion, substantial evidence for the role of ACOX1-mediated pFAO in CLL. At the same time, my work opens up new perspectives on a metabolic specificity of CLL B cells that could be targeted alone or as part of combination therapy. Although pFAO inhibition appears as a potential therapeutic option, little is known about its effect *in vivo*. Therefore, whether using TRCDA or other pFAO inhibitors, this metabolic specificity deserves further investigation. Future studies are also needed to confirm the potential therapeutic interest of peroxisomes, organelles that are still little considered in the cancer research field.

Conclusions & Perspectives

Conclusions and perspectives

This thesis highlights a potential role of fatty acid oxidation mediated by peroxisomal ACOX1 as a metabolic signature of CLL cells. Peroxisomal metabolism and its implication in cancer is an underexplored field, with only a handful of studies addressing the contribution of this organelle and its metabolic pathways to tumor cell survival. Here we provide evidence for the contribution of ACOX1-mediated pFAO to powering the mitochondrial ATP generation process in CLL. It appears to be an essential mechanism for supplying the energy required to carry out the functions of these tumor cells. Unraveling metabolic vulnerabilities of malignant cells may engender innovative therapeutic strategies that specifically target and eliminate these cells. One of the strengths of this thesis is that ACOX1-mediated pFAO is a metabolic vulnerability characteristic of CLL, irrespective of the disease stage, the IGHV mutational status, or the genetic profile. In a disease characterized by its heterogeneity which affects its treatment options, we suggest ACOX1-mediated pFAO as a common metabolic signature of CLL. Therefore, targeting this metabolic pathway may be an effective therapeutic approach against this leukemia.

TRCDA, a highly selective ACOX1 inhibitor, induced a caspase-dependent programmed cell death of CLL primary cells, sparing healthy B lymphocytes, T cells, and NK cells. This reveals the exclusivity of this metabolic pathway for CLL B cells. Our work revealed that CD5⁻ B cells have glucose-mediated metabolism whereas CD5⁺ B cells from CLL cells are able to revert from FAO to glucose metabolism by down-regulating the rate-limiting pFAO enzyme ACOX1, but are unable to reprogram their metabolism with complete pharmacological inhibition of this enzyme. This provides additional evidence of the importance of the pFAO pathway in CLL. While our studies were done *in vitro* on CLL primary cells, it would be interesting to test the effect of TRCDA on tumor growth and progression *in vivo*, for example in the **E μ -TCL1** mouse model. Another option could be OSU-CLL cells **xenograft** into a nude mouse model. This *in vivo* approach can also determine any potential toxicities of TRCDA (or other newly developed pFAO inhibitors).

Targeting cancer cells from multiple angles as part of combination therapies can maximize response while allowing dose adjustments to minimize drug-related toxicities. In this way, this thesis provides evidence of a synergistic effect when combining TRCDA with BTK inhibitors (ibrutinib and acalabrutinib) but not with fludarabine or venetoclax. It would be interesting to further test the combination of TRCDA/rituximab, TRCDA/selinexor, etc. The

combination therapy TRCDA/BTKi can also be tested *in vivo* to validate its potential therapeutic benefit.

From a metabolic point of view, we were able to detect the end products of ACOX1-mediated pFAO in abundance in CLL compared to healthy B cells. What is lacking in our study is detecting the substrate that fuels this metabolic pathway. Several very-long chain FAs can be oxidized by peroxisomes, therefore identifying which one is preferentially catalyzed by CLL cells remains a question to be further investigated. This also includes identifying the source of this or these FAs, whether it is serological or synthesized by the cells. Once a specific FA metabolized by peroxisomes is identified, we can follow its metabolic fate by a metabolomics fluxomic analysis using a ¹³C-labeled FA. Identifying ¹³C labelling in the TCA cycle intermediates can confirm our hypothesis about the connection between pFAO and mitochondrial metabolism.

Several other metabolic pathways also take place in peroxisomes. In 2013, a study by Ollberding et al. demonstrated an increased risk of CLL as well as other non-Hodgkin's lymphomas, associated with increased intake of phytanic acid in the diet (a type of branched-chain FA) that is metabolized by peroxisomes³⁷⁹. Therefore it can be interesting to investigate the peroxisomal metabolic pathways that metabolize branched-chain FAs (such as ACOX2-mediated pFAO). The limitation of this study would be the pharmacological targeting of ACOX2, given that no inhibitor has yet been identified against this enzyme. The approach can be complemented by the knockdown or knockout of the enzyme by genetic engineering.

Our study introduces the peroxisomes and the peroxisomal metabolism in CLL physiology. A larger investigation is needed to elucidate the general role of this organelle in the disease. In fact, most cancer metabolism research takes into consideration mitochondria but little research is done on peroxisomes. Several aspects can be investigated to examine peroxisomes as pro-tumor organelles in CLL. These include a comparison of **peroxisome abundance** between CLL and healthy B cells. This can be achieved by staining peroxisomes with a fluorescent dye that targets a specific peroxisomal protein (e.g. PMP70). In addition, the identification of one or more peroxisome biogenesis factors overexpressed in CLL may provide further evidence of a possible increase in peroxisome biogenesis that may be associated with disease progression.

In conclusion, most of the objectives of this doctoral work have been achieved. Notably, I was able to identify a specific metabolic feature in the CLL cells that can be used as a potential therapeutic target. Indeed, depriving the malignant cell of its energy source by targeting the

pFAO metabolic pathway, alone or in combination with current clinical approaches, could represent an innovative metabolic therapeutic strategy against CLL.

Appendix



Review

Current Status of Novel Agents for the Treatment of B Cell Malignancies: What's Coming Next?

Mariana Tannoury, Delphine Garnier , Santos A. Susin and Brigitte Bauvois *

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Simple Summary: Since the identification of a large variety of biomarkers in B cell malignancies as being driving factors for tumor progression and patient prognosis, their targeting may confer valuable options for the treatment of these diseases. Over the past 20 years, the permanent development of a multitude of inhibitors acting on survival-associated biomarkers has made it into clinical evaluation in B lymphoid tumors. Although certain drugs approved by the US Food and Drug Administration improve clinical outcome, some patients do not respond and others relapse. This review summarizes the current state-of-the-art, provides a summary of new, safer, more selective inhibitors currently under evaluation in clinical trials, and highlights the emerging positioning of metabolic drugs in tumor B cell biology as a promising strategy to be translated into clinical practice.

Abstract: Resistance to death is one of the hallmarks of human B cell malignancies and often contributes to the lack of a lasting response to today's commonly used treatments. Drug discovery approaches designed to activate the death machinery have generated a large number of inhibitors of anti-apoptotic proteins from the B-cell lymphoma/leukemia 2 family and the B-cell receptor (BCR) signaling pathway. Orally administered small-molecule inhibitors of Bcl-2 protein and BCR partners (e.g., Bruton's tyrosine kinase and phosphatidylinositol-3 kinase) have already been included (as monotherapies or combination therapies) in the standard of care for selected B cell malignancies. Agonistic monoclonal antibodies and their derivatives (antibody–drug conjugates, antibody–radioisotope conjugates, bispecific T cell engagers, and chimeric antigen receptor-modified T cells) targeting tumor-associated antigens (TAAs, such as CD19, CD20, CD22, and CD38) are indicated for treatment (as monotherapies or combination therapies) of patients with B cell tumors. However, given that some patients are either refractory to current therapies or relapse after treatment, novel therapeutic strategies are needed. Here, we review current strategies for managing B cell malignancies, with a focus on the ongoing clinical development of more effective, selective drugs targeting these molecules, as well as other TAAs and signaling proteins. The observed impact of metabolic reprogramming on B cell pathophysiology highlights the promise of targeting metabolic checkpoints in the treatment of these disorders.

Keywords: leukemia; lymphoma; biomarker; inhibitor; signaling; survival; drug resistance; antibody; therapy; metabolism



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1. Background and Introduction

The balance between cell death and cell proliferation contributes to the maintenance of tissue homeostasis. Cell death evasion is a hallmark of human cancers (including B cell malignancies; Table 1) and might contribute to treatment resistance [1]. A number of molecular pathways underpin cell growth and cell survival in B cell malignancies (Figure 1). For example, anti-apoptotic proteins from the B-cell lymphoma/leukemia 2 (BCL2) family (such as Mcl-1, Bcl-2, Bcl-x_L, and Bcl-w) are overexpressed in most B cell malignancies (Table 1) as a result of genetic lesions or changes in signal transduction [2,3]. This expression

is associated with a poor prognosis [3–6]. Anti-apoptotic BCL2 proteins interact with the pro-apoptotic “BH3-only” proteins (such as Bim, Bid, Puma, Bad, and Noxa) or bind and sequester the apoptotic effectors Bax and Bak in an inactive form (Figure 1a) [3,7]. The pro-apoptotic proteins induce apoptosis by activating Bax and Bak either directly or indirectly via inhibition of the anti-apoptotic proteins (Figure 1a) [3,7]. Active B-cell receptor (BCR) signaling also contributes to cell survival, proliferation, and resistance to apoptosis in B cell malignancies [8–12] (Table 1). BCR-mediated proliferation of malignant B cells may be caused by BCR pathway mutations and/or chronic active stimulation of the BCR [8–12]. The mechanism of BCR pathway activation in B cell malignancies is complex and has been well reviewed by Burger and Wiestner [13]; it includes continuous BCR stimulation by microbial antigens or autoantigens, activating mutations within the BCR complex or downstream signaling partners, and antigen-independent BCR signaling [13]. The BCR pathway includes a large array of kinases, adaptor proteins and transcription factors (such as NF- κ B); in particular, Bruton tyrosine kinase (BTK) and phosphatidylinositol-3 kinase (PI3K) are critical signaling enzymes involved in uncontrolled B cell proliferation and survival [10–13] (Figure 1b). Lastly, high levels of expression of certain tumor-associated antigens (TAAs; CD19, CD20, CD22, and CD38) in most B cell malignancies are correlated with a high proliferation rate and disease progression (Table 1) [14–17]. These TAAs are physically associated with the BCR [18–21] and are involved in modulating BCR-dependent (Figure 1b) and BCR-independent proliferation/survival signals (Figure 1c) [13,15–18,20–26].

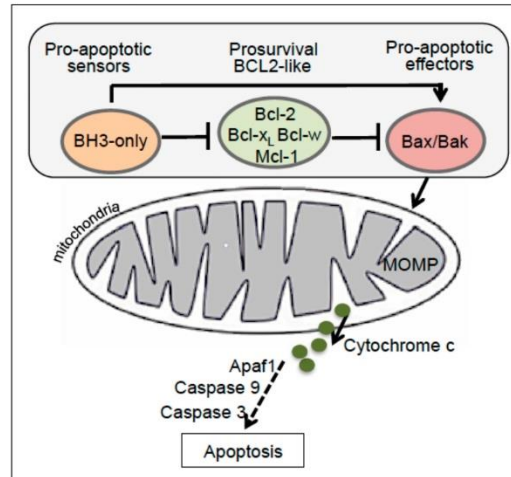
Over the last 25 years, greater knowledge of the detrimental roles of these survival-associated proteins in malignant B cell pathologies have prompted the development of a large array of inhibitors. These novel anticancer agents include small molecules that target BCL2 members, BTK, or PI3K, and mAbs and their derivatives (mono/bispecific Ab, antibody–drug conjugate/ADC, antibody–radioisotope conjugate/ARC, bispecific T cell engager/BiTE, and chimeric antigen receptor-modified T cells/CAR-T) against TAAs. The use of some of these novel agents have been authorized by the US Food and Drug Administration (FDA) [27]. Although these drugs improve clinical outcomes when used alone or in combination with other treatments, some patients do not respond, and others relapse after an initial response [28–34]. As such, efforts to develop more effective, selective inhibitors against the same targets or new targets in malignant B cells must be continued. Specifically, novel mAbs (acting on other TAAs) and small-molecule inhibitors (acting on survival-associated proteins, such as signaling proteins, proteases, immune checkpoints, etc.) are in preclinical or clinical development. Here, we first review the current management of B lymphoid tumors with FDA-approved drugs targeting the above-cited TAAs, Bcl-2, or BTK/PI3K. We then focus on the clinical efficacy of newly developed drug candidates for inhibiting current and novel protein targets and overcoming some forms of resistance. Lastly, we review perspectives for therapy, with emphasis on the importance of metabolic reprogramming in tumor B cell biology and opportunities for inhibiting this process.

Table 1. Characteristics of the main B cell malignancies.

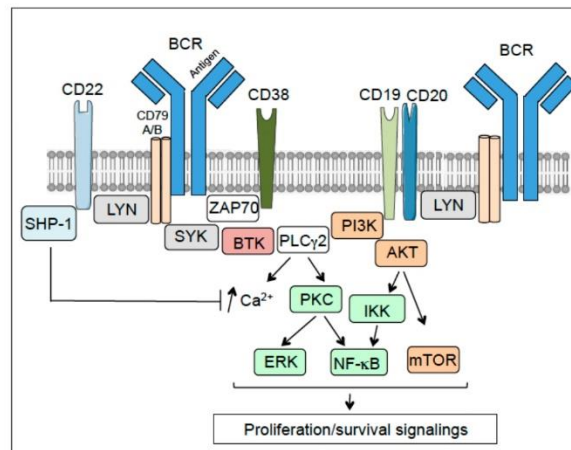
B Cell Malignancy	Pathophysiology	Expression of BCL2 Prosurvival Proteins	Active (Pre-)BCR Signaling	Surface Markers That Are Strongly Expressed	References
B-ALL	Transformation and expansion of lymphatic B progenitor cells	Bcl-2, Mcl-1, Bcl-x _L	pre +	CD19 CD20 CD22 CXCR4	[7,35–41]
HCL	Accumulation of CD5 ⁺ B cells most in the blood, bone marrow and spleen	Bcl-2 > Mcl-1	+	CD19 CD20 CD22 CD38 CXCR4	[2,11,40,42,43]
CLL	Accumulation of CD5 ⁺ B cells primarily in the blood and bone marrow	High Bcl-2 > Mcl-1 >> Bcl-x _L	+	CD19 CD20 CD38 (poor prognosis) BAFF-R ROR1 CXCR4	[3,7,36,38,40,41,44,45]
SLL (B-NHL)	Accumulation of CD5 ⁺ B cells most in the lymph nodes	Bcl-2, Mcl-1, Bcl-x _L	+	CD19 CD20 CD38 (poor prognosis) ROR1	[40,44–47]
MM	Accumulation of clonal, Ig secreting plasma cells in the bone marrow	High Bcl-2, Mcl-1, Bcl-x _L , Bcl-w	+	CD19 CD22 CD38 BCMA CD13 ADAM17 ROR1 CXCR4	[3,7,40,41,48–51]
FL (B-NHL)	Extensive proliferation and accumulation of abnormal B cells in lymph nodes	High Bcl-2, Bcl-w and Bcl-x _L > Mcl-1	–/+	CD19 CD20 CD22 CD38 CXCR4	[3,7,40,41,45,48,52]
MCL (B-NHL)	Development of abnormal B cells in the mantle zone of lymph nodes, spleen, bone marrow, blood, and gastrointestinal tract	High Bcl-w and Bcl-2 > Mcl-1	+	CD19 CD20 CD22 CD38 ROR1 CXCR4	[3,7,40,41,47,52,53]
MZL (B-NHL)	Development of abnormal B cells in the marginal zones of lymph tissue	High Bcl-2 and Bcl-w > Bcl-x _L	+	CD19 CD20 CD22 CXCR4	[3,10,40,41,52,54,55]
DLBCL (B-NHL)	Development of abnormal B cells in germinal centers of secondary lymphoid organs	Bcl-2 and Bcl-w > Mcl-1, Bcl-x _L	+	CD19 CD20 CD22 CD38 CXCR4	[3,7,36,40,41,45,52,56,57]
WM (B-NHL)	Proliferation of clonal, IgM-secreting plasma cells in the lymph nodes and bone marrow	High Bcl-2 > Bcl-x _L and Mcl-1	+	CD19 CD20 CD38 CXCR4 (mutation)	[8,40,46,58,59]

ADAM17, a disintegrin and metalloprotease 17; B-ALL, B-acute lymphoblastic leukemia; BAFF-R, B-cell activating factor receptor; BCMA, B cell maturation antigen; BCR, B-cell receptor; B-NHL, B cell non-Hodgkin lymphoma; CLL, chronic lymphocytic leukemia; CXCR4, CXC chemokine receptor 4 specific for stromal cell-derived factor-1. DLBCL, diffuse large B cell lymphoma; FL, follicular lymphoma; HCL, hairy cell leukemia; MCL, mantle cell lymphoma; MM, multiple myeloma; MZL, marginal zone lymphoma; ROR1, receptor tyrosine kinase-like orphan receptor; SLL, small lymphocytic lymphoma; WM, Waldenström's macroglobulinemia. Present on precursor B lymphocytes, pre-BCR differs from conventional BCRs in that it possesses a germ line-encoded surrogate light chain which is associated with the signal transduction machinery via heavy chain proteins that have been generated by productive rearrangement of the immunoglobulin HC genes.

(a) The BCL2 family protein-intrinsic mitochondrial pathway



(b) The BCR-dependent signaling pathway



(c) The BCR-independent signaling pathway

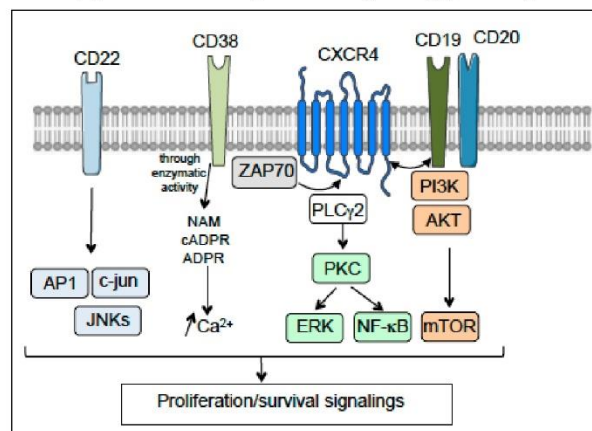


Figure 1. Models for the signaling pathways regulated by BCL2 family proteins, BCR components, and TAAs (CD19, CD20, CD22, CD38) in B cell malignancies. (a) Pro-survival BCL2-like proteins (Bcl-2, Bcl-x_L, Bcl-w, and Mcl-1) interact with the proapoptotic members called BH3-only proteins (Bim, Bid,

Puma, Bad, and Noxa), or bind to the apoptotic effectors Bax and Bak and sequester them in an inactive form. The pro-apoptotic proteins induce apoptosis by activating Bax and Bak either directly or indirectly through inhibition of the anti-apoptotic proteins. Once activated, Bax and Bak form oligomers; this leads to pore formation, mitochondrial outer membrane permeabilization (MOMP), and then the release of cytochrome c and other pro-death proteins from mitochondria, resulting in caspase activation and cell apoptosis. (b) Upon BCR ligation and activation, Lyn phosphorylates CD79A/B, which activates SYK. In turn, activated SYK phosphorylates and recruits the B-cell linker protein (BLNK), which binds to BTK and PLC γ 2 and catalyzes the cleavage of membrane phosphatidyl inositol bisphosphate into inositol triphosphate and diacyl glycerol. This releases Ca²⁺ from intracellular stores and activates PKC β and downstream proteins. The positive BCR coreceptors CD19 and CD20 are phosphorylated by LYN during BCR signaling, leading to the recruitment of PI3K to the BCR. Together, these signaling pathways activate the ERK, NF- κ B, AKT/mTOR pathways. The negative B cell coreceptor CD22 is phosphorylated by LYN and inhibits the BCR signal by recruiting SHP-1, which dephosphorylates and inactivates BLNK and CD19 and thus leads to a decrease in the cytoplasmic Ca²⁺ concentration. SHP-1 also decreases the cytoplasmic Ca²⁺ concentration by activating the plasma membrane Ca²⁺-ATPase (PMCA4) and thus promoting Ca²⁺ efflux. CD38 activation leads to the phosphorylation of ZAP70 and further sustains the signal mediated by the BCR. (c) Independently of the BCR signaling pathway, CD19, CD20, CD22, and CD38 have a role in activating proliferation/survival pathways in malignant B cells. CD38 (via ZAP70 activation), CD19 and CD20 are linked to the proliferation/survival signaling pathways controlled by CXCR4. Through CD38's enzymatic activity, the reaction products (nicotinamide adenine dinucleotide, cyclic ADP-ribose, and ADP-ribose) are used inside the cells to open different Ca²⁺ stores, which leads to an increase in the cytoplasmic Ca²⁺ concentration independently of the conventional IP3 pathway. CD22 ligation activates AP-1, c-jun, and the c-jun NH2-terminal kinases (JNKs).

2. Current Treatments and Clinical Trials of B Cell Malignancies Using Drugs That Target BCL2, BTK, PI3K Proteins, and TAAs (CD19, CD20, CD22, CD38)

The drugs currently approved by the FDA for the treatment of B cell malignancies [27] still include the first-in-class anti-CD20 agent (rituximab), the first-in-class Bcl-2 inhibitor (venetoclax), the first-in-class BTK inhibitor (ibrutinib), and the first-in-class PI3K inhibitor (idelalisib). Figure 2 charts these key discoveries, which led to the development of more efficacious treatments (Figure 2a) and new indications for clinical evaluation (Figure 2b). These agents include BCL2, BTK, and PI3K inhibitors, together with mAbs and their derivatives (ADC, ARC, bispecific Ab, and CAR-T).

(a) FDA-approved drugs used currently to treat B cell malignancies

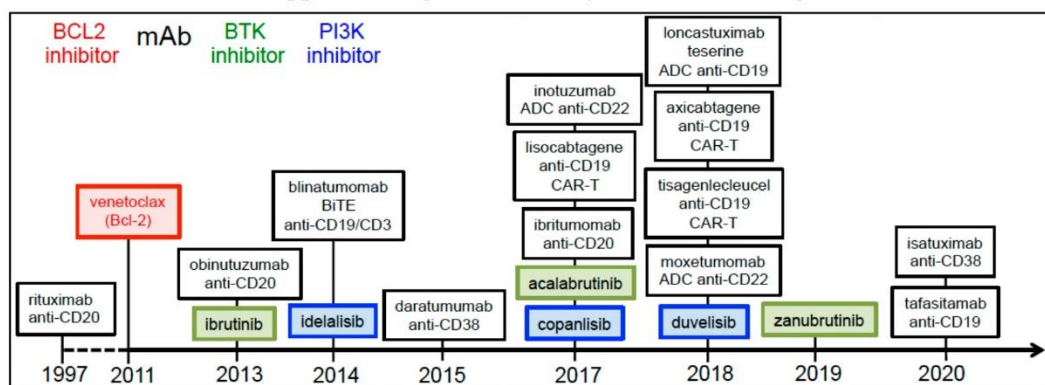


Figure 2. Cont.

(b) Investigational drugs currently in clinical development for B cell malignancies

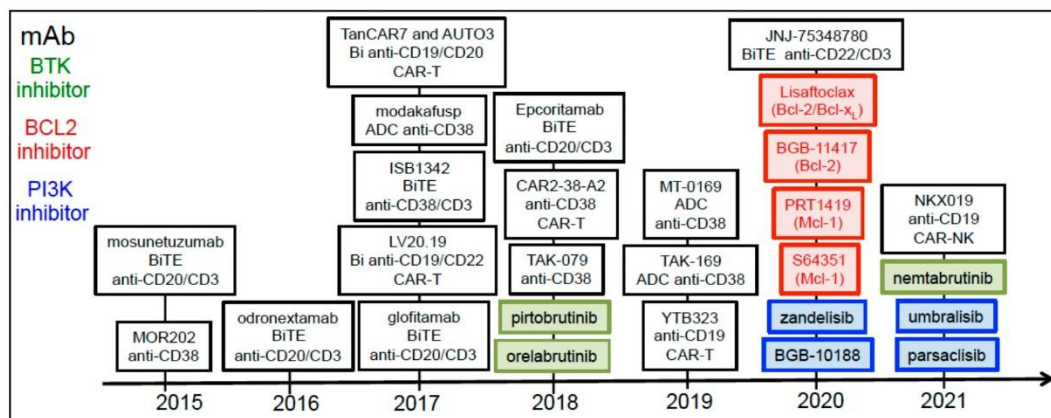


Figure 2. Timeline from 1997 onwards, showing the development of therapeutics for B cell malignancies. (a) (1997–2020) Drugs currently approved by the FDA for the treatment of B cell malignancies; (b) investigational drugs in clinical development (2013–2021) for B cell malignancies. ADC, antibody–drug conjugate; BCL2, B-cell lymphoma/leukemia 2; BiTE, bispecific T-cell engager, BTK, Bruton tyrosine kinase; CAR-T, chimeric antigen receptor-T cells; PI3K, phosphatidylinositol-3 kinase.

2.1. BCL2 Inhibitors

Over the last 20 years, significant efforts have been made to develop the therapeutic potential of agents targeting BCL2 family members [3,30,60]. The broad panel of small molecules developed to mimic the action of anti-apoptotic proteins includes navitoclax/ABT-263 (targeting Bcl-x_L, Bcl-2, and Bcl-w), obatoclax/GX15-070 (targeting Bcl-2, Bcl-x_L, Mcl-1, and Bcl-w), and venetoclax/ABT-199 (targeting Bcl-2 only); all have entered clinical trials [3,61,62]. Navitoclax and obatoclax induced severe adverse events, and the trials were stopped [3,61,62]. Venetoclax was approved in 2016 for treatment of previously treated CLL patients with a 17p deletion [3,60]. Resistance to venetoclax appeared related (at least in part) to upregulation of anti-apoptotic members Mcl-1 and Bcl-x_L, acquired Bcl-2 mutations, and the post-translational phosphorylation of Bcl-2 [61]. Thus, rational combinations of venetoclax with other agents were expected to produce a better clinical response [61]. In 2018, FDA expanded venetoclax's market authorization so that it could be combined with the anti-CD20 rituximab for the treatment of patients whose CLL had progressed after a single course of treatment, regardless of their 17p deletion status [61]. When combined with rituximab or obinituzumab (also an anti-CD20 agent), venetoclax is now an approved standard of care for treatment- and relapsed CLL disease (Figure 2a and Table 2) [30]. Further, many clinical trials have combined venetoclax with other chemotherapeutic agents in CLL; for recent reviews, see [60,63]. Three ongoing phase II trials are evaluating the venetoclax + ibrutinib combination in treatment-naïve CLL patients (NCT02910583) [64] and patients with relapsed/refractory (R/R) CLL/small lymphocytic lymphoma (SLL) (NCT0275689) [65], and the venetoclax + ibrutinib + obinituzumab combination in treatment-naïve CLL patients with a p53 deletion (17p-) and/or mutation (NCT02758665).

Bcl-2 is expressed strongly and constitutively in most B-NHL (Table 1). In this context, venetoclax has also been evaluated in patients with MM, FL, MCL, WM, MZL or even B-ALL or DLBCL (where ≈30% of tumors express high levels of Bcl-2) [3,60]. While venetoclax exhibited marked single-agent activity in MCL and WM, its outcome in treating the other B neoplasms was not as satisfactory [3,60,66]. Many clinical trials have started to evaluate various combination regimens of venetoclax in NHL and B-ALL (reviewed in [3,30,59–61,63]). Two phase II/III trials are evaluating the efficacy of venetoclax + ibrutinib in patients with relapsed MCL (NCT03112174) [67] and in WM with a specific MYD88

gene mutation (NCT04273139). In 2018, a phase I clinical trial of venetoclax + lenalidomide + rituximab started in patients with previously untreated MCL (NCT03523975). Unexpectedly, a phase III trial of venetoclax in R/R MM patients receiving bortezomib and dexamethasone as their standard therapy (NCT02755597) was suspended in 2022 [68]. A phase II study (NCT02055820) showed that venetoclax was efficacious and safe when combined with R-CHOP in DLBCL patients expressing high levels of Bcl-2 protein [69]. There are few trials of combinations of venetoclax with other targeted agents in FL, MZL and B-ALL [60]. With regard to B-ALL, the Bcl-2 and Bcl-x_L dependence of cell lines and primary cells has prompted clinical trials of BCL2 antagonists as single agents or combination therapies [70,71]. A retrospective study confirmed the safety and efficacy of venetoclax when compared with conventional treatments of ALL in pediatric patients [72]. In a phase I study (NCT03181126), a combination of venetoclax with low-dose navitoclax and chemotherapy was well tolerated in both pediatric and adult patients with R/R B/T-ALL [73]. Two phase I/II trials (NCT03236857, NCT04029688) of venetoclax alone or combined with chemotherapeutics are being initiated for pediatric patients with relapsed B/T-ALL [74,75]. As a whole, these clinical trial data suggest that the addition of venetoclax to conventional chemotherapies improves response rates for NHL and B-ALL.

Treatment with venetoclax can be impeded by frequent gastrointestinal adverse events, neutropenia, and the emergence of specific, resistance-inducing BCL2 mutations [30,60]. Recently, two new Bcl-2 inhibitors were designed and developed: BGB-11417 targets Bcl-2 only, whereas lisaftoclax/APG-2575 targets Bcl-2 and Bcl-x_L. In phase I trials, both compounds were found to be safe and effective in CLL/SLL and B-NHL (Table 3) [76,77]. These findings warrant further clinical investigation.

The anti-apoptotic protein Mcl-1 is a major factor in resistance to cancer chemotherapies and contributes to the onset of venetoclax resistance in some Mcl-1-overexpressing tumor cells [78]. Since 2012, many Mcl-1 inhibitors have been developed [79–84]. AMG176 [81], AMG-397 [82], and AZD5991 [84] were suspended due to the occurrence of serious adverse events in phase I trials for hematological malignancies, while the trial of ABBV-467 in R/R MM was terminated in 2021 for strategic reasons. A phase I study is now evaluating a novel Mcl-1 inhibitor (PRT1419) in patients with R/R MM and NHL (Table 3). Interestingly, the combination of another Mcl-1 inhibitor (S64315, also referred to as MIK665) with azacytidine is starting to be assessed in patients with acute myeloid leukemia (AML) [83]; the clinical value of S64315 in B lymphoid malignancies has yet to be assessed.

Table 2. Current treatments of B cell malignancies with FDA-approved drugs targeting Bcl-2, BTK, PI3K and TAAs (CD19/CD20/CD22/CD38), alone or in combination and as first- or second-line therapies.

B Cell Malignancy	Current Treatment Options	References
B-ALL	Conventional chemotherapies *	[85–89]
	Tyrosine kinase inhibitors imatinib or dasatinib for patients with Philadelphia chromosome-positive Blinatumomab Tisagenlecleucel Inotuzumab ozogamycin (adults)	
CLL/SLL	Fludarabine, cyclophosphamide and rituximab (FCR) Bendamustine and rituximab (BR) Rituximab and chlorambucil (RCb) Rituximab , cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) Obinutuzumab and chlorambucil Ibrutinib or acalabrutinib , alone Idelalisib alone and with rituximab Venetoclax + rituximab (or obinutuzumab)	[60,90–97]

Table 2. Cont.

B Cell Malignancy	Current Treatment Options	References
HCL	Purine analogs (cladribine, pentostatin) alone and with rituximab Moxetumomab pasudotox	[98,99]
MM	Conventional chemotherapies ** Daratumumab (or isatuximab) + lenalidomide (or pomalidomide) + dexamethasone Belantamab mafodotin	[34,49,100–102]
FL	BR Rituximab , cyclophosphamide, vincristine, and prednisone (R-CVP) R-CHOP Rituximab + hyaluronidase human Rituximab + lenalidomide Obinutuzumab + bendamustine Idelalisib or copanlisib alone Ibritumomab tiuxetan (Yttrium-90) Axicabtagene ciloleucel	[103–107]
MCL	R-CVP, R-CHOP Rituximab + lenalidomide Rituximab + bortezomib Ibrutinib or acalabrutinib or zanubrutinib , alone Ibrutinib + rituximab	[53,108,109]
MZL	R-CVP, R-CHOP, FCR, BR Fludarabine and rituximab (FR) Rituximab + lenalidomide Ibrutinib or zanubrutinib	[54,110–112]
DLBCL	R-CHOP R-CHOP + etoposide Rituximab Tafasitamab + lenalidomide Loncastuximab teserine Tisagenleucel Axicabtagene ciloleucel	[89,113–117]
WM	Alkylating drugs and proteasome inhibitors both in combination with rituximab Ibrutinib or acalabrutinib or zanubrutinib , alone and with rituximab	[59,118–122]

* Various combinations of vincristine, dexamethasone or prednisone, and an anthracycline drug (doxorubicin or daunorubicin); fludarabine, cytarabine and G-CSF; high-dose cytarabine; cytarabine and mitoxantrone; bortezomib. ** Proteasome inhibitors (bortezomib, carfilzomib, and ixazomib), immunomodulatory drugs (lenalidomide, pomalidomide, and thalidomide), alkylating agents (cyclophosphamide and bendamustine), steroids (prednisone and dexamethasone), a nuclear export inhibitor (selinexor), and bisphosphonates, alone or in various combinations. R-CHOP, rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone. The FDA-approved drugs listed in Figure 2a are written in bold type.

Table 3. Selected active or recruiting clinical trials of novel inhibitors of BCL2/BTK/PI3K proteins or TAAs (CD19/CD20/CD22/CD38), evaluated alone or in combination as first- or second-line therapies for B cell malignancies.

Drug	Class	Disease Setting	Phase	Study	References
BGB-11417	Bcl-2 inhibitor	CLL/SLL, B-NHL	I	NCT04277637 Recruiting, 2020–2023 Alone or combined with zanubrutinib	[76]

Table 3. Cont.

Drug	Class	Disease Setting	Phase	Study	References
Lisafoclax/ APG-2575	Bcl-2/Bcl-x _L inhibitor	CLL/SLL, MZL, MCL	I	NCT03913949 Recruiting, 2019–2023	[77]
		CLL/SLL	Ib/II	NCT04494503 Recruiting, 2020–2023 Alone or combined with rituximab or ibrutinib	[123]
PRT1419	Mcl-1 inhibitor	R/R MM R/R B-NHL	I	NCT04543305 Active, 2020–2022	[124]
S64351/ MIK-665	Mcl-1 inhibitor	AML	I/II	NCT04629443 Recruiting, 2020–2024 + azacytidine	
Orelabrutinib /ICP-022	BTK inhibitor	MCL CLL/SLL	I/II	NCT03494179 NCT03493217 Active, 2018–2022	[125]
		FL, MZL, MCL, CLL/SLL	I/II	NCT04014205 Recruiting, 2019-	
Nemtabrutinib/ MK-1026/ARQ-531	BTK inhibitor	R/R MZL, R/R WM, DLBCL	II	NCT03797456 NCT04440059 NCT04438005 Recruiting, 2020-	
		CLL/SLL, MCL, R/R MZL R/R FL, R/R WM	II	NCT04728893 Recruiting, 2021–2027	
Pirtobrutinib/ LOX-305	BK inhibitor	Previously treated CLL/SLL, WM, MCL, MZL	I/II	NCT03740529 Recruiting, 2018–2023 Combined with venetoclax + rituximab	
		Untreated CLL/SLL	III	NCT05023980 Recruiting 2021-	
Parsaclisib/ INCB500465/ IBI-376	PI3Kδ inhibitor	Previously treated CLL/SLL	III	NCT04965493 Recruiting, 2021- Combined with venetoclax + rituximab	
		CLL, DLBCL	I/II	NCT04809467 Recruiting, 2021–2023 Alone or combined with tafasitamab	
Zandelisib/ PWT143/ME-401	PI3Kδ inhibitor	R/R FL, R/R MZL	III	NCT04745832 Recruiting, 2021–2031 Combined with rituximab	[126]
		R/R CLL	II	NCT05209308 Recruiting, 2022–2026 Combined with rituximab + venetoclax	

Table 3. Cont.

Drug	Class	Disease Setting	Phase	Study	References
BGB-10188	PI3K δ inhibitor	R/R CLL, FL, MCL, DLBCL, MZL	I/II	NCT04282018 Recruiting, 2020–2025 Alone or combined with zanubrutinib and tiselizumab	
Umbralisib/ TGR-1202	Dual PI3K δ /CK1 ϵ inhibitor	R/R CLL, R/R MCL	I/Ib	NCT02268851 Active, 2014–2023 Combined with ibrutinib	[127]
		R/R CLL, R/R B-NHL	I	NCT03283137 Active, 2017–2024 Combined with pembrolizumab	
Mosunetuzumab/ BTC4465A	BiTE anti-CD20/CD3	CLL, B-NHL	I/II	NCT02500407 Recruiting, 2015- Alone or combined with atezolizumab (anti-PD-L1)	[128]
		B-NHL	I/II	NCT03671018 Recruiting, 2018- Alone or combined with polatuzumab (anti-CD79B-monomethyl auristatin E)	
Odronextamab/ REGN1979	BiTE anti-CD20/CD3	R/R DLBCL, FL, MZL, WM	I/II	NCT02651662 Active, 2016–2026 Alone or combined with cemiplimab (anti-PD-1) NCT03888105 Recruiting, 2019–2028	[129]
Epcoritamab/ GEN301	BiTE duoBody-anti-CD20/CD3	R/R or progressive DLBCL, MCL, FL, MZL, SLL	I/II	NCT03625037 Recruiting, 2018–2024	[130,131]
		R/R DLBCL	III	NCT04628494 Recruiting, 2020–2024	
Glofitamab (RO7082859)	BiTE anti-CD20/CD3	R/R B-NHL	I/II	NCT03075696 Recruiting, 2017–2023 Alone or combined with obinutuzumab	[132]
			Ib/II	NCT03533283 Recruiting, 2018–2024 Alone or combined with atezolizumab, obinutuzumab, tocilizumab polatuzumab vedotin	

Table 3. Cont.

Drug	Class	Disease Setting	Phase	Study	References
Tafasitamab/ MOR208/	Anti-CD19	R/R FL, R/R MZL	III	NCT04680052 Recruiting, 2020- Combined with rituximab + lenalidomide compared with rituximab + lenalidomide	
		DLBCL	III	NCT04824092 Recruiting, 2021–2026 Combined with lenalidomide + R-CHOP	[133]
CAR-20.19-T	Anti-CD19/CD20 CAR-T/CD28/ 4-1BB/CD3ζ	CLL/SLL, R/R B-NHL	I	NCT03019055 Active, 2017–2022	[134]
CD19-CAR-NK using blood cord NKs	Anti-CD19 CAR- NK/OX40/CD3ζ/IL- 15	B-ALL CLL/SLL, R/R B-NHL	I/II	NCT03056339 Active, 2017–2022 after treatment with fludara- bine/cyclophosphamide lymphodepletion	[135,136]
NKX019 using peripheral blood NKs	Anti-CD19 CAR-NK/OX40/ CD3ζ/IL-15	B-ALL CLL/SLL, R/R B-NHL	I	NCT05020678 Recruiting 2021–2023	[137]
C-CAR066/ CBM.20-CAR-T	Anti-CD20 CAR-T	NHLs resistant to rituximab or anti-CD19 CAR-T	I	NCT04036019 Recruiting, 2019–2021 NCT04316624 Recruiting, 2020-	[138]
Inotuzumab ozogamicin	ADC anti-CD22- calicheamicin	B-ALL, B-NHL	III	NCT03959085 Recruiting, 2019–2029 post chemotherapy	
JNJ-75348780	BiTE anti-CD22/CD3	CLL, B-NHL	I	NCT04540796 Recruiting, 2020–2024	
CD22-CAR	Anti-CD22 CAR-T/CD8/4- 1BB/CD3ζ	R/R B-ALL, B-NHL	I	NCT02315612 Recruiting, 2014–2040	[139]
LV20.19	Bi anti-CD19/CD22 CAR-T/4- 1BB/CD3ζ	CLL/SLL, B-NHL	I	NCT03019055 Active, 2017–2022	[134,140]
AUTO3	Bi anti-CD19 (OX40 costim)/CD22 (4-1BB costim) CAR-T	R/R DLBCL	I/II	NCT03287817 Active 2017-	[141]
CD19-22.BB.z-CAR	Bi anti-CD19/ CD22 CAR-T	R/R B-ALL, DLBCL	I	NCT03233854 Recruiting, 2017–2025 Combined with NKTR-255 (IL-15 receptor agonist)	[142]
		Pretreated B-ALL	I	NCT03448393 Recruiting, 2018–2040 following cyclophos- phamide/fludarabine treatment	[143]

Table 3. Cont.

Drug	Class	Disease Setting	Phase	Study	References
CD20-22 CAR	Bi anti-CD20/CD22 CAR-T	B cell malignancies	I	NCT04283006 Recruiting, 2020–2028	
Felzartamab/ MOR202/TJ202	Anti-CD38	R/R MM	II	NCT03860038 Active, 2019–2022 Combined with dexamethasone	
			III	NCT03952091 Active, 2019–2022 Combined with lenalidomide + dexamethasone	
TAK-079	Anti-CD38	R/R MM	I/IIa	NCT03499280 Completed, 2018–2022 Alone or combined with pomalidomide and dexamethasone	
		MM	I	NCT03984097 Active, 2019–2023 Combined with lenalidomide + dexamethasone	
TAK-573/ modakafusp	ADC anti-CD38 delivering attenuated IFN- α 2b	R/R MM	I/II	NCT03215030 Recruiting, 2017- Alone or combined with dexamethasone	[144]
TAK-169/ MT-0169	ADC anti-CD38 delivering Shiga-like toxin	R/R B-NHL	I	NCT04017130 Recruiting, 2019-	[145]
ISB 1342/ GBR 1342	BiTE anti-CD38/CD3	R/R MM	I	NCT03309111 Recruiting 2017–2024	

2.2. BTK Inhibitors

Of the many BTK inhibitors developed in the last 10 years, three (ibrutinib/PCI-32765, acalabrutinib/ACP 196, and zanubrutinib/BGB 3111) are now used in the clinic to treat B cell malignancies (Figure 2a and Table 2). Ibrutinib and acalabrutinib are authorized for untreated and R/R CLL/SLL and CLL with 17p deletion [146–149], ibrutinib and zanubrutinib are authorized for R/R MZL patients who have received at least one line of an anti-CD20-based regimen [149], and ibrutinib, acalabrutinib and zanubrutinib are authorized for WM patients and for MCL patients who have received at least one line of treatment [119,147–150]. These three agents are orally administered alone or in combination with an anti-CD20 mAb such as rituximab (Table 2). Acalabrutinib and zanubrutinib are better tolerated than ibrutinib, although the first two drugs have not been on the market as long as the latter [149]. All three drugs inhibited BTK irreversibly by forming a covalent bond with cysteine-481 (Cys-481) in the active site [28,151]. The first-generation BTK inhibitor ibrutinib has off-target kinase inhibition activities [28], whereas the second-generation BTK inhibitors acalabrutinib and zanubrutinib are more selective for BTK [28,149].

Several completed phase I trials have demonstrated the safety of ibrutinib and acalabrutinib in R/R MM, either alone or combined with other therapeutics [152]. A phase III trial (NCT01855750) showed a survival benefit of the addition of ibrutinib to R-CHOP chemotherapy in younger patients with DLBCL [153], while a phase II study (NCT01779791)

did not find any benefit of ibrutinib in R/R FL [154]. In a phase III trial (NCT03734016), zanubrutinib significantly improved response rates and delayed disease progression in patients with R/R CLL/SLL (relative to ibrutinib) and did so with less toxicity [155]. The efficacy and safety of zanubrutinib are being evaluated in treatment-naïve CLL/SLL patients with and without a 17p deletion (NCT03336333). Adding zanubrutinib to obinutuzumab improves responses and progression-free survival in R/R FL (NCT03332017, 2017–2023) [156]. In a phase II study (NCT03145064), zanubrutinib monotherapy led to modest responses in R/R DLBCL patients with both CD79B and MYD88 mutations [157].

The resistance of lymphoid tumors to treatment with first- and second-generation BTK inhibitors and subsequent relapse appear to be in part related to the acquisition of mutations in BTK [149,158]. The most common is the Cys-481 to serine (C481S) mutation, which disrupts inhibitor-BTK binding [149]. To overcome this resistance problem, third-generation noncovalent BTK inhibitors have been developed to inhibit the kinase activities of both BTK and BTK^{C481S} [28,149]. These drugs include vecabrutinib/SNS-062, fenebrutinib/GDC-0853, orelabrutinib/ICP-022, nemtabrutinib/MK1026/ARQ-531, and pirtobrutinib/LOX-305. The completed phase I trials with vecabrutinib in CLL/SLL and B-NHL (NCT03037645) and with februnitib in R/R NHL and CLL (NCT01991184) showed that these drugs were well tolerated but exhibited low levels of antitumor activity; hence, the studies were discontinued [159]. Orelabrutinib, pirtobrutinib, and nemtabrutinib are undergoing clinical development as monotherapies or combination therapies in most B cell malignancies (Table 3).

2.3. PI3K Inhibitors

PI3K isoforms (PI3K α , PI3K β , PI3K δ and PI3K γ) are commonly found in B cell malignancies and are activated by BCR signaling [160,161]. This is why targeting the PI3K pathway has promise in treating B cell malignancies. At present, two PI3K inhibitors are used in routine clinical practice for the treatment of patients with CLL and FL: idelalisib (an orally administered PI3K δ inhibitor) and copanlisib (an intravenously administered pan-PI3K inhibitor) [162,163] (Figure 2a and Table 2). As the first-in-class PI3K δ inhibitor, idelalisib showed efficacy in patients with a 17p deletion and p53 mutation, unmutated IGHV status, and R/R CLL [161]. The drug is currently used in combination with rituximab (Table 2) [164]. Idelalisib and copanlisib have been approved for the treatment of R/R FL in patients having received two or three lines of treatment (Table 2) [107,164–166]. A recent, retrospective, Italian multicenter study confirmed the effectiveness of idelalisib in R/R FL patients treated in routine clinical practice [167]. Compared with idelalisib, copanlisib was associated with a higher complete remission rate and a more favorable safety profile [107]. It should be noted that the oral dual PI3K δ / γ inhibitor duvelisib (initially approved in 2018 for the treatment of patients with R/R FL having received at least lines of systemic treatment [168]) was withdrawn from the market by its developer Secura Bio in December 2021, following the occurrence of fatal and/or serious infections [169].

Despite the recent withdrawal of duvelisib in the indication of FL, several clinical trials in CLL/SLL are still evaluating duvelisib as combination treatments and with different dosing regimens [161,162]. A phase III trial (NCT02004522) of duvelisib vs. ofatumumab (an anti-CD20) in patients with R/R CLL/SLL was terminated in 2021 after the occurrence of serious adverse events, including severe infections and diarrhea/colitis [161]. Clinical evaluations of the efficacy of idelalisib as a monotherapy and of duvelisib combined with anti-CD20 (obinutuzumab) in R/R MM were recently reviewed by Grimont et al. [119]. Idelalisib is associated with significant hepatotoxicity, which may limit the drug's use in WM [118]. Four phase I/II studies are evaluating copanlisib as part of a combination therapy for CLL, FL, MZL and DLBCL (NCT03884998), DLBCL (NCT03484819), untreated FL (NCT03789240), and DLBCL and R/R grade 3b FL (NCT03789240) (reviewed in [107,164]). In an ongoing phase III trial (NCT02367040), copanlisib was found to be safe when combined with rituximab for the treatment of R/R NHL [170].

Given the potentially limiting toxicities reported for idelalisib and copanlisib [161], further improvements are required to develop new, safer, and more selective PI3K inhibitors. Many PI3K inhibitors have been developed over the last five years, and some of them are being tested in the clinic as monotherapies or combination therapies (reviewed in [12,160,161]). For example, the oral PI3K δ inhibitor piasclisib (used alone or in combination with chemotherapy) demonstrated antitumor activity and an acceptable safety profile in R/R B-NHL (the phase II NCT02998476 and phase III NCT02018861 both completed in 2021) [171,172]. Following a business decision in February 2022, Incyte withdrew piasclisib as a treatment for patients with R/R MCL, MZL and FL [173]. However, a combination of piasclisib with the anti-CD19 mAb tafasitamab is still being evaluated in patients with R/R-CLL and DLBCL (Table 3). Furthermore, two potent, selective PI3K δ inhibitors (zandelisib and BGB-10188) are in clinical development for the treatment of patients with R/R CLL and B-NHL. Zandelisib (alone or in combination with rituximab) was found to be well tolerated in all R/R neoplasms CLL/SLL, R/R FL, MZL and DLBCL (NCT02914938) [174]. A multicenter, randomized phase III study is now investigating the safety and efficacy of the zandelisib + rituximab combination vs. standard immunochemotherapy in patients with R/R FL or MZL (Table 3) [126], while a phase II trial has been initiated with a zandelisib + rituximab + venetoclax combination in patients with R/R CLL (Table 3). In a phase I/II study, BGB-10188 is being evaluated as a monotherapy and a combination therapy with zanubrutinib and tislelizumab (an anti-PD1) in patients with CLL and B-NHL (MCL, FL, DLBCL, and MZL) (Table 3). Lastly, umbralisib/TGR1202 inhibits both PI3K δ and casein kinase 1 ϵ (CK1 ϵ); the latter is involved in the translation of the c-Myc oncogene and the regulation of the Wnt pathway [175]. Umbralisib is associated with less immune-mediated toxicity than the other PI3K δ inhibitors (because it disables WNT signaling by inhibiting CK1 ϵ) and exerts less detrimental effects on regulatory T cells' immunosuppressive functions [175]. Umbralisib was found to be efficacious and safe as a monotherapy for patients with R/R CLL and B-NHL [176] and for CLL patients who do not tolerate BTK or PI3K δ inhibitor therapy [177]. The drug's favorable toxicity profile was confirmed when it was combined with ibrutinib in patients with R/R CLL and MCL [127] or with ublituximab (an anti-CD20) in patients with CLL/SLL and B-NHL [178]. In January 2022, however, the FDA placed a partial clinical hold on studies of umbralisib and ublituximab [160,161] in an indication of CLL or B-NHL [179]. Studies of a combination of umbralisib with ibrutinib or pembrolizumab (an anti-PD1) in patients with R/R CLL and R/R B-NHL are ongoing (Table 3).

2.4. mAbs and CAR-T Cells

Antibody-based cancer treatments have developed quickly over the past 25 years. Following on from the first generation of therapeutic murine mAbs (-momabs), a variety of second-generation mAbs were engineered: chimeric mAbs (-ximabs), humanized mAbs (-zumabs), and human mAbs (-umabs) [180]. Chimeric mAbs possess murine variable regions, whereas the rest of the Ab is of human origin. Humanized mAbs retain murine hypervariable segments, whereas fully human mAbs have a native Fc region [180]. Third-generation mAbs have an engineered Fc region, which is designed to improve therapeutic performance by adapting their effector functions [180]. The main effector pathways commonly employed by mAbs are complement-dependent cytotoxicity (CDC), Ab-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and programmed cell death (PCD) [181,182].

To enhance their therapeutic value, mAbs have also been conjugated with toxins (giving ADCs), radioisotopes (giving ARCs), immunomodulatory cytokines, and costimulatory molecules (giving CAR-T cells) [180,182–185]. An ADC combines selectivity and cytotoxic potency and so can target a given TAA with high specificity [183,186]. After binding to a TAA, ADCs enter the cell via receptor-mediated endocytosis and release the lethal drug. ADCs can also activate ADCC and ADCP signaling by immune effector cells [186]. Multivalent Abs simultaneously target a TAA and an activating receptor on the effector cell

(typically a T cell) [187]. Most engineered, multispecific Abs lack an Fc region and therefore do not mediate CDC or ADCC [184]. Bispecific T-cell engagers (BiTEs) are bispecific, single-chain Abs that specifically recognize the T cell antigen CD3 and a TAA, which also stimulate cytotoxic T cells for targeted tumor cell lysis [184,185]. CAR-T therapy is based on antibody recognition of TAAs [188,189]. CAR-T cells are cytotoxic T cells that have been modified genetically to express an artificial chimeric antigen receptor (CAR). The CAR comprises an extracellular target-binding domain (composed of a single-chain variable fragment (scFv) from antibody light- and heavy-chain variable regions and which bind to the TAA), a spacer/hinge region, transmembrane domains, and intracellular domains (comprising two or more costimulatory domains (CD28 and/or 4-1BB/CD137/TNF-RSF9) and a T cell signaling domain (with CD3 ξ) [14,190,191]. The first generation of CAR constructs included CD3 ξ ; the second generation included CD28 and CD3 ξ , or 4-1BB and CD3 ξ ; the third generation included CD28, 4-1BB, and CD3 ξ ; the fourth generation included a cytokine transgene initiated through NFAT signaling, and the fifth generation included IL-2R β (to initiate JAK/STAT signaling) [14,190,191]. Following expansion in the laboratory and then infusion into the patient, CAR-T cells bind to their cognate targets on tumor cells through their extracellular domain and initiate signal transduction that triggers the effector functions of host cytotoxic T cells, which then kill the tumor cells [188,189].

2.4.1. CD20 mAbs and CD20-Targeting CAR-T

Within the last 20 years, a variety of strategies have been used to develop a large number of anti-CD20 mAbs. The FDA's approval of rituximab in 1997 marked a breakthrough in the treatment of B cell malignancies (Figure 2a). Rituximab is still part of the standard of care for most B cell tumors (Table 2). Rituximab engages the main effector pathways employed by mAbs (i.e., CDC, ADCC, and ADCP) and even directly activates PCD [182,192]. Many phase I/II/III trials have evaluated the combination of other novel drugs (including ibrutinib, idelalisib, acalabrutinib, venetoclax, ipilimumab (an anti-CTLA4), chromatin modulators, and other drugs) with rituximab in B cell malignancies [15,193]. Based on the efficacy, relative safety and ubiquitous use of rituximab in B cell malignancies, attempts have been made to develop more efficacious and/or less toxic CD20 mAbs [15,184,185,194,195]. Indeed, the CD20 mAbs obinutuzumab and ibritumomab tiuxetan (radiolabeled with ⁹⁰Yttrium) are now used to treat CLL and FL (Figure 2a and Table 2), and four BiTE anti-CD20/CD3 mAbs (mosunetuzumab, odronextamab, glofitamab and epcoritamab) are in clinical development (Figure 2b and Table 3) [128,130,131].

Obinutuzumab's mechanisms of action are similar to those of rituximab [182,195]. ⁹⁰Y-ibritumomab binds to the CD20 antigen on tumor B cells, and the long range of β particles from the ⁹⁰Y means that neighboring tumor cells can be killed without direct antibody binding [195]. Compared with rituximab in the induction therapy of B-NHL, obinutuzumab was associated with significantly greater progression-free survival but a greater incidence of adverse events, and ⁹⁰Y-ibritumomab was associated with a greater overall response rate [33].

Mosunetuzumab, odronextamab and glofitamab monotherapies have already show manageable safety profiles and given lasting responses in heavily pretreated patients with R/R B-NHL (Table 3) [128,132,196]. Compared with intravenously infused mosunetuzumab, odronextamab or glofitamab, the subcutaneously administered epcoritamab is usefully associated with (i) lower peak cytokine levels, (ii) a lower treatment burden for patients, and (iii) less resource use at the treatment facility [130,131]. Mosunetuzumab is currently in phase I/II trials, where it is combined with mAbs targeting the programmed cell death protein 1 (PD-1) immune checkpoint, the latter's ligand (programmed death-ligand 1, PD-L1), or CD79B (an antigen associated with the BCR; see Figure 1b) (Table 3). Other novel BiTE anti-CD20/CD3 mAbs are being evaluated in phase I trials [184].

Several clinical studies have focused on the potential of autologous, first-generation anti-CD20 CAR-T cell therapies in patients with B cell malignancies (reviewed in [138,197,198]). Two phase I clinical trials are underway to evaluate the efficacy of C-CAR066 (a novel,

second-generation, anti-CD20 CAR-T cell therapy) in patients with R/R NHL (including cases of DLBCL) after the failure of rituximab or CD19 CAR-T therapy (Table 3) [138]. All the enrolled B-NHL patients who were previously R/R to rituximab achieved various clinical responses, and the level of toxicity was tolerable [138].

2.4.2. CD19 mAbs and CD19-Targeting CAR-T

Many models based on CD19 mAbs have been built and evaluated in the treatment of B cell malignancies [89,199,200]. Several strategies have been implemented, and four types of CD19 mAbs are currently used to treat B-ALL, DLBCL and FL (Table 2): blinatumomab (a BiTE), tafasitamab (an Fc-engineered and Fab affinity-matured Ab), loncastuximab tesirine (ADC), and CAR-T therapies (lisocabtagene maraleucel, axicabtagene ciloleucel and tisagenlecleucel) (Table 2) [89,201–206].

By binding both CD19 on B cells and CD3 on T cells, blinatumomab triggers ADC-dependent cytotoxicity [89]. As the first FDA-approved BiTE for the treatment of B-ALL, blinatumomab (developed by Amgen) induces remissions in cases of R/R B-ALL that had failed to respond to other therapies (Table 2) [201,207]. However, blinatumomab has a short half-life and must be continuously infused intravenously [201]. Of the many other CD19 mAbs developed [199], tafasitamab (MOR208) is combined with lenalidomide in the treatment of R/R DLBCL (Table 2) [115,116]. Tafasitamab mediates both tumor B cell apoptosis, ADCC and CDC [208,209]. The main adverse event associated with tafasitamab is bone marrow suppression, with anemia, neutropenia, and thrombocytopenia [115]. At present, two phase III clinical trials are recruiting patients; a combination of tafasitamab and first line-chemotherapeutic agents will be used to treat DLBCL (NCT04824092) [14,89,133] or R/R FL and R/R MZL (NCT04680052) [14,89,133]. Loncastuximab tesirine (ADCT-402) is a humanized CD19 mAb linked to tesarine (SG32499); lysosomal degradation delivers a pyrrobenzodiazepine dimer that crosslinks the tumor cell's DNA [117,202]. Based on the phase II LOTIS-2 trial, loncastuximab tesirine was recently granted fast-track approval for the therapy of adult patients with R/R DLBCL and who have already received two or more lines of treatment (Table 2) [210]. An ongoing phase I clinical trial (NCT03684694) is investigating a combination of loncastuximab and ibrutinib in the treatment of patients with DLBCL and MCL.

The three CD19-CAR-T cell therapies on the market (tisagenlecleucel, axicabtagene ciloleucel, and lisocabtagene maraleucel) use autologous T cells [89,203,205,211]. CAR-T engagement of CD19⁺ tumor cells results in T-cell activation and proliferation, the secretion of inflammatory cytokines and chemokines, and thus tumor cell lysis [212]. Tisagenlecleucel is composed of an anti-CD19 scFv, a CD8- α hinge region, 4-1BB, and CD3 ζ [213]. Axicabtagene ciloleucel contains an anti-CD19 scFv, an IgG4 hinge region, CD28, and CD3 ζ [212]. Lisocabtagene maraleucel contains an anti-CD19 scFv, an IgG4 hinge region, and CD28, 4-1BB and CD3 ζ domains [214]. Tisagenlecleucel has been approved for the treatment of children and young adults with R/R B-ALL (Table 2) [88,213,215–217]. In recent phase II/III studies of pediatric/young adult patients with Down-syndrome-associated ALL (NCT02435849, NCT02228096, and NCT03123939), tisagenlecleucel treatment was associated with high remission rates, manageable side-effects, and promising long-term outcomes [216]. From the third line of treatment onwards, both tisagenlecleucel and axicabtagene ciloleucel are currently recommended for patients with DLBCL who have failed autologous transplantation or have relapsed after two previous lines of treatment (Table 2) [32,212,215]. A recent retrospective evaluation of the safety and efficacy of axicabtagene ciloleucel and tisagenlecleucel in DLBCL outside the setting of a clinical trial found that although the two cohorts did not differ significantly with regard to any of the baseline characteristics, cytokine release syndrome and neurologic events were more frequent in the axicabtagene group [218]. An ongoing phase II trial is looking at tisagenleucel CAR-T cell therapy for FL (NCT04094311, 2019–2023). At present, there are three active phase II clinical trials evaluating axicabtagene alone in FL (NCT03105336) [219] and in combination with first line-chemotherapeutic agents (cyclophosphamide/fludarabine) in

DLBCL (NCT03761056, NCT03391466) [220]. In 2022, lisocabtagene was approved as a second-line treatment of patients with R/R DLBCL and FL and who were not eligible (due to comorbidities or age) for hematopoietic stem cell transplantation (Table 2) [214]. Two phase I/II studies will evaluate the efficacy and safety of lisocabtagene in combination with other chemotherapeutic agents in R/R CLL/SLL (NCT03331198, with ibrutinib or venetoclax) [221], and high-risk DLBCL and FL (NCT03310619: lisocabtagene alone or combined with ibrutinib or durvalumab). Relative to standard care, the lisocabtagene + ibrutinib combination yielded significantly higher event-free survival and response rates in patients with R/R CLL/SLL [221]. Thus, CD19-CAR-T therapy appears to have promising outcomes and a tolerable safety profile in R/R B cell malignancies [222,223]. Further modifications of the CAR's structure (resulting in second/third-generation anti-CD19/CAR-T agents) and the therapeutic strategy are being tested as treatments for B cell malignancies (reviewed in [31,223,224]).

Given that the emergence of CD19⁺ tumor cells substantially increases the risk of relapse [32], another approach seeks to optimize single-chain, bispecific CARs (biCARs) that trigger robust cytotoxicity against tumor cells expressing either CD19 or CD20 [134,225,226]. Dual targeting may improve response rates and sustained responses while limiting antigen escape [134,225,226]. In the first-in-human biCAR phase I/IIa trial (NCT03097770, completed in 2020), a bispecific anti-CD19/CD20 tandem receptor (tanCAR7 with 4-1BB/CD3 ζ) elicited a potent, lasting antitumor response in the absence of grade 3 or higher cell-related encephalopathy syndrome in patients with R/R NHL [226]. Similarly, another anti-CD19/anti-CD20 CAR-T (CAR-20.19-T) showed low toxicity and high efficacy as single agent in a phase I study with R/R B-NHL (Table 3) [134].

CAR-engineered natural killer (CAR-NK) cells constitute another CAR-based approach to cancer therapy [227,228]. The first anti-CD19 CAR-NK cell construct was composed of an anti-CD19 scFv, CD28, a costimulatory OX40 domain, CD3 ζ , and a membrane-bound form of IL-15 [135]. The addition of IL-15 enhanced the proliferation, persistence and activity of NK cells in preclinical models [135]. In a phase I/II trial, HLA-mismatched anti-CD19 CAR-NK cells derived from cord blood were administered to patients with R/R B-NHL, CLL/SLL and B-ALL after standard fludarabine/cyclophosphamide lymphodepletion (Table 3) [135,136]. The majority of the patients responded to the CAR-NK treatment and did not develop serious adverse events [135,136]. More recently, a similar anti CD19-NK cell therapy (NKX019, using HLA-mismatched anti-CD19 CAR-NK cells obtained from peripheral blood) entered a phase I study as a monotherapy for patients with R/R B-NHL, CLL and B-ALL (Table 3) [137]. NKX019 treatment has already shown long-term NK cell persistence and potent antitumor activity [137]. Additional clinical trials for CAR-NK cells for the treatment of B cell malignancies are being set up [14,228].

2.4.3. CD22 mAbs and CD22-Targeting CAR-T

CD22-targeting mAbs have emerged as promising treatment options with proven therapeutic value [16,21,98]. Two ADC-triggering anti-CD22 mAbs (inotuzumab ozogamicin (CMC544) and moxetumomab pasudotox) have been approved for the treatment of adult patients with B-ALL and R/R HCL, respectively (Table 2) [99,229–232]. Inotuzumab ozogamicin combines an anti-CD22 mAb with the DNA-binding drug calicheamicin, which leads to DNA breakage and apoptosis [16,21]. Moxetumomab pasudotox is an anti-CD22 scFv conjugated to a truncated 38 kDa fragment of *Pseudomonas* exotoxin A (PE38) [233]. In a phase I/II study (NCT01371630, 2011–2025), a combination of inotuzumab ozogamicin with mini-hyperfractionated cyclophosphamide, vincristine, dexamethasone and (in some cases) blinatumomab was found to be highly effective in patients with B-ALL after their first relapse [234]. A phase II study (ITCC-059; EUDRACT 2016-000227-71; NTR5736) provided further evidence of inotuzumab's activity in pediatric patients with R/R B-ALL [235]; the study is ongoing and is testing a combination of inotuzumab with chemotherapy in pediatric patients with R/R ALL. In an ongoing phase III randomized trial, inotuzumab ozogamicin is being tested as a first-line treatment with high-risk B-ALL

and B-lymphoblastic lymphoma (Table 3). However, phase I/II studies of inotuzumab ozogamicin alone or in combination with rituximab for the treatment of B-NHL did not give promising results [236–238]. Based on a phase I study of patients with R/R ALL, moxetumomab pasudotox's clinical activity appears to be moderate [231,239]. Only one phase I study with a BiTE construct targeting CD22 and CD3 (JNJ-75348780) is currently assessing patients with R/R NHL and R/R CLL (Table 3).

Over the last 10 years, many CD22-targeting CARs have been developed and tested in phase I/II clinical trials [16,240]. Two anti-CD22 CARs containing anti-CD22 scFv, IgG4 (or CD8), 4-1BB and CD3 ζ domains gave high response rates in patients with B-ALL who had previously failed chemotherapy and/or CD19-targeting CAR-T treatment (Table 3) [139]. Moreover, many bispecific anti-CD19/CD22 CAR-T cells have been developed to prevent relapse by minimizing antigen escape and have been assessed in phase I studies for the treatment of B-cell malignancies [16]. The first anti-CD19/CD22 CAR-T cell construct (4-1BB-CD3 ζ , LV20.19 CAR-T) contained a CD19-binding domain derived from a clinically active CD19-CAR, together with a CD22 binding domain [241]. In a phase I trial, LV20.19 showed low toxicity and high efficacy in R/R NHL and CLL (Table 3) [134]. On the same lines, the combination of another dual CD19/22-targeting CAR-T cell therapy (AUTO 3) with pembrolizumab led to complete remission in patients with R/R DLBCL (Table 3) [141]. A new anti-CD19/CD22-CAR-T construct (CD19-22.BB.z, alone or combined with an IL-15 receptor agonist) is being assessed in patients with R/R DLBCL or B-ALL in a phase I trial (Table 3) [142]. According to the initial results, almost all the patients with ALL achieved complete remission [142]. The results of a phase I trial recently confirmed the safety and efficacy of CD19.22.BB.z in heavily pretreated children and young adults with B-ALL (Table 3) [242]. On the basis of these preliminary data, it appears that anti-CD19/CD22 CAR-T cells may improve clinical responses by mitigating target antigen downregulation as a relapse mechanism. Another novel, bispecific anti-CD20/CD22 CAR-T is now being tested for safety in B-cell malignancies (Table 3). A phase I study of monotherapy with anti-CD22-NK cells is planned in patients with R/R NHL and who previously received anti-CD19 treatment (NCT03692767: not yet recruiting).

2.4.4. CD38 mAbs and CD38-Targeting CAR-T

Although the development of therapeutic anti-CD38 mAbs started in the 1990s and has progressed slowly since then, two such mAbs (daratumumab and isatuximab) are now used as combination chemotherapies for patients with R/R MM (Figure 2a and Table 2) [243–246]. In MM, the two Abs induce ADCC, CDC and ADCP as part of their antitumor mechanism of action [247,248]. Furthermore, isatuximab directly induces both lysosome-dependent death and caspase 3/7-dependent apoptosis of MM cells—even those bearing a p53 mutation [249]. Unlike daratumumab, isatuximab binds to a CD38 epitope that encompasses the CD38 ectoenzyme's catalytic site, but it is not known whether isatuximab exerts its therapeutic effect by inhibiting CD38's NADase activity [17]. Usefully, daratumumab is available in formulations for intravenous administration and subcutaneous administration [34,195]. One advantage of isatuximab over daratumumab is the need for less frequent dosing [34,195]. The most common adverse reactions to these two anti-CD38 Abs are neutropenia, pneumonia, upper respiratory tract infection, and diarrhea [34,195].

Following the observation of daratumumab's and isatuximab's efficacy in R/R MM, several clinical trials have evaluated the addition of one mAb or the other to standard-of-care regimens in newly diagnosed MM patients [250–252]. Based on these studies, the combination of daratumumab with bortezomib/thalidomide/dexamethasone (VRd) was recently approved by the FDA as treatment for these transplant-eligible patients [253]. Since then, two phase III trials (NCT03710603, NCT03652064) have compared the daratumumab-VRd combination with VRd in non-transplant-eligible patients and as an induction treatment before autologous stem cell transplantation in transplant-eligible patients. Other phase III trials are assessing the benefit of combining isatuximab with (i) lenalidomide + bortezomib + dexamethasone or lenalidomide + carfilzomib induction treatment in

patients with newly diagnosed MM (NCT03617731, NCT03319667, and NCT04483739), (ii) carfilzomib in patients with R/R MM (NCT03275285, 2017–2023), and (iii) lenalidomide in patients with high-risk smoldering MM (NCT04270409).

Data on the efficacy of daratumumab and isatuximab in other B cell malignancies are scarce. Daratumumab has been tested in phase I trials on patients with CLL and other B-NHL. In CLL, the prognostic value of CD38 expression in patients is well proven (Table 1), and preclinical studies (ex vivo experiments and CLL mouse models) have demonstrated the antitumor efficacy of daratumumab monotherapy or combination therapy [254,255]. The combination of daratumumab with ibrutinib in CLL patients with a poor prognosis (e.g., 17p deletion and/or p53 mutation) is now being studied (NCT03447808 and NCT03734198). The putative efficacy of daratumumab in B-ALL and other types of NHL has not yet been tested. A phase I/II study of the safety and tolerability of a combination of isatuximab with the anti-PD1 cemiplimab in patients with DLBCL started in 2018 (NCT03769181). The fact that CD38 is strongly expressed by some tumor cell subsets in pediatric patients with B-ALL [256] makes this antigen a potential target for the treatment of ALL in children. Isatuximab has significant antitumor activity in vitro and in ALL xenograft models, with robust ADCC- and ADCP-mediated effects [256]. A now-recruiting phase II multicenter study is evaluating the antitumor activity, safety, and pharmacokinetics of isatuximab combined with standard salvage chemotherapies in children after one or two relapses of B-ALL (NCT03860844) [257].

Along with daratumumab and isatuximab, several novel CD38-targeting strategies are being developed (mostly for MM); these include the use of naked anti-CD38 mAbs, anti-CD38 ADCs, anti-CD38/CD3 BiTEs, and CD38-targeting CAR-T cells [195,247,251,258]. Felzartamab (MOR202; TJ202) and TAK-079 are both fully human IgG1 mAbs [251,259]. Although felzartamab showed promising efficacy in MM (both alone and combined with immunomodulators; NCT01421186) [260], the drug's sponsor (MorphoSys AG) unexpectedly stopped this development in 2020 [259]. However, two ongoing trials are evaluating the effect of a combination of felzartamab and conventional therapies in patients with R/R MM (Table 3). The results of a multicenter phase I study showed that monotherapy with subcutaneously administered TAK-079 is safe and generally well tolerated in patients with R/R MM (Table 3) [261]. Two other clinical trials are evaluating a combination of TAK-079 with polalidomide or lenalidomide + dexamethasone in R/R MM and newly diagnosed MM (Table 3).

Two ADC-triggering anti-CD38 drugs (TAK-573 and TAK-169) have recently entered clinical development in the treatment of R/R NHL (Table 3) [144,145]. TAK-573 (modakaf-sup) is a CD38 mAb fused to attenuated interferon (IFN)- α ; it demonstrates potent anti-MM activity in patients with R/R MM [144]. TAK-169 is an engineered Ab with a deimmunized form of the ribosome-inactivating Shiga-like toxin A subunit genetically fused to a CD38-binding Ab fragment [145]. TAK-169 is internalized by CD38⁺ MM cells, inactivates ribosomes, abrogates protein synthesis and thus kills cells directly [145]. The full results of these clinical trials are eagerly awaited.

Among the many humanized anti-CD38/CD3 BiTEs having been through preclinical development, AMG-424 and ISB 1342 (GBR 1342) have shown strong antitumor effects in MM models [262,263]. A phase I study (NCT03445663) of AMG424 in R/R MM began in 2018 but was terminated by the sponsor for business reasons in 2020. Since ISB 1342 contains a full Fc domain with a reduced effector function, its mechanism of tumor cell killing critically relies on the engagement and activation of T lymphocytes [263]. ISB 1342 is currently in phase I trial for the treatment of patients with MM who have relapsed or who do not respond to standard therapies (e.g., proteasome inhibitors, immunomodulators, and daratumumab) (Table 3).

As is the case for CD19, CD20, and CD22, CD38 is an attractive target for CAR-T therapy. However, given the widespread expression of CD38 (e.g., in plasma cells, precursor B cells, T cells, NK cells, myeloid precursor cells, and various organs), anti-CD38 CAR-T cell constructs can induce various syndromic adverse events [251]. For instance, the

administration of anti-CD38 CAR-T cells to a patient with relapsed B-ALL (after the failure of bispecific anti-CD19/CD22 CAR-T cell treatment) was associated with target-mediated toxicity and severe cytokine release syndrome [264]. Some novel anti-CD38 CAR-T cells have been developed with a modified light chain and a high-affinity scFv [251]. A phase I study has evaluated the safety and efficacy of a novel CD38 A2 CAR-T cells construct in patients with R/R MM (NCT03464916, completed in 2022) but it is not known whether this construct has been efficacious in phase II/III trials. The strategies assessed in MM could also be investigated in the treatment of other CD38⁺ lymphoid tumors, including poor-prognosis CLL/SLL, HCL, and other B-NHL. Lastly, the promising preclinical efficacy seen with CD38-targeting NK cells suggests that clinical development is warranted [265,266].

3. Clinical Trials of Novel Anti-TAA Inhibitors in the Treatment of B Cell Malignancies

3.1. B Cell Maturation Antigen (BCMA) and B-Cell Activation Factor Receptor (BAFF-R)

BCMA (also known as tumor necrosis factor receptor superfamily member 17/TNFRSF17) is a transmembrane protein that contains cysteine-rich extracellular domains and lacks a signal peptide [267]. Along with the related TNFR superfamily member BAFF-R, BCMA critically regulates B cell proliferation, survival and differentiation into plasma cells [102,267,268]. BCMA is expressed at significantly higher levels by MM cells (Table 1) than by normal tissues (except for normal plasma cells) [269]. Thus, BCMA has become an important therapeutic target in MM, with four treatment modalities in development: ADCs, BITEs, CAR-T therapies and CAR-NK therapies [102,184,228,269–271]. In 2021, the ADC mAb belantamab mafodotin (GSK2857916) was the first anti-BCMA therapy to obtain approval in an indication of R/R MM (Table 2) [272]. Belantamab mafodotin is a humanized anti-BCMA IgG1 mAb linked to the antimetabolic drug monomethyl auristatin F [267]. Its efficacy is being evaluated in three clinical trials (Table 4) [273]. MEDI2228 is another ADC, in which an anti-BCMA mAb is covalently linked to the pyrrolobenzodiazepine dimer tesirine. This toxin induces DNA crosslinking and the DNA damage response [274]. The results of a recent phase I study (NCT03489525) [275] demonstrated that MEDI2228 had much the same level of efficacy as belantamab mafodotin [276]. The ADC AMG224 is a BCMA-targeting IgG1 mAb coupled to the tubulin inhibitor mertansine. The results of the first phase I dose-expansion and -escalation study in R/R MM (NCT02561962, 2015–2023) evidenced significant thrombocytopenia [277]. Two BiTE anti-BCMA/CD3 agents (teclistamab and elranatamab) are currently being evaluated in as monotherapies and in combination with other treatments (Table 4) [278]. The initial results for the anti-BCMA CAR-T therapy idecabtagene vicleucel showed clinical activity and the expected adverse effects in patients with R/R (Table 4) [279]. Accordingly, the anti-BCMA CAR-T therapy ciltacabtagene autoleucel was associated with significantly better outcomes than conventional therapies in patients with R/R MM (NCT033548207) [280]. In an early phase I study, the first dual-target anti-BCMA/CD38 CAR-T (BM38) gave a high response rate, a low recurrence rate, and a manageable cytokine release syndrome in patients with R/R MM (Table 4) [281]. A now-recruiting phase I/II study is set to evaluate the safety and antitumor efficacy of BCMA-CAR-NK92 cells in patients with R/R MM (Table 4). Three phase I trials are respectively evaluating adverse events associated with the anti-BAFF-R mAb lanalumab in patients with CLL, and another trial is evaluating anti-BAFF-R-CAR-T cells in B-ALL and R/R MCL (Table 4).

Table 4. Selected ongoing and now-recruiting clinical trials of novel anti-TAA inhibitors in B cell malignancies.

Drug	Class	Disease Setting	Phase	Study	References
Belantamab mafodotin/ GSK2857916	ADC anti-BCMA- monomethyl auristatin F	R/R MM	I/II	NCT03848845 Active, 2018- Alone or combined with pembrolizumab	[273]
			I/II	NCT04126200 NCT03544281 NCT03715478 Recruiting, 2018- Alone or combined with current therapies	
Teclistamab/ JNJ-64007957	BiTE anti-BCMA/CD3	R/R MM	I/II	NCT04557098 Active, 2017–2024	[278]
			I	NCT04586426 Recruiting, 2019–2024 Combined with talquetamab or daratumumab or pomalidomide	
			III	NCT05083169 Recruiting, 2021–2026 daratumumab compared with current therapies	
Elranatamab/ PF-06863135	BiTE anti-BCMA/CD3	Refractory MM	II	NCT04649359 Active, 2020–2024	
Idecabtagene vicleucel/ bb2121	Anti-BCMA CAR-T	R/R MM	II	NCT03361748 Active, 2017–2024	[279]
BM38	Dual anti-BCMA/CD38 CAR-T	R/R MM	I	ChiCTR1900026286 Active, 2019-	[281]
BCMA-CAR-NK92	Anti-BCMA CAR-NK	R/R MM	I/II	NCT03940833 Recruiting, 2019–2022	[282]
Lanalumab/VAY-736	Anti-BAFF-R Ab	CLL	I	NCT03400176, Recruiting, 2018- Alone or combined with ibrutinib	
BAFFR-CAR T	Anti-BAFF-R CAR-T	B-ALL, R/R MCL	I	NCT04690595, NCT05370430 Recruiting, 2020–2024	
		CLL	II	NCT04501939 Recruiting, 2020- Combined with venetoclax	
Cirmtuzumab/ UC961 newly renamed Zilovertamab	Anti-ROR1 Ab	CLL, SLL, MCL	I/II	NCT03088878 Active, 2018–2025 Combined with ibrutinib	
		R/R MCL	III	NCT05431179 Recruiting, 2022- Combined with ibrutinib	

Table 4. Cont.

Drug	Class	Disease Setting	Phase	Study	References
Pembrolizumab/MK-3475	Anti-PD1 Ab recruiting immune cells to attack tumor B cells	R/R CLL, B-NHL	II	NCT02332980 Active, 2015–2023 Alone or combined with ibrutinib or idelalisib	[283]
		High-Risk or R/R CLL/SLL	II	NCT03204188 Active, 2017–2030 Combined with ibrutinib, fludarabine	
		R/R WM	II	NCT03630042 Recruiting, 2018- Combined with rituximab	
		R/R B-ALL	I/II	NCT03512405 Recruiting, 2018- Combined with blinatumomab	[284]
Cemiplimab/REGN2810	Anti-PD1 Ab recruiting immune cells to attack tumor B cells	R/R DLBCL	I	NCT03340766 Active, 2017- Combined with blinatumomab	
		B-NHL	I	NCT02651662 Active, 2016- Combined with REGN1979 (anti-CD20-CD3)	
		R/R MM	I/II	NCT03194867 Active, 2016- Combined with isatuximab	
Atezolizumab	Anti-PD-L1 Ab	DLBCL	I/II	NCT03769181 Active, 2018- Combined with isatuximab	[285]
		DLBCL	I/II	NCT02926833 Active, 2016–2022 Alone or combined with axicabtagene ciloleucel	[286]
Ulocuplumab/BMS-936564	Anti-CXCR4 Ab	WM	I/II	NCT03225716 Active, 2017–2025 Alone or combined with ibrutinib	[287]
Mavoxifafor/AMD070	CXCR4 antagonist (blocks CXCL12 binding)	WM	I	NCT04274738 Active, 2020–2025 Alone or combined with ibrutinib	
CS1-CAR-T	CS1-CAR-T/IL7-CCL19	R/R MM	I	NCT03778346 Recruiting, 2018–2022	
CS1-CAR-T	CS1-CAR-T/41BB-tEGFR	R/R MM	I	NCT03710421 Recruiting, 2018–2023 following chemotherapy	

Table 4. Cont.

Drug	Class	Disease Setting	Phase	Study	References
J1/Melflufen	Melphalan-p-fluoro-L-phenylalanine (aminopeptidase substrat)	MM refractory to lenalidomide	III	NCT03151811 Active, 2017-combined with dexamethasone compared to pomalidomide + dexamethasone	[288]
Brentuximab vedotin	ADC anti-CD30-mono methyl auristatin E	R/R B-NHL	II	NCT03646123 Active, 2018–2026	
Lilotomab/tetulumab/betalutin	ARC anti-CD37- ¹⁷⁷ lutetium	Relapsed B-NHL	I/II	NCT01796171 Recruiting, 2013–2026	[289]
GEN3009	DuoHexaBody-CD37	R/R B-NHL	I	NCT04358458 Recruiting, 2020–2025	
CART30	Anti-CD30 CAR-T	B-NHL	I	NCT02259556 Recruiting, 2016–2029 Combined with cyclophosphamide and fludarabine	[290]
CD30.CAR-Ts	Anti-CD30 CAR-T	B-NHL	I/II	NCT02690545 Recruiting, 2016–2038 Combined with fludarabine and bendamustine	[291]
CD30.CAR T	Anti-CD30 CAR-T	B-NHL	I	NCT02917083 Recruiting, 2018–2036	
ICAR30	Anti-CD30 CAR-T	B-NHL	I	NCT03383965 Recruiting, 2017–2025	
ATLCAR.CD30	Anti-CD30 CAR-T	B-NHL	I	NCT026663297 Active, 2016–2037	
		R/R B-NHL	I/II	NCT02953509 Active, 2016–2026 Combined with rituximab and (in some cases) with chemotherapy	[292]
Magrolimab/Hu5F9-G4	Anti-CD47	R/R MM	II	NCT04892446 Recruiting, 2021–2024 Combined with daratumumab, or pomalidomide and dexamethasone, or bortezomib and dexamethasone	
		R/R B-NHL	II	NCT04788043 Recruiting, 2021–2027 Combined with pembrolizumab	
IBI322	Bi anti-CD47/PD-L1	ALL, CLL/SLL B-NHL	I	NCT04795128 Recruiting, 2021–2024	

Table 4. Cont.

Drug	Class	Disease Setting	Phase	Study	References
TG-1801/ NI-1701	Bi anti-CD47/CD19	B-NHL	I	NCT03804996 Active, 2019–2022 Combined with ublituximab	[293]
		B-NHL, CLL	Ib	NCT04806035 Recruiting, 2021–2023 Alone or combined with ublituximab	
CPO107/ JMT601	Fusion protein Anti-CD20-SIRP α	Advanced B-NHL	I	NCT04853329 Recruiting, 2021–2024	
STRO-001	ADC anti-CD74- maytansoid	DLBCL, FL, MM	I	NCT03424603 Recruiting, 2018–2023	[294]
Polatuzumab vedotin	ADC anti-CD79b- monomethyl auristatin E	DLBCL	I	NCT04231877 Recruiting, 2020–2031 Combined with rituximab, prednisone, etoposide, doxorubicin, cyclophosphamide	
		R/R DLBCL	I	NCT04739813 Recruiting, 2021–2027 Combined with venetoclax, ibrutinib, prednisone, obinutuzumab, and lenalidomide	
		R/R B-NHL	Ib/II	NCT03533283 Recruiting, 2018–2024 Combined with glofitamab	
Cevostomab/ BFCR4350A	BiTE-anti- FcRL5/CD3	R/R MM	I	NCT03275103 Recruiting, 2017–2023	[295]
Talquetamab/ JNJ-64407564	BiTE duoBody-anti- GPRC5D/CD3	R/R MM	I/II	NCT03399799 Recruiting, 2018–2025	[296]

ADAM17, a disintegrin and metalloprotease 17; ADC, antibody drug conjugate; BAFF-R, B-cell activating factor receptor; BCMA, B cell maturation antigen FcRL5, Fc receptor-like 5; GPRC5D, G-protein coupled receptor family C group 5 member D; ROR1, receptor tyrosine kinase-like orphan receptor 1; tEGFR, truncated epidermal growth factor receptor.

3.2. Receptor Tyrosine Kinase-like Orphan Receptor (ROR1)

Aberrant expression of ROR1 is seen in CLL/SLL, MCL, and MM [47]. In CLL, ROR1 expression is associated with relatively short treatment-free and overall survival times [297,298]. As a receptor for Wnt5a [298], ROR1 can stimulate ROR1-dependent CLL cell activation of Rho-GTPases [299]. High expression levels of ROR1 and strong ROR1 signaling are associated with venetoclax resistance in CLL [300]. A phase I/II study in newly treated or R/R CLL patients showed that the mAb anti-ROR1 cirmtuzumab (recently renamed as zilovetamab) was safe and efficacious in inhibiting tumor cell ROR1 signaling in the context of R/R disease (NCT02860676, NCT02222688) [301]. Since then, several clinical trials of a combination of zilovetamab with venetoclax or ibrutinib have been initiated in patients with CLL/SLL or MCL (Table 4).

3.3. Program Cell Death 1 (PD1) and PD-Ligand 1 (PD-L1)

The immunosuppressive costimulatory signal receptor PD-1 is expressed on activated T and B cells [302,303]. PD-L1 is strongly expressed by various solid tumors, B tumor cells (including DLBCL and MM cells), and BM stromal cells [303,304]. As the tumor develops, both PD-1 and PD-L1 cause tumor immune escape—mainly by inhibiting the activity of tumor-infiltrating lymphocytes [303,304]. Several mAbs against PD1 (nivolumab, pembrolizumab, and cemiplimab) or PD-L1 (e.g., atezolizumab and durvalumab) have been approved for the treatment of various solid tumors [305,306]. Anti-PD-1 mAbs promote tumor cell apoptosis by binding to T-cell PD-1 receptors and disrupting the interaction with PD-L1 molecules on tumor cells [302]. However, no significant objective responses were observed in two phase I/Ib trials of nivolumab as a monotherapy or a combination therapy in patients with R/R MM [307,308]. Pembrolizumab exhibits selective efficacy in patients with B-NHL and CLL [283], and is currently being tested in combination with ibrutinib or fludarabine (Table 4). In a phase I/II study, a pembrolizumab + blinatumomab combination had a good safety profile (manageable adverse events) in R/R B-ALL (Table 4) [284]. However, a phase III study of a combination of pembrolizumab with pomalidomide and dexamethasone in R/R MM (NCT02576977) found an unfavorable risk-benefit ratio and was terminated [170,309]. Three clinical studies are evaluating the safety and tolerability of cemiplimab in combination with odronextamab (an anti-CD20/CD3 BiTE) or with isatuximab in patients with B-NHL (including R/R MM and DLBCL) (Table 4) [285]. A phase I study of atezolizumab alone or combined with axicabtagene ciloleucel showed a manageable safety profile in patients with R/R DLBCL and thus supported a move into phase II (Table 4) [286]. Durvalumab as a monotherapy or a combination therapy with tremelimumab (an anti-CTLA-4 mAb) or danvatirsen (AZD9150, an antisense oligonucleotide that inhibits STAT3) was well tolerated but showed limited efficacy in R/R DLBCL or FL (NCT02733042) [310,311]. The combination of durvalumab with JCAR014 (CD19/4-1BB CAR-T cells) for the treatment of patients with aggressive B-cell NHL appeared safe; although complete responses were observed in patients at initial restaging after JCAR014 infusion, and in patients continuing durvalumab therapy after initially failing to achieve complete remission (NCT02706405), the study was terminated in 2021 due to slow accrual [312].

3.4. C-X-C Chemokine Receptor Type 4 (CXCR4)

CXCR4 is the natural chemokine receptor specific for CXCL12, also termed stromal cell-derived factor-1 (SDF-1), which is released by the stromal cells residing in the thymus and in the bone marrow [313]. The overexpression of CXCR4 in solid and hematological tumors is related to disease progression [41]. In hematological malignancies, high expression of CXCR4 is observed in B cell malignancies (Table 1) [313]. Highly recurrent somatic mutations in CXCR4 gene are found in up to 40% of patients with WM [313]. Alterations in CXCR4 are associated with activation of PI3K and JAK/STAT signaling processes, which promote drug resistance by impacting BTK-inhibitor response [313]. Thus, WM patients with mutated CXCR4 are less likely to respond to ibrutinib or ibrutinib/rituximab combination therapy than WM patients with CXCR4 wild-type disease [287]. Ulocuplumab (BMS-936564) is a fully human IgG4 anti-CXCR4 mAb [314] that exhibits antitumor activity in a WM xenograft model [315]. A phase Ib/II trial showed that ulocuplumab is safe and leads to a high response rate in combination with lenalidomide and dexamethasone in patients with R/R MM (NCT01359657) [316]. An ongoing phase I/II clinical trial evaluating the combination of ibrutinib and ulocuplumab, in patients with WM, already shows that ulocuplumab dose-escalation did not impact adverse events (Table 4) [287]. Interestingly, an ongoing multicenter phase I trial is assessing the safety and tolerability of mavorixafor (AMD070, an orally bioavailable CXCR4 allosteric antagonist) in combination with ibrutinib in WM patients with CXCR4 mutation (Table 4).

3.5. CS1/Signaling Lymphocyte Activation Molecule Family 7 (SLAMF7)

CS1/SLAMF7 is overexpressed in MM cells, NK cells, plasma cells, and (to a lesser extent) some subsets of B and T cells [317]. Elotuzumab is a humanized IgG1 Ab that directly activates NK cells through the CS1 pathway and Fc receptors, thus facilitating NK-mediated ADCC [317]. In 2015, the US FDA approved elotuzumab in combination with lenalidomide and dexamethasone for the treatment of patients with MM who received one to three prior therapies, with the following warnings and precautions: infusion reactions, infections, second primary malignancies, hepatotoxicity, and interference with the determination of complete response [318]. Two phase I clinical studies are evaluating two different CS1-CAR-T constructs in patients with R/R MM (Table 4).

3.6. Other TAAS

3.6.1. CD13

The cell surface protease aminopeptidase N (APN/CD13) is overexpressed in various tumors including hematological diseases [319,320]. Based on its ability to bind to the Asn-Gly-Arg (NGR) motif, CD13 has proven to be a key for targeted delivery of NGR-bound chemotherapeutic drugs to both CD13⁺ tumor cells and CD13⁺ tumor-associated endothelium [320]. Among drugs coupled to the NGR motif, is the anti-angiogenic truncated tissue factor (tTF) that targets CD13⁺ tumor-associated vasculature [320]. A recent phase I trial with NGR-tTF was initiated for patients with CD13⁺ R/R MM (NCT02902237, completed in 2020) indicating that NGR-tTF is safely applicable with this regimen; imaging showed selective reduction of tumor blood flow and intratumoral hemorrhage and necrosis [321]. This innovative approach deserves to be pursued. A second therapeutic strategy exploited the enzymatic activity of CD13 for the activation of the alkylating pro-drug melflufen (J1; dipeptide consisting of melphalan and *p*-fluoro-L-phenylalanine) into an active cytotoxic drug, melphalan [322]. In phase I/II studies, melflufen plus dexamethasone demonstrated encouraging clinical activity and a manageable safety profile in heavily pretreated patients with R/R MM (NCT02963493, completed in 2021) [323]. A randomized phase III study is evaluating the efficacy and safety of melflufen/dexamethasone vs. pomalidomide/dexamethasone (Table 4) [288]; melflufen plus dexamethasone shows clinically meaningful efficacy and a manageable safety profile in patients with heavily pretreated R/R MM, including those with triple-class-refractory and extramedullary disease (Table 4) [288].

3.6.2. CD16

NK cells express a CD16 (FcγRIII) V158 variant, which can exert ADCC by binding to the Fc fragment of Ig present on tumor cells [223]. Based on it, one approach was the engineering of T cells expressing CD16 CARs so that they are capable of mediating ADCC [324]. The constructs included the high-affinity CD16 V158 variant, CD8α hinge, and transmembrane domains, along with signaling domains from CD3ζ and CD28 or 4-1BB signaling domains [191,223]. Two completed first-in-human pilot studies already showed the efficacy (safety and antitumor activity) of infusing autologous T-cells engineered to express CD16V-41BB-CD3 ζ, in combination with rituximab, in patients with CLL and B-NHL (NCT02315118), and R/R B-NHL (NCT02776813) [325].

3.6.3. CD30

This antigen belongs to TNF receptor superfamily, and CD30 signaling is involved in regulation of cytokine secretion, cell proliferation and survival [326]. CD30 is highly expressed in most B-NHL [326–328]. Brentuximab vedotin, an ADC comprising a human anti-CD30 chimeric Ab covalently bound to the microtubule-disrupting agent monomethyl auristatin E, was approved in R/R B-NHL after autologous stem cell transplantation and anaplastic large-cell lymphoma [326]. One phase II trial is now evaluating brentuximab vedotin in combination with three chemotherapy drugs (doxorubicin, vinblastine, and dacarbazine) for B-NHL (Table 4). Anti-CD30 CAR-T cells therapies have demonstrated significant clinical responses in early clinical trials of patients with B-NHL (Table 4) [290,291].

There are other ongoing clinical trials with different CD30 CAR-T cell constructs in R/R lymphomas addressing ways to improve outcome (Table 4) (reviewed in [329,330]).

3.6.4. CD37

CD37 is expressed almost exclusively on hematopoietic cells with high expression on mature B-cells, including their malignant counterparts [331]. A large number of CD37 targeting agents have been developed including mAbs, ARCs, ADCs, and CAR-T cells [332–335]. Among them, the anti-CD37 BI856826, initially reported as a valid therapeutic target in B-NHL (NCT01403948) [336] and CLL (NCT02759016) [334], did not undergo further clinical development upon the decision of sponsors to discontinue the trials. A phase I/II study is evaluating the safety and efficacy of the ARC ¹⁷⁷Lutetium-lilotomab satetraxetan (betalutin) for treatment of relapsed B-NHL (Table 4) [289]. In contrast to other newly developed CD37 targeting agents, the duoHexaBody-CD37 (GEN3009, a novel biparatopic CD37-bispecific IgG1 with an E430G hexamerization-enhancing single point mutation in the Fc-domain), mediates potent CDC in addition to ADCC and cellular phagocytosis [131]. A first-in-human clinical trial is evaluating GEN3009 in patients with R/R B-NHL (Table 4). Naratuximab emtansine (IMGN529, Debio1562) is an ADC anti-CD37 mAb conjugated to maytansine-derived microtubule disruptor; it was investigated alone in CLL and R/R B-NHL (NCT01534715) [337], and in combination with rituximab in R/R DLBCL, FL and MCL (NCT02564744), where the treatment was well-tolerated and resulted in complete response rates and a manageable safety profile [338]. The second ADC, AGS67E, is an anti-CD37 mAb conjugated to monomethyl auristatin E, that showed a favorable safety profile in a phase I clinical trial in patients with CLL and R/R B-NHL (NCT02175433) [339]. Extended studies are awaited.

3.6.5. CD46

CD46 (also referred to as membrane cofactor protein, MCP) is coexpressed as four predominant isoforms on almost all cell types, and is overexpressed on a variety of human tumor cells [340]. Clinical and experimental data support an association between increased CD46 expression, malignant transformation, and metastasizing potential [340]. More recently, CD46 has been reported as a driver of metabolic processes [340]. The ADC anti-CD46-monomethyl auristatin E (FOR46) demonstrated an acceptable toxicity profile in a phase I study to treat patients with R/R MM (NCT03650491) [341].

3.6.6. CD47

This ubiquitous antigen belongs to the immunoglobulin superfamily [342], and exhibits various cellular functions with multiple binding partners, such as inhibition of phagocytosis through an interaction with signal-regulating protein alpha (SIRP α) on the surface of phagocytic cells [343]. Many clinical trials have started to evaluate numerous anti-CD47 mAbs (and derivatives) in the treatment of solid tumors and hematological neoplasms including B cell diseases [343,344]. Monotherapy phase I clinical trials of anti-CD47 Abs often yielded limited signs of efficacy compared with combination strategies [343]. Among them, the humanized anti-CD47 mAb magrolimab selectively binds to CD47 expressed on tumor cells and blocks the interaction of CD47 with SIRP α , thus allowing the activation of macrophages, resulting in specific tumor cell phagocytosis [345]. Furthermore, blocking CD47 signaling activates an antitumor T-lymphocyte immune response and T-mediated cell killing [345]. Of interest, the phase II study (NCT04778397) enrolling patients with AML or myelodysplastic syndrome who received magrolimab in combination with azacytidine, was paused in 2022 following suspicions of safety issues [346]. Thus, particular attention should be paid to magrolimab, which is currently tested in combination with standard therapies in several trials in B-NHL (Table 4). A second anti-CD47 (JMT601/CPO107) is a bispecific fusion protein in which the CD47 binding fragment SIRP α is covalently fused to ofatumumab (an anti-CD20); it thus targets both CD20 and CD47 on tumor cells and leads

to ADCC and CDC [344]. A phase I/II multicenter clinical trial in CD20⁺ B-NHL patients is starting (Table 4).

3.6.7. CD56

This antigen, primarily expressed in cells of neuroendocrine origin as well as in NKs and some T cell subtypes, is overexpressed in several solid and hematologic malignancies, including MM [347]. The ADC lorvotuzumab mertansine (IMGN901) combines the anti-CD56 Ab lorvotuzumab covalently bound to the cytotoxic maytansinoid effector. A phase I trial of lorvotuzumab mertansine provided evidence of safety and signals of clinical activity for this agent in R/R MM (NCT00346255) [348], warranting its further clinical development as part of combination regimens for the management of MM.

3.6.8. CD74

Otherwise known as HLA-DR-associated invariant chain, CD74 functions as an MHC class II chaperone and as a high affinity receptor for the pro-inflammatory cytokine macrophage migration inhibitory factor [349]. CD74 is overexpressed in some B-NHL [349]. A phase I study with the anti-CD74 mAb milatuzumab [350] showed no objective responses in R/R MM (NCT00421525) [351]. ASTRO-001 is a fully human ADC anti-CD74 incorporating maytansinoid [352]; a phase I study of ASTRO-001 is recruiting patients with advanced B cell malignancies including DLBCL, FL and MM (Table 4) [294].

3.6.9. CD79B

CD79 is the BCR's signaling component (Figure 1b) and is composed of a heterodimer of CD79A and CD79B, both of which induce internalization of the BCR [353]. CD79 is strongly expressed in most types of B-NHL and CLL but weakly expressed on normal cells (with restriction to pre-B and mature B cells) [353]. Polatuzumab vedotin is an ADC humanized anti-CD79B Ab conjugated to monomethyl auristatin E, that showed significant clinical activity in phase I trials to treat patients with FL and DLBCL [353]. In 2019, it was FDA-approved in combination with bendamustine and rituximab for patients with R/R DLBCL after at least two prior therapies [353]. Two phase I trials are evaluating polatuzumab vedotin in combination with several conventional chemotherapies for untreated or R/R patients with DLBCL and other B-NHL (Table 4).

3.6.10. CD156/a Disintegrin and Metalloprotease 17 (ADAM17) (Also Referred to as TNF- α Converting Enzyme/TACE)

The cell surface protease ADAM17 is overexpressed in certain solid and hematological tumors [354], and the development of a large variety of inhibitors against the enzymatic activity of ADAM17 has made it into clinical evaluation in cancers [320]. Among these ADAM17 inhibitors, aderbisib (INCB7839) [355] entered a phase I/II clinical trial, to be used along with rituximab as consolidation therapy after an autologous hematopoietic cell transplant for patients with DLBCL (NCT02141451) (Table 4). The short-term results suggest applicability as relapse preventing therapy for DLBCL [356].

3.6.11. CD307/Fc Receptor-like 5 (FcRL5)

CD307/FcRL5, specifically expressed within the B cell lineage [357], belongs to transmembrane FcRL family proteins with sequence homology to classical Fc receptors, which can promote B cell proliferation and activation [317]. Present on malignant cells from patients with MM, CLL and MCL, CD307 may be therefore a potential therapeutic target for the treatment of these diseases [358]. Cevostomab (BFCR4350A) is a BiTE anti-FcRL5/CD3 Ab being evaluated in an ongoing phase I, dose escalation and expansion trial (Table 4) [295].

3.6.12. G-Protein Coupled Receptor Family C Group 5 Member D (GPCR5D)

GPCR5D is ubiquitously expressed on healthy and malignant plasma cells, and high expression on MM cells associated with adverse prognosis [359]. Talquetamab is a BiTE

duobody anti-GPRC5D/CD3 currently evaluated in a phase I study, in which it is administered both intravenously or subcutaneously in patients with R/R MM (Table 4); it already shows a high clinical response rate and appears well-tolerated, allowing the next exploration of dosing strategies [296].

4. Targeting Metabolic Reprogramming in B Cell Malignancies—Preclinical and Clinical Studies

4.1. Metabolic Reprogramming in B Cell Malignancies

4.1.1. The Metabolism in Cancer Cells, the Warburg Theory

The main characteristics of tumor cells (e.g., evading cell death, insensitivity to anti-growth signals, limitless replicative potential, tissue invasion and metastasis, sustained angiogenesis) were defined two decades ago as “the hallmarks of cancer” [360]. Ten years later, the same authors updated this model of acquired capabilities of cancer by including two extra features that emerged as crucial factors for cancer growth and progression, e.g., metabolic reprogramming and immune evasion [361]. When considering the current treatments of B cell malignancies, both hallmarks are very promising and intensively studied sources of inspiration for the development of new therapeutic strategies. Besides the excitement for immunotherapy with a tremendous number of engineered CAR-T/CAR-NK cells strategies (Section 3.4), the discovery of metabolic vulnerabilities in tumor cells is also likely to profoundly impact the development of new therapeutic options [362].

To compensate for its elevated bioenergetic needs associated to increased proliferation and biosynthesis, tumor cells must adapt and reprogram their metabolism to increase nutrients import. In particular, a dramatic increase in glucose uptake and a shift toward its oxidation through aerobic glycolysis (referred to as the “Warburg effect”) is observed in most tumor cells (Figure 3) [362]. Glucose is first converted into pyruvate through 10 enzymatic reactions, the first of which is catalyzed by hexokinase 2 (HK2) (Figure 3). Pyruvate, the final product of glycolysis, is further transformed into lactate by the lactate dehydrogenase-A (LDHA) (Figure 3). Another major alternative to produce energetic ATP occurs through mitochondria, where the oxidation of various nutrients (including amino acids such as glutamine, or lipids such as free fatty acids/FFA) can feed the tricarboxylic (TCA) cycle (Figure 3). The TCA can then activate mitochondrial respiration through the electron transport chain, which promotes oxidative phosphorylation (OXPHOS) and silences the Warburg effect and the shift to glycolysis (Figure 3).

4.1.2. Specific Metabolic Features of B Cell Malignancies

While the Warburg effect describes the typical metabolic response in aggressive B-NHL [363], other B cell malignancies favor mitochondrial respiration, such as CLL.

Glycolysis: the expression of the rate limiting enzyme (HK2, an indicator of glycolytic activity) is often upregulated in B-NHL [364,365]. No change in glycolysis is observed in CLL, although an increase in glycolytic capacity and reserve appears linked to drug resistance [366]. As a consequence, the blockade of the GLUT4 glucose transporter by the HIV protease inhibitor ritonavir leads to in vitro cell death in CLL [367] and potentiates the efficacy of metformin in MM cell lines and xenograft models [368]. A recent clinical trial demonstrated the safety, tolerability and feasibility of metformin + ritonavir combination therapy in patients with R/R MM or CLL (NCT02948283) [369]. Furthermore, the expression of lactate dehydrogenase A (LDHA) is upregulated in NHL [370] including MM [210]. Because of the involvement of glycolysis in B-NHL progression, the targeting of lactate trafficking through the blockade of monocarboxylate transporter 1 (MCT-1) by AZD3965 shows very promising results in NHL [371], including DLBCL [372]. A phase I clinical trial of AZD3965 combined to rituximab in DLBCL has recently been completed (NCT01791595) [373]. Independently of glycolysis, one regulatory mechanism has been described in B-cell malignancies by which glucose is redirected toward glycolysis or the pentose phosphate pathway (PPP), offering new options with the LB-100 inhibitor [374].

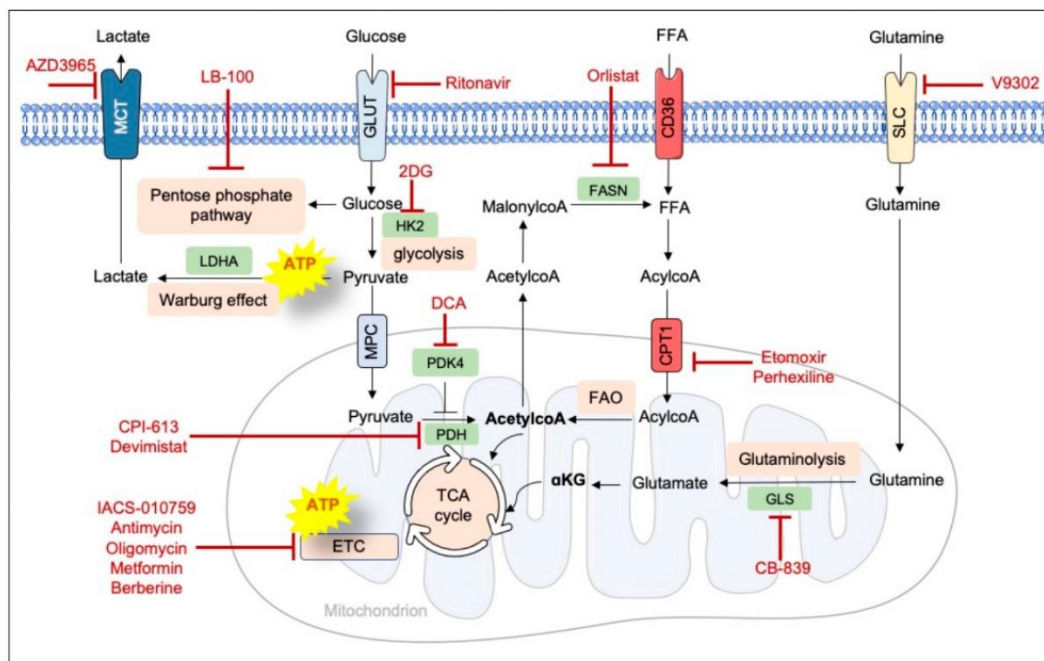


Figure 3. The cell's main metabolic pathways and the corresponding inhibitors. Glucose, free fatty acids (FFAs) and glutamine enter cells via glucose transporters (GLUT), CD36, and solute carrier (SLC) transporters respectively. Once inside the cells, these substrates are catabolized by various metabolic reactions in the cytosol and the mitochondria in the tricarboxylic acid cycle (TCA). Cofactors produced by the TCA are used by the electron transport chain (ETC) to produce ATP. Some cancer cells shift their metabolism (and specifically glycolysis) towards lactate production; ATP is generated outside the mitochondria. Lactate can then be exported from the cells via monocarboxylate transporters (MCTs). Inhibitors are shown in red, key enzymes are labeled in green, and metabolic pathways are shown in pink. α KG, α -ketoglutaric acid; FASN, fatty acid synthase; FAO, fatty acid oxidation; GLS, glutaminase; HK2, hexokinase 2; LDHA, lactate dehydrogenase A; PDH, pyruvate dehydrogenase; PDK4, pyruvate dehydrogenase kinase 4.

Mitochondrial OxPhos: an increase in OxPhos has been observed in MCL [375], MM [376] and CLL [377,378]. The increase in OXPHOS in CLL leads to increase in the oxidative stress [379], and forcing mitochondrial respiration through PI3K/AKT hyperactivation leads to toxic accumulation of ROS, resulting in CLL cell death [380]. In DLBCL, the combination of AZD3965 with the mitochondrial respiration inhibitor IACS-010759 in B cell lines and xenografts suggests a clear clinical advantage compared with monotherapy [381]. The novel mitochondrial disrupter CPI-613 (devimistat, a lipoate analog) inhibits the mitochondrial enzymes pyruvate dehydrogenase and α -ketoglutarate dehydrogenase in the TCA cycle [382,383]. A phase II study with CPI-613 is now recruiting patients with R/R NHL, including those with high-risk translocations of Myc, BCL2 and/or BCL6 (NCT03793140).

Lipids: beside carbohydrates metabolism, the metabolism of lipids is also affected in B-cell malignancies [264]. Lipid metabolism is upregulated in BL [384]. In CLL, high expression of lipoprotein lipase LPL is associated with a shorter treatment-free survival and a trend of a shorter median overall survival [385]. Its overexpression causes an increase in the β -oxidation of FFAs [386], while its inhibition by orlistat induces apoptosis of CLL cells [387]. Orlistat also induces cell death in MCL by inhibiting fatty acid synthase (FASN) [388] while pharmacological or genetic inhibition of FASN in DLBCL also induces apoptosis [389]. The antigen CD36 that facilitates FFA uptake by cells, is upregulated in CLL, and its inhibition by anti-CD36 neutralizing Abs or sulfosuccinimidyl oleate (SSO)

leads to apoptosis [390]. Similarly, the blockade of FFAs import into mitochondria via their β -oxidation by perhexiline (inhibitor of carnitine palmitoyltransferases (CPT), essential for transporting long chain fatty acid into mitochondria) [391] or the irreversible inhibitor of CPT-1, etomoxir [377], induces CLL cell death.

Amino acids: amino acids play a crucial role in the progression of B-cell malignancies. In peculiar, addiction of tumor cells to glutamine is observed in many tumors including MM [392] and CLL with 11q deletion [393], and in the Burkitt lymphoma cell line P493 [394]. The glutaminase inhibitor CB-839 (telaglenastat) has been tested in phase I clinical trial in MM and WM, but the results are not yet available (NCT02071888).

4.1.3. Involvement of the Tumor Microenvironment in the Adaptive Metabolism

Often, tumor cells depend on microenvironmental non-malignant cells for survival. Indeed, while some intrinsic common metabolic characteristics can be observed in B cell malignancies, metabolism can be modified by the contact of tumor cells with the adjacent tumor microenvironment (TME) [395,396]. For instance, interaction of tumor CLL cells with surrounding bone marrow (BM) stromal cells is shown to induce a shift toward glycolysis [379,397], that is in part mediated by Notch/c-Myc signaling [379]. Protection by the TME involves the transcriptional regulation of genes involved in redox homeostasis [398]. Furthermore, the protection of CLL cells from oxidative stress is also handled through a metabolic collaboration with stromal cells which transform cystine to cysteine, and cysteine transport to CLL cells allows the synthesis of GSH to promote cell survival and drug resistance [399]. As recently reviewed by Nie et al. [378], the agents targeting metabolic enzymes or signal pathways activated in CLL by lymph nodes (LN) microenvironment (converging to glucose, purine and glutamine metabolism) may effectively impede the progression of CLL [400]; these data provide further insight into the crucial metabolic changes driven by the CLL LN microenvironment and the prominent use of amino acids as fuel for the TCA cycle, in view of new therapeutic vulnerabilities. A high mitochondrial respiration in MM and B-ALL cells appears to be the consequence of the direct transfer of mitochondria from stromal to tumor cells [401,402]; this phenomenon in MM cells is driven by CD38 [402]. Tumor-associated adipocytes support MM cells through various mechanisms, including metabolic reprogramming of MM cells which involves MM cell-induced lipolysis in BM adipocytes, upregulation of FA transporters 1 and 4 on MM cells which then mediate the uptake of secreted free FFAs, leading to enhanced MM proliferation [403].

In contrast, tumor-associated stromal cells can metabolically stimulate the growth and metastasis of cancer by producing a “reverse Warburg effect” [404,405]. Thus, stromal cells shift from glycolysis to mitochondrial respiration in cases of B-NHL [406,407]; in particular, the lactate trafficking that promotes tumor growth in NHL is caused by the upregulation of MCT4 in tumor-associated macrophages and the concomitant upregulation of MCT-1 in tumor cells [371,408]. The blockade of lactate by AZD3965 leads to NHL cell apoptosis [371,408].

In conclusion, these examples of metabolic vulnerabilities open up new opportunities for the treatment of B cell malignancies by cutting off the tumor’s supplies of energy substrates.

4.2. Targeting Metabolism in B Cell Malignancies: Clinical Perspectives

4.2.1. Metabolic Features as Biomarkers of B Cell Malignancies

Metabolomic approaches on plasma of patients with B cell malignancies may allow to identify metabolic biomarkers for early diagnosis and prediction, prognosis and treatment [409]. Although many metabolic biomarkers have already shown their clinical validity, many others have not yet been subjected to extensive testing to demonstrate their clinical usefulness in B-cell malignancies. For instance, three metabolic subtypes have been identified in nine B-NHL model cell lines according to their preferential use of glucose, glutamine or both as a source of energy [410]. As an attempt to perform a classification

of B-NHL according to their specific metabolic profile, a pilot study was performed on the plasma samples of 66 NHL patients, compared with 96 matching controls [411]. A significant difference was observed between the metabolites produced by DLBCL, CLL, MM, NHL patients' plasma (but not FL) and their respective control population, allowing to discriminate these patients and healthy controls [411]. Although the NHL cohort size was rather small, the results already suggest a classification of NHL subtypes based on plasma metabolites [411]. In MM, the serum metabolic profiling shows a specific signature after complete remission [412]. A similar analysis of serum from 47 childhood ALL patients lead to the identification of 30 potential biomarkers (17 detected in positive mode and 13 in negative mode) [413]; in particular, deregulated glycerophospholipid metabolism in ALL patients appeared to represent an underlying metabolic pathway associated with disease progression [413]; this non-invasive diagnosis approach may especially be relevant as diagnosing ALL requires BM puncture procedure.

In CLL, IGHV mutational status correlates with disease-related metabolic profiles [366,414]. Several key glycolytic genes (*PFKP*, *PGAM1* and *PGK1*) were found to be down-regulated in samples harboring mutated immunoglobulin variable heavy-chain. Furthermore, 8q24 copy number gains, 8p12 deletions, 13q14 deletions and ATM mutations were identified as determinants of cellular respiration [366]. Such heterogeneity in the energy metabolism of CLL cells could be therapeutically exploited in the selection of therapeutic strategies. In DLBCL, a genome-wide array study identified three clusters including one defined by increased OXPHOS and expression of genes involved in mitochondrial oxidative phosphorylation (OxPhos-DLBCL). OxPhos-DLBCL also overexpresses proteasome and apoptosis regulators, suggesting a better response to drugs targeting mitochondrial function, proteasome and BCL2 family [415]. A further characterization of these three DLBCL subtypes detailed the metabolic signature of each of the subtypes [415]. The blockade of FAO and GSH synthesis is selectively toxic to OxPhos-DLBCL [416]. Conversely, the absence of functional BCR signaling in OxPhos-DLBCL renders it insensitive to BCR inhibition contrary to non-OxPhos-DLBCL [417], resulting in enhanced glycolysis [416]. More recently, a very comprehensive study identified the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a biomarker for distinguishing between OxPhos-DLBCL (GAPDH^{low}) and non-OxPhos-DLBCL (GAPDH^{high}) and a clinical marker for predicting overall survival in patients treated with R-CHOP [418].

4.2.2. New Strategies for Targeting Metabolism in B Cell Malignancies and Optimizing Current Treatments

While metabolic inhibitors have been used for decades for the treatment of metabolic disorders, the acknowledgement of metabolic reprogramming as a crucial feature of cancer biology has recently started to open new perspective for cancer treatment, with the repositioning of commercial metabolic drugs, or the design of new inhibitors evaluated in clinical trials [419,420]. As the inhibition of major metabolic pathways may have a dramatic impact on both tumor and normal cells, current experience shows that the best potential of metabolic inhibitors to treat tumor, comes from their combination with classically used drugs, as a way to overcome drug resistance [421]. Importantly, while tumor progression correlates to a change in metabolic profile, acquisition of resistance to drug is, most of the time, also linked to a change in bioenergetics. Thus, resistance to chemo-immunotherapy in CLL correlates to the activities of glycolytic enzymes LDHA and pyruvate kinase M2 (PKM2), leading to the development of a metabolic score as a diagnostic tool in CLL [422]. A larger CLL screening study revealed that the metabolic state of leukemic cells is associated with drug sensitivity; in particular, higher glycolytic activity is linked to increased resistance towards drugs (including venetoclax, ibrutinib, R-CHOP, rituximab, proteasome inhibitors) thus shedding some light on metabolic vulnerabilities displayed by resistant cells [366].

Venetoclax: two main metabolic patterns emerge in venetoclax resistant B-NHL and CLL. First, mitochondrial OxPhos is increased in response to venetoclax in B-NHL, ren-

dering them more sensitive to respiration inhibitors such as antimycin, oligomycin, IACS-010759, metformin or berberine [423–426]. Second, inhibition of LN-derived glutamine import with V9302 attenuates LN-induced resistance of CLL cells to venetoclax [400]; a higher glycolytic activity is also part of the resistant phenotype [366].

Ibrutinib: ibrutinib treatment which blocks BCR signaling also interferes with metabolic pathways in CLL, decreasing mitochondrial oxidative respiration, glycolysis and glutamine metabolism [400,427,428]. An increase in glutamine uptake is also observed in ibrutinib-resistant CLL cells, and etomoxir treatment (that blocks the mitochondrial import of FAs and their β -oxidation), has a cytotoxic effect on ibrutinib resistant CLL cells [377]. In contrast, ibrutinib-resistant MCL cells display increased OxPhos, and combining ibrutinib to IACS-010759 blocks tumor growth [375]. Ibrutinib resistance also correlates to higher glutaminolysis, that renders glutaminase inhibitor CB-839 efficient at killing ibrutinib-resistant MCL cells [429].

R-CHOP: Low GAPDH expression level correlates to DLBCL patients' resistance to R-CHOP treatment [418,430], and their sensitivity to metabolic inhibitors targeting mammalian target of rapamycin complex 1 (mTORC1) signaling, OxPhos and glutaminolysis [418]. In a preliminary clinical trial, four DLBCL patients with GAPDH^{low} tumors received a combination therapy involving kidrolase (asparaginase), metformin, and temsirolimus (mTOR inhibitor), tumor regression was reported in three out of four patients for the first two weeks of treatment, but relapsed after longer treatment, with a median response duration of six months [430]. This preliminary study suggests that combination of metabolic inhibitors may offer a treatment strategy for metabolically active cancers [430].

Rituximab: acquisition of resistance to rituximab appears related to an increase in glycolysis in BL and DLBCL cell lines, and downregulation of HK2 by 2-Deoxy-D-glucose (2DG) glucose competitive inhibitor, metformin, idelalisib or temsirolimus overcomes resistance and enhances the cytotoxic effect of rituximab [431]. Similarly, the blockade of lactate transporter MCT1 by AZD3965 combined to rituximab leads to tumor regression in a BL mouse xenograft [432] as well as in patients with DLBCL (phase I NCT01791595, completed in 2020) [373]. An enhancement of rituximab response is observed in DLBCL when combined with metformin [433] or pyruvate dehydrogenase kinase 4 (PDK4) inhibitor dichloroacetate (DCA) [434]. Both temsirolimus and everolimus are already FDA-approved for the combined treatment with rituximab of R/R MCL, DLBCL and FL [435].

Proteasome inhibitors: in MM cell lines and a MM mouse xenograft, the response to proteasome inhibitors is increased when combined to the glutaminase inhibitor CB-839 [436] or to DCA [437].

4.2.3. Metabolism as a Tool to Boost Immunotherapy

In the last years, the development of immunotherapy has been quite impressive for the treatment of various tumors including B-cell malignancies. Despite some very promising results, this new therapy is facing long term efficacy issues, due to the difficulty to maintain the proliferation of immune cells in an immunosuppressive TME. One major limitation is the availability of nutrients in the TME, for which immune and tumor cells compete; the high avidity of tumor cells for energy substrates, especially glucose, often results in exhaustion of immune cells, ending with tumor immune evasion [438]. Moreover, the accumulation of immune suppressive metabolites in the TME has negative effects on the immune system, in particular lactate production by tumor cells can inhibit the activity of B and T cells [439]. Complementing immunotherapy with molecules that modulate metabolism therefore emerges as a solution of choice to optimize immunotherapies, including chimeric antigen receptor (CAR) T cells [440,441]. For instance, glutaminolysis in DLBCL interferes with immunotherapy by regulating positively PD-L1 expression [442]. Two phase I clinical trials in B-NHL evaluated the combinations of pembrolizumab with troriluzole (BHV-4157), an inhibitor of glutamate (NCT03229278, completed in 2020), or ciforadenant, an antagonist of the adenosine A2A receptor (NCT03454451, 2018–2025). These innovative approaches deserve to be pursued.

5. Concluding Remarks and Perspectives

Today, a key challenge in using FDA-approved drugs in the current treatment of B cell malignancies (Figure 2a) is that these therapies must simultaneously eliminate tumor cells and preserve/reestablish normal hematopoiesis. However, the effectiveness of these anticancer therapies is sometimes limited by primary and acquired resistance [1]. Drug-mediated T cell exhaustion may lead to the possibility of immune escape of hematological malignancies [1]. Residual disease and/or resistance in patients treated with Bcl-2/BTK/PI3K inhibitors (venetoclax, ibrutinib, idelalisib, etc.) is associated with genomic resistance (mutations, activation, instability, p53 aberrations.) as well as non-genomic, acquired resistance through (re)activation of signaling survival pathways (PI3K, NF- κ B, MYC, etc.), cancer stem cells, and the tumor microenvironment [60,443–447]. The main adverse effects of mAbs therapies include cytokine release syndrome, neurotoxicity, and on-target/off-tumor toxicity resulting from a direct attack on normal tissues that have shared expression of the target antigen [223,448]. To improve clinical outcomes (residual and progressive disease) and immune function in patients with B cell malignancies, while evading the emergence of drug resistance, newer drugs that inhibit the same targets as the current treatments are in continuous clinical development. For instance, ongoing trials focus on overcoming venetoclax resistance by targeting the BCL2 family members Mcl-1 and Bcl-x_L, and evaluate more highly selective BTK and PI3K δ inhibitors with fewer off-target effects. These second-generation drugs, although more potent, are not likely to be used only as single agents. Drug combinations can favor compounds with synergistic antitumor effects and ensure manageable toxicity. Thus, the combinations of BTK inhibitors with BCL2 inhibitors and/or rituximab (anti-CD20 mAb), or PI3K inhibitors with BCL2 inhibitors, are of interest.

Importantly, this review outlines the increasing importance of therapeutic mAbs (mono-/bi-specific, ADC, BiTE, CAR-T cells, Bi-CAR-T cells, CAR-NK cells) as the predominant treatment modality for B cell malignancies. To face the resistance mechanism, BiTEs and CAR-T cells redirect T cells to target antigen-expressing tumors; novel BiTEs and CAR-T cells with various costimulatory signals and delivery systems, as well as Bi-CAR-T cells (anti-CD19/CD20 CAR-T, anti-CD19/CD22 CAR-T, and anti-CD20/CD22 CAR-T) may therefore represent an effective solution to the challenge of antigen escape in immunotherapy (Table 3). As already planned for the treatment of solid tumors [449], BiTEs and CAR-T cells could be combined with standard chemotherapy and/or targeted therapies of B cell malignancies to reduce the tumor burden and/or modulate the immune response. Recently, CAR-T cells and BiTEs have been engineered into a single immunotherapy platform (BiTE-secreting CAR-T) for T-cell-directed therapy of solid tumors [450,451]. BiTEs secreted by CAR-T cells exhibit potent antitumor activity in vitro and in vivo with significant sensitivity and specificity, demonstrating a promising strategy in solid tumor therapy [450,451]. This approach could have therapeutic application in hematological malignancies. Lastly, compared with CAR-T cells, CAR-NK cell constructs offer advantages, including better safety, and multiple mechanisms for activating cytotoxic activity [452–454]. For therapy of B cell malignancies, equipping anti-CD19 CAR-NK cells with on-board cytokines or chemokines might improve clinical efficacy by enhancing both persistence and cytotoxicity against tumor cells.

The consideration of other TAAs (including BCMA, BAFF-R, ROR1, CXCR4, CS1, FcRL5, GPRC5D, etc.) as druggable targets for B cell malignancy immunotherapy, has promoted the development of novel inhibitors, with some of them under single or combined evaluation in clinical trials for a variety of patients with B cell malignancies (Table 4). Most TAAs show their potential for ADC, BiTE, CAR-T, and CAR-NK therapies. Regarding the PD1/PD-L1 axis responsible in part for cancer immune escape, immune checkpoint blockade therapies with anti-PD1/anti-PD-L1 mAbs in combination with BTK (ibrutinib), anti-CD38 (isatuximab) or anti-CD19 CAR-T (axicabtagene ciloleucel) may optimize the landscape of B-NHL therapy.

Targeting additional intracellular signaling pathways contributing to B malignant cell dysfunction may also prove efficacious. For example, ruxolitinib, a potent JAK1/2 inhibitor, demonstrates antitumor activity as a single agent in MM (NCT03110822) [455]. Anvatirsen, an antisense oligonucleotide inhibiting STAT3, combined with durvalumab + tremelimumab is well-tolerated in patients with R/R DLBCL (NCT02807454) [311]. The efficacy and safety of a combination of panobinostat (a pan-histone deacetylase inhibitor) with bortezomib + dexamethasone is being evaluated in MM patients having received two or more lines of treatment (NCT02654990). Tazemetostat/EPZ-6348, which inhibits the histone methyl transferase EZH2, shows safety and antitumor activity in patients with DLBCL (NCT02889523) and FL (phase I/II NCT01897571) [456–458]. Combination therapy with temsirolimus, an inhibitor of the oncogenic kinase mTOR, and lenalidomide demonstrates encouraging activity in patients with DLBCL and FL (NCT01076543) [459]. Ponatinib is a third-generation tyrosine kinase inhibitor with a wide spectrum of kinase inhibition [460]; it targets BCR-ABL1, an abnormal tyrosine kinase that is expressed in chronic myeloid leukemia and Philadelphia chromosome-positive (Ph⁺) ALL; ponatinib shows clinical activity in R/R Ph⁺ (BCR-ABL) ALL [461], and in the first-line setting in combination with standard chemotherapy (NCT01641107, [462]; NCT02776605, [463]) or blinatumomab (NCT03263572) [464]. A Phase III study is comparing the efficacy and the safety of the first in-class selective inhibitor of nuclear export, selinexor, in combination with bortezomib + dexamethasone vs. bortezomib + dexamethasone in patients with R/R MM (NCT03110562) [465]. Pharmacological inhibition of the bromodomain and extra-terminal (BRD/BET) family proteins block downstream components of BCR signaling, downregulate Bcl-2 transcription, and suppress NF- κ B signaling in CLL and B-NHL [466]; among novel single-molecules cotargeting BRD4 and other tumor targets recently developed, SRX3177 targeting CDK4/6-PI3K-BRD4 and SRX3305 targeting BTK-PI3K-BRD4, demonstrate pre-clinical activity against MCL [467], CLL [468], and MCL [469]. These studies underscore the potential effectiveness of these novel multi-action small molecule inhibitors, alone or combined, for potential treatment of B tumors.

Last, but not least, metabolic changes in tumor cells represent a novel opportunity for combination therapy approaches [470]. Metabolic reprogramming is linked to oncogenic transformation. One hallmark of BCL2, PI3K, BTK, and mTOR proteins and some TAAs such as CD38, concerns their participation in tumor metabolism. Activation of these oncogenic pathways makes tumors more metabolically active, and conversely, active metabolism upregulates BCL2, PI3K, BTK, and mTOR proteins, inhibiting susceptibility to cell death. In a consistent way, a few clinical trials have started to include metabolic inhibitors (targeting for instance glycolysis, OxPhos, amino acids) with venetoclax, ibrutinib and other drugs, to overcome the limitations of targeted therapeutic strategies in lymphoid malignancies. As metabolic reprogramming is closely linked to tumor B cells' microenvironment and to immunoevasion, strategies targeting these crosstalks may also open new avenues for overcoming therapeutic resistance.

To conclude, most therapeutic drugs have underscored our advancement in the understanding of the biology of malignant B cells, and has improved outcomes for many patients. In the search for better efficacy and safety, the status of therapies in B cell malignancies is continually advancing with emerging concepts in therapy and evolving results from clinical protocols. A large variety of more effective and selective inhibitors and targeted combinations are being evaluated in these diseases, and it is expected that some of these new compounds will proceed into the clinic in the near future.

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Review

Extracellular Vesicles in Chronic Lymphocytic Leukemia: Tumor Microenvironment Messengers as a Basis for New Targeted Therapies?

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Simple Summary: Chronic lymphocytic leukemia (CLL) is characterized by the accumulation of abnormal B lymphocytes in the peripheral components of the immune system. Despite the development of new therapies for CLL, drug resistance and disease relapse still occur. In the bone marrow and secondary lymphoid tissues, the trafficking, survival, and proliferation of leukemic B cells are regulated by interactions with the microenvironment (via cell-extracellular matrix interactions, cell-cell contacts, and the exchange of soluble factors) and contribute to treatment resistance. Here, we review the biology of the extracellular vesicles released into this microenvironment with cross-talk between neoplastic B cells and neighboring or remote target cells. A better understanding of the extracellular vesicles' role in CLL progression and drug resistance might open up opportunities for the development of novel therapeutics that target the pro-survival dialogue between tumor cells and the tumor microenvironment.

Abstract: In addition to intrinsic genomic and nongenomic alterations, tumor progression is also dependent on the tumor microenvironment (TME, mainly composed of the extracellular matrix (ECM), secreted factors, and bystander immune and stromal cells). In chronic lymphocytic leukemia (CLL), B cells have a defect in cell death; contact with the TME in secondary lymphoid organs dramatically increases the B cells' survival via the activation of various molecular pathways, including the B cell receptor and CD40 signaling. Conversely, CLL cells increase the permissiveness of the TME by inducing changes in the ECM, secreted factors, and bystander cells. Recently, the extracellular vesicles (EVs) released into the TME have emerged as key arbiters of cross-talk with tumor cells. The EVs' cargo can contain various bioactive substances (including metabolites, proteins, RNA, and DNA); upon delivery to target cells, these substances can induce intracellular signaling and drive tumor progression. Here, we review recent research on the biology of EVs in CLL. EVs have diagnostic/prognostic significance and clearly influence the clinical outcome of CLL; hence, from the perspective of blocking CLL-TME interactions, EVs are therapeutic targets. The identification of novel EV inhibitors might pave the way to the development of novel combination treatments for CLL and the optimization of currently available treatments (including immunotherapy).

Keywords: chronic lymphocytic leukemia; extracellular vesicles; exosomes; tumor microenvironment



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1. Introduction

1.1. Chronic Lymphocytic Leukemia

Chronic lymphocytic leukemia (CLL) is the most common leukemia in Western countries and is characterized by the accumulation of malignant CD5+ CD19+ B cells with a defect in apoptosis and a very low proliferation rate [1]. The disease is heterogeneous in both molecular and clinical terms. Some cases of CLL are indolent and do not require

treatment, whereas others are very aggressive. Clinically, patients are typically classified according to the Rai [2] and Binet [3] staging systems, which are based on blood cell counts and other variables. On the molecular and genetic levels, several chromosomal alterations are indicative of a poor prognosis; these include the deletion of the short arm of chromosome 17 (del17p), the long arm of chromosome 11 (del11q), trisomy 12, del13q, *TP53* point mutations, and an unmutated immunoglobulin heavy chain region (*UM-IGHV*) [1].

The CLL B cells' proliferation rate is high when the cells reside in lymphoid organs such as the bone marrow (BM), lymph nodes (LNs), and spleen, but their proliferation rate is low in the peripheral blood (PB) [4,5]. The survival and proliferation of CLL cells are highly dependent on the B cell receptor (BCR) pathway. Once activated by external antigens or self-antigens, the BCR recruits spleen tyrosine kinases (SYKs) and Lck/Yes novel tyrosine kinase (LYN) and thus induces a phosphorylation cascade that activates Bruton's tyrosine kinase (BTK), phosphoinositide 3-kinase (PI3K), protein kinase C, and Ras-dependent extracellular signal-regulated kinase [4]. The end result is the upregulation of nuclear factor kappa B (NF- κ B) and CLL cell survival and proliferation [4]. As a result of genetic lesions or changes in signal transduction, anti-apoptotic proteins from the B-cell lymphoma/leukemia 2 (*BCL2*) family (Bcl-2, Mcl-1, and Bcl-xL) are overexpressed in CLL and are associated with a poor prognosis [6]. High expression levels of several tumor-associated antigens (including CD20, CD19, and CD22) in CLL are correlated with a high proliferation rate and disease progression and modulate BCR-dependent and BCR-independent proliferation/survival signals [6].

Current treatments for CLL include broad-spectrum cytostatic agents, targeted therapies, and combinations thereof [1,6,7]. The cytostatic agents include chlorambucil, fludarabine, and bendamustine. Novel inhibitors of BCR-associated kinases (i.e., the BTK inhibitors ibrutinib, acalabrutinib, zanubrutinib, and pirtobrutinib and the PI3K inhibitor idelalisib) have been approved in the USA and Europe for relapsed CLL or first-line treatment of a CLL patient bearing a *TP53* abnormality [6,7]. First-line treatment with idelalisib has been withdrawn as a result of adverse drug events (including hepatotoxicity) but is still used in combination with other drugs [6,7].

Another treatment option is venetoclax, a BH3 mimetic that inhibits Bcl-2's survival function and has been approved for the treatment of relapsed CLL patients—including those bearing a del17p [7]. Lastly, two anti-CD20 agents are currently approved by the US Food and Drug Administration for the treatment of CLL: rituximab is particularly effective when combined with chemotherapy, and obinutuzumab is less toxic than rituximab [1,6,7].

However, the treatment of CLL remains a challenge in the clinic because a significant proportion of patients are either refractory to the available first-line treatment or relapse after treatment. Although ibrutinib is one of the most frequent first-line treatments, continuous treatment leads to relapse and the acquisition of resistance; this is mainly associated with mutations in BTK or phospholipase C gamma 2 [6,8,9]. A similar process is observed with venetoclax, resistance to which is correlated with the acquisition of a mutation in *BCL2* [9]. Novel therapies are therefore needed to overcome resistance to existing drugs, and the identification of new therapeutic targets in CLL is of general interest.

1.2. The CLL Tumor Microenvironment

In addition to intrinsic genomic alterations (including oncogenic mutations and chromosomal rearrangements), tumor progression and drug resistance are greatly influenced by the interactions between CLL cells and the surrounding tumor microenvironment (TME) [10–12]. The normal cells in the TME (mainly immune cells (T cells and monocyte-derived cells) and stromal cells (including mesenchymal stromal cells (MSCs), endothelial cells, and fibroblasts)) form a niche in which CLL cells are protected from apoptosis, as evidenced by the high proportion of cells that die spontaneously when CLL cells are separated from the TME and cultured *in vitro* [13]. CLL cell survival is promoted by tumor-associated macrophages known as nurse-like cells through the secretion of a proliferation-inducing ligand (APRIL), a B-cell activating factor (BAFF), the C-X-C motif chemokine CXCL12

(also known as stromal cell-derived factor 1 (SDF-1)), and soluble CD14 or the lymphocyte function-associated antigen 3 (LFA-3)/CD2 interaction [12]. Similarly, endothelial cells are activated by CLL cells and then secrete BAFF and APRIL which, in turn, promote CLL cell survival [14]. MSCs protect tumor cells against spontaneous and drug-induced apoptosis by activating BCR and changing the expression of antiapoptotic molecules, SDF-1, and vascular endothelial growth factor (VEGF) [10,15]. Interestingly, stromal cells (such as MSCs and fibroblasts) in contact with the tumor can transdifferentiate into “activated” cancer-associated fibroblasts (CAFs). The CAFs further contribute to tumor progression through a phenotypic shift that notably regulates the formation of metastases, the production of the extracellular matrix, and the release of cytokines and growth factors [16].

One of the best illustrations of the TME’s influence on CLL biology is the difference in proliferation between quiescent CLL cells in PB and actively proliferating CLL cells in lymphoid secondary organs (especially LNs) [4]. Activated CD4 T cells can stimulate CLL cells and induce their proliferation through CD40 ligand (CD40L)-mediated cell–cell interactions [17,18].

The fact that cell metabolism reprogramming is clearly involved in tumor progression and dissemination has prompted growing interest in cancer research and drug development over the last decade [19]. In the particular context of CLL, the results of several studies have demonstrated that the TME can increase tumor cell survival by modulating tumor cells’ mitochondrial oxidative phosphorylation and nucleotide synthesis [20], favoring protection against oxidative stress by promoting glutathione synthesis [21] and causing a glycolytic switch through Notch-c-Myc signaling [22]. The increase in the glycolytic phenotype of CLL cells in LNs is BCR-dependent, although a specific subpopulation of CLL cells with a del17p (often correlated with *TP53* mutation) appears to spontaneously display this metabolic characteristic [23]. Chen et al. recently showed that the LN microenvironment induces dramatic metabolic changes in CLL tumor cells. Interestingly, the inhibition of glutamine metabolism in CD40/BCR-activated CLL cells abrogated resistance to venetoclax [24].

Another means of favoring tumor cell expansion involves the interaction between CLL cells and the TME and then the establishment of an immunosuppressive milieu [11]. For example, the presence of CLL cells is correlated with T cell exhaustion (by impeding glucose metabolism [25,26]) and T-cell dysfunction (via overexpression of the programmed death ligand (PD-L)1 in CLL cells). In CLL, macrophages display pro-tumor M2 differentiation, and the combination of impaired glucose metabolism and programmed death 1 (PD-1) activation results in monocyte dysfunction and a subsequent defect in immune surveillance [11].

Given the importance of the TME’s stimulation of CLL B cell survival and expansion, CLL-TME interactions have become therapeutic targets [27]. The TME’s signals include direct cell–cell and cell–extracellular matrix (ECM) contacts, the release of soluble factors (chemokines, interleukins, growth factors, and matrix metalloproteinase 9), and the release of small extracellular vesicles (EVs). A growing body of evidence shows that the EVs released by normal and tumor B cells are key components of the cancer-supporting TME [10,11]. Here, we review the biology of EVs in general, data from the current literature on the EVs’ expression profiles and roles in CLL, and the EVs’ putative functional value in countering drug resistance in CLL.

2. EV Generalities

EVs are small vesicles known to mediate intercellular communications in local and distant microenvironments under physiological and pathological conditions [28–31]. They carry a large variety of proteins, metabolites, DNA, RNA, microRNAs (miRNA), and long non-coding RNAs, and are secreted in biological fluids (urine, blood, ascites, and cerebrospinal fluid) [28–31].

Given the explosion of papers about EVs published in the last decade, the corresponding variety of protocols to purify them, and the continuous discovery of new vesicle types and characteristics, leading to the evolution of their classification, some guidelines have

been edited by the International Society for Extracellular Vesicles (ISEV) for the clarification of EV nomenclature and protocols. The Minimal Information for Studies of Extracellular Vesicles (MISEV) guide was first published in 2014 [32], updated in 2018 [33], and is about to be updated again.

2.1. Nomenclature and Biogenesis

Based on MISEV 2018 [33], EVs are defined as nucleus-free particles with a bilayer lipid membrane, and they are released by cells in the extracellular space. EVs were initially separated into two main categories [28,31]:

- Exosomes (EXOs) are 30–150 nm vesicles generated through endosome maturation, the formation of multivesicular bodies (MVBs), intraluminal vesicles, and the fusion of MVBs with the plasma membrane. The secretion of EXOs is regulated by the endosomal sorting complexes required for transport (ESCRT) machinery. Therefore, some common EXO markers include ESCRT proteins such as Tsg101 and Alix. Tetraspanins CD9, CD81, and CD63 are also amongst the most popular EXO markers.
- Microvesicles (MVs) (previously referred to as ectosomes or microparticles) are 150–1000 nm vesicles resulting from the blebbing of the plasma membrane.

Despite intense research efforts developed to precisely describe the two different biogenesis pathways, there is still no consensus about the markers that can segregate these two populations, most likely because these processes are not exclusive. As some confusion has been seen over time in research articles regarding the nature, denomination, or origin of EVs, the ISEV was led to recommend the use of the generic name “extracellular vesicles”. When dealing with EV subtypes, a proposed alternative is to name EVs according to their size (small, medium/large EVs), density, surface expression markers, and/or any source/condition parameter that defines them [33]. With respect to research articles about EVs in CLL and other malignancies, most studies relate to EXOs, which are mainly defined as small-size vesicles isolated by ultracentrifugation. Independent of the EV’s nature, vesiculation can be modulated by different factors, including treatment with Ca^{2+} ionophores, temperature, pH, oncogenic transformation, cytoskeleton remodeling, or stress signals such as hypoxia [28].

2.2. Purification

EVs can be prepared from cell culture supernatants or biological fluids. Many different protocols have been developed. However, the gold standard remains purification by differential ultracentrifugation; that is, a series of centrifugations at different speeds to collect sequentially intact cells, dead cells and cell debris, then MVs, and finally EXOs [34]. An optimization of this protocol is density gradient ultracentrifugation, which improves the purity of collected EVs. Other techniques include polymer-based precipitation, ultrafiltration, size-exclusion chromatography, or immunoaffinity-based methods (ELISA and beads) [34]. Parameters that differ between these different techniques are purity, cost, time, yield, and specificity (the specific selection of EV markers or, on the contrary, a broad size selection). The choice of the optimal protocol also depends on the quantity of EV material available, the type of EVs, the volume of sample to analyze, and the application chosen [33].

2.3. Uptake of EVs and Transfer of Their Cargo to Recipient Cells

EV targeting to recipient cells can be mediated by cell surface receptors that specifically recognize EVs or through unspecific processes (such as micropinocytosis or macropinocytosis) [29,31,35]. While the fusion of EVs with the plasma membrane can result in the release of their content into the cytoplasm of recipient cells, EVs can also be internalized and fused with intracellular endosomes.

Following their internalization in recipient cells, the EV cargo can activate a variety of signaling pathways that regulate distinct biological functions in tumor cells related to proliferation, differentiation, migration, metabolism, drug resistance, or the survival/cell death

balance [30]. Communication through EVs can therefore ultimately influence metastasis formation, immune escape, and a multitude of signals involving the TME.

3. CLL EVs and Their Role in the TME

CLL EVs have diagnostic and prognostic value in CLL. The secretion of EVs into the bloodstream contributes to the progression of CLL [10,36–38]. Thus, by targeting these EVs, one can reasonably expect to disrupt the CLL–TME interaction and increase the effectiveness of cancer treatments [39,40].

3.1. Purification and Characterization of CLL EVs

Several research groups have compared and optimized various protocols for the purification and characterization of CLL EVs from cell culture supernatants or patients' plasma [41–43]. Other than the conventional EV markers, several CLL-specific surface markers have been identified; these include components of the BCR pathway (such as IgM, CD19, and Lyn) and other molecules (such as HLA-DR, CD82, CD37, CD54, CD20, CD5, and CD52) [41,42,44–49] (Figure 1).

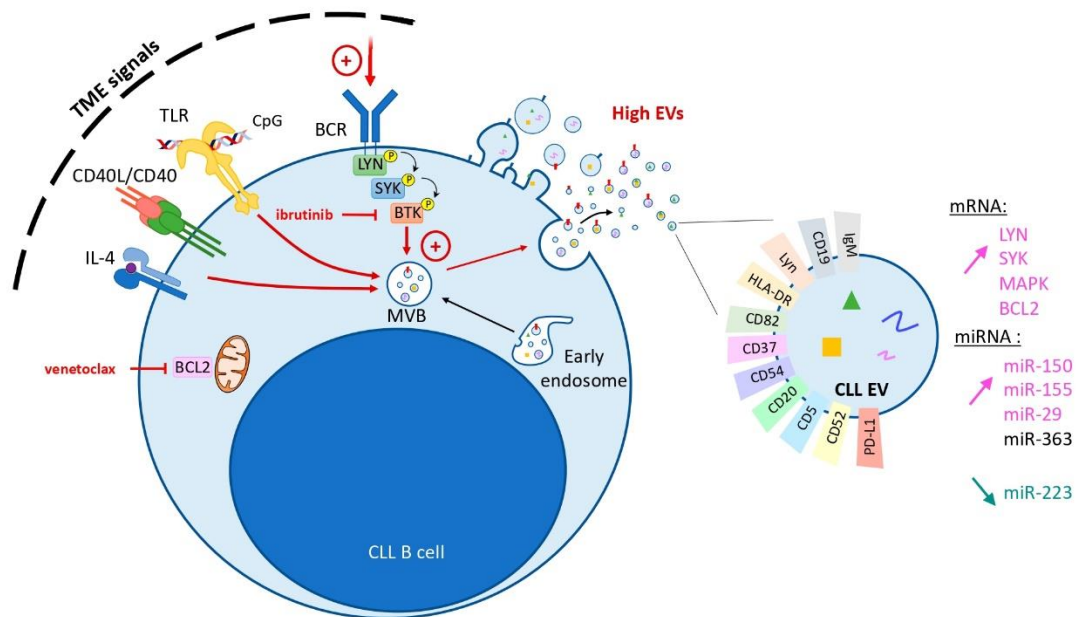


Figure 1. Modulation of EVs in CLL. The fusion of MVBs (multivesicular bodies) with plasma membrane or the budding of the plasma membrane leads to the release of CLL EVs from CLL B cells. EVs' release can be increased by TME (tumor microenvironment) signals recapitulated by the stimulation of the BCR (B-cell receptor), TLR (Toll-like receptors) signaling, or CD40/IL-4 (left panel). CLL EVs are characterized by the exposure of specific surface markers (right panel). Compared to normal B cell EVs, CLL EVs contain a higher level of LYN, SYK, MAPK and BCL2 mRNAs, as well as a higher level of miR-150, miR-155, and miR-29 but a lower level of miR-223. TME stimulation increases their miR-363 content (BTK—Bruton's tyrosine kinase; LYN—Lck/Yes novel tyrosine kinase; SYK—spleen tyrosine kinase).

Here, we review the involvement of EVs (EXOs or MVs) in CLL biology in general. Technical details concerning the types of EV considered and the purification methods used in the research reviewed are summarized in Table 1. Conflicting results have been obtained by laboratories using different EV purification protocols, and techniques have evolved rapidly over the last few years.

Table 1. Summary of discoveries and protocols used in the main articles about CLL EVs discussed in the review (UC—ultracentrifugation; CLL cell lines: EHEB—HG3—MEC-1—PGA-1; endothelial cell lines: HMEC-1—HUVEC; human BM cell lines: HS5; monocytic cell line: THP-1; primary fibroblasts: HDFn; ↑: increase; ↓: decrease).

Donor Cells	EVs	EV Markers	Cargo	Purification Protocol	Target Cells	Results	Ref
Untreated CLL patients (<i>n</i> = 34) and healthy donors, MEC-1	EXO	TSG101, MHC I/II, IgM, Lyn kinase, CD81, CD37, ITGA4	miR-202-3p	10 min at 500× <i>g</i> , 10 min at 4000× <i>g</i> , 30 min at 18,000× <i>g</i> , filtration (Immuno-magnetic isolation), UC 90 min at 100,000× <i>g</i> (×2)	HS-5	CLL EXOs ↑ expression of genes such as c-fos and ATM; ↑ proliferation of recipient HS-5 cells; CLL EXOs enriched in certain miRNAs	[44]
Primary CLL B-cells and normal B cells, plasma (<i>n</i> = 33 CLL/ <i>n</i> = 9 treatment-naïve CLL patients/ <i>n</i> = 5 CLL patients under ibrutinib)	MV	CD52	-	20 min at 2500× <i>g</i> (×3); 1 h at 16,000× <i>g</i> at 4 °C	-	↑ CD52+ MVs with BCR stimulation in CLL B-cells; ↑ plasma CD52+ MVs correlated to tumor progression; ↓ plasma CD52+ MVs after ibrutinib therapy	[45]
Primary CLL and healthy B cells	EV	CD63, CD9, CD54, CD82	TCL1A-mRNA	10 min at 300× <i>g</i> , 15 min at 6800× <i>g</i> (×2), UC 90 min at 100,000× <i>g</i> (×2)	HDFn and THP-1	CLL-CpG-EVs contain disease-relevant mRNA; ↑ CLL-EVs compared to healthy B cells	[46]
CD19+ B cells from CLL patients and healthy donors	EXO	CD63, CD9, CD37	miR-155, miR-150	10 min at 300× <i>g</i> , 10 min at 2000× <i>g</i> , 30 min at 10,000× <i>g</i> at 4 °C, UC 70 min at 100,000× <i>g</i> at 4 °C (×2)	-	↑ EXOs in CLL patients' plasma; ↑ EXOs with BCR activation by α-IgM in CLL B cells; ibrutinib impedes α-IgM-stimulation EXO release; ↑ EXO miR-150 and miR-155 with BCR activation	[47]
Serum (<i>n</i> = 131 CLL/ <i>n</i> = 28 healthy controls)	MV	CD19, CD37	-	20 min at 2000× <i>g</i> at 4 °C, 30 min at 10,000× <i>g</i> at 4 °C, UC 70 min at 100,000× <i>g</i> at 4 °C (×2)	-	↑ MVs in CLL patients' plasma; CD19+ CD37+ MVs correlate to tumor progression; total MVs predict for overall survival and time to treatment	[48]

Table 1. Cont.

Donor Cells	EVs	EV Markers	Cargo	Purification Protocol	Target Cells	Results	Ref
Plasma ($n = 60$ CLL, $n = 5$ healthy controls)	MV	CD19		20 min at $2500 \times g$ ($\times 3$), 1 h at $16,000 \times g$ at 4°C	HS-5, primary BMSCs	\uparrow MVs in CLL patients' plasma; \uparrow VEGF, B-catenin pathway, cyclin D1, and c-myc in CLL-BMSCs	[50]
CD19+ CD5+ B cells CLL patient and healthy donors; plasma CLL patients	EV comprise EXO	-	miR-363, miR-155, miR-374b	5 min at $250 \times g$ at 4°C , 10 min at $2000 \times g$ at 4°C , 30 min at $10,000 \times g$ at 4°C , UC 110 min at $100,000 \times g$	CD4+ T cells from CLL patients	CD40/IL-4-stimulated CLL cells released specific EV miRNAs; \uparrow migration; proliferation of CD4+ T cells; immunological synapse signaling	[51]
Primary CLL ($n = 21$), MEC-1	EXO	ALIX, TSG101, HLA-DR		10 min at $400 \times g$ ($\times 2$), 20 min at $2000 \times g$, filtration $0.45 \mu\text{m}$, UC at $110,000 \times g$ at 4°C , flotation on Optiprep cushion (Axis-Shield, 17%) for 75 min at $100,000 \times g$ at 4°C , filtration $0.45 \mu\text{m}$	Human BM-MSCs, HMEC-1, HS5	CLL-EXO transfer protein and miRNA into stromal cells that induce a CAF-like phenotype; uptake by endothelial cells \uparrow angiogenesis	[52]
MEC-1	EXO	CD63, CD9	miR-146a	10 min at $400 \times g$ ($\times 2$), 20 min at $2000 \times g$, filtration $0.45 \mu\text{m}$, UC 70 min at $110,000 \times g$ ($\times 2$), 75 min at $100,000 \times g$ at 4°C , filtration $0.45 \mu\text{m}$	Human BM-MSCs	CLL cells deliver miR-146a to BM-MSCs that induce CAFs phenotype by down-regulation of USP16 mRNA expression	[53]
Human BM-MSCs	EV	CD63		10 min at $300 \times g$ ($\times 2$), concentrated on 3 K centrifugal device, UC 1 h at $150,000 \times g$ at 4°C ($\times 2$)	Primary CLL	BM-MSCs \downarrow B CLL spontaneous apoptosis and \uparrow chemoresistance to fludarabine, ibrutinib, idelalisib and venetoclax; \uparrow CLL B cells migration	[54]

Table 1. Cont.

Donor Cells	EVs	EV Markers	Cargo	Purification Protocol	Target Cells	Results	Ref
Plasma CLL patients, MEC-1	EXO	CD63, CD81, TSG101	CLIC1	20 min at 400× g, 40 min at 2000× g, filtration 0.45 μm, UC 70 min at 110,000× g at 4 °C, floatation on Optiprep cushion (Axis-Shield, 17%) 75 min at 100,000× g at 4 °C, filtration 0.45 μm	HUVECs	MEC-1 EXO invasion; metastasis and angiogenesis of HUVECs by transferring CLIC1	[55]
Plasma CLL patients and healthy donors, MEC-1	EXO	RAB5a, HSP70, HLA-DR, CD81	noncoding YRNA hY4	300× g and 10,000× g, UC at 100,000× g, UC on 40% sucrose cushion	Human monocytes or murine BM-MDSCs	↑ release of cytokines, such as CCL2, CCL4, and interleukin-6, and expression of PD-L1	[56]
Untreated CLL patients (n = 26, aggressive/indolent) and healthy donors, MEC1 and HG3	EXO	CD63, CD81	NAMPT	15 min at 3000× g, ExoQuick-TC reagent overnight at 4 °C, 30 min at 1500× g	Primary monocytes (CD14+ CD16+)	CLL-EXO transfer NAMPT to monocytes; ↑ NAD+ (nicotinamide adenine dinucleotide) which activated SIRT1-C/EBPβ signaling pathway in monocytes	[57]
CLL patients (n = 56) and healthy donors, EHEB and MEC1, serum CLL patients and healthy donors	EXO	CD63, CD81		10 min at 500× g, 20 min at 16,500× g, filtration 0.2 μm, UC 70 min at 110,000× g at 4 °C (×2) and filtration 0.2 μm	CD14+HLA-Drlow monocytes (MDSCs)	miR-155 in CLL-EXO induces MDSCs; is disrupted by vitamin D	[58]
PB and plasma samples, CLL patient and healthy donors, MEC-1, HG-3, EHEB, and PGA1	EV	CD9, CD63, CD81, CD19, CD20, CD40	ICs	filtration 0.2 μm, UC 70 min at 110,000× g at 4 °C	T-cells	CLL-EV contain ICs that may hamper T-cell viability, proliferation, activation, and metabolism	[59]

Table 1. Cont.

Donor Cells	EVs	EV Markers	Cargo	Purification Protocol	Target Cells	Results	Ref
E μ -TCL1 CLL murine model (WT B cells from C57BL/6 mouse)	sEV (EXO)	Alix, TSG101, CD63, CD9, CD81	miR-150, -155, -21, -146a, -378a, and -27a, IC ligands	5 min at 400 \times g, 20 min at 400 \times g, 40 min at 2000 \times g, 60 min at 10,000 \times g, filtration 0.2 μ m, UC 70 min at 110,000 \times g at 4 $^{\circ}$ C, flotation on 17% Optiprep cushion, 75 min at 100,000 \times g at 4 $^{\circ}$ C, UC 70 min at 110,000 \times g at 4 $^{\circ}$ C, filtration 0.45 μ m and 0.22 μ m	CD8+ T cells	small EVs secreted by CLL cells in mouse model inhibit CD8+ T-cell immune response against tumor cells	[60]
Serum ($n = 9$ CLL/ $n = 18$ healthy controls)	EV			-	-	\uparrow miR-155 in CLL EVs	[61]

3.2. Modulation of CLL Vesiculation by TME Signals

Despite their low proliferation rate *in vitro*, B cells from CLL patients spontaneously release MVs and EXOs in this setting. Furthermore, the plasma or serum levels of these EVs are higher in CLL patients than in healthy controls [45,47,48,50,62] (Table 1). Similarly, PB levels of EVs are abnormally high in patients with other hematological malignancies, including Waldenström's macroglobulinemia, Hodgkin's lymphoma, multiple myeloma, and acute myeloid leukemia [62].

EV release can be modulated by stimulating or blocking TME signals (Figure 1 and Table 1). For example, *in vitro* BCR stimulation with anti-IgM antibodies induces EXO release by CLL B cells; conversely, BCR inactivation with ibrutinib prevents this induction [47]. BCR ligation with anti-IgM treatment also increases CD52+ MV release by CLL B cells, although the total amount of MVs was not significantly influenced by ibrutinib treatment of CLL cells *in vitro* [45]. The treatment of CLL cells with idelalisib also blocks the EXO secretion resulting from anti-IgM stimulation [47], demonstrating that BTK/PI3K are involved in EXO release. Interestingly, the release of MVs *in vitro* and the release of plasma EXOs after BCR stimulation was greater in CLL cells from patients with a poor prognosis UM-IGHV, even though the difference vs. mutated-IGHV patients was not statistically significant [45,47].

Although it is clear that EXOs and MVs are released after BCR stimulation *in vitro* and *in vivo*, the data on inhibition by ibrutinib are contradictory. As observed *in vitro*, Yeh et al. found lower plasma EXO levels in CLL patients treated for one month with ibrutinib [47]. In the study by Boysen et al. of CLL patients, the plasma level of CD52+ MVs was low after three months of ibrutinib treatment; this might reflect a decrease in the total number of tumor cells after response to ibrutinib [45]. In the long term, however, an opposing trend was observed, with higher levels of CD52+ MVs in most patients [45]. This relative increase was not correlated with disease progression [45]. It is noteworthy that an electron microscopy analysis revealed that the purification method used by Boysen et al. resulted in a mixture of MVs and EXOs, which might explain the interstudy difference in profiles. More recently, the quantification of EXOs in a small cohort of ibrutinib-treated CLL patients did not evidence a significant decrease after 3 or more months of treatment [63]. However, it is important to note that several variables can influence EV release. Firstly, the latter three studies indicated changes over time in EV release: in all three cases, there was an initial decrease after ibrutinib treatment, despite some differences thereafter. Secondly, interindividual variability in small cohorts of CLL patients ($n = 9, 5,$ and 14 in the studies by Yeh et al. [47], Boysen et al. [45], and Ishdorj et al. [63], respectively) would require a greater number of plasma samples for more precision. Most importantly, the studies differed in the selectivity of the technique used to quantify the EVs. EVs in the blood of ibrutinib-treated patients were variously measured in (i) a 100 k ultracentrifugation fraction, using NanoSight nanoparticle tracking [47], (ii) a 16 k fraction assayed for CD52 [45], and (iii) clarified supernatant assayed for CD9 [63]. These differences in purification and analysis techniques highlight the difficulty in comparing studies. Lastly, these interstudy differences probably also result partly from combinations of biological variabilities: the blood EV level might reflect not only BCR activation/inhibition but also the tumor cell count and levels of therapy-induced cell death. Nevertheless, the above-cited studies have clearly evidenced a link between BCR activation and EV release both *in vitro* and *in vivo*. Further investigation of the EVs' involvement in anti-BCR therapy is therefore warranted.

The activation of other mediators of the CLL–TME interaction can also influence EV secretion (Figure 1). The stimulation of normal B cells' interleukin (IL)-4 receptor and CD40 mimics activation by T cells and increases the release of EVs [64]. In CLL B cells, however, the CD40/IL-4 activation leads to a change in the EVs' miRNA content but not in the EV count [51]. Treatment with CpG and thus stimulation of Toll-like receptor (TLR) signaling is associated with greater EV secretion by CLL cells; next-generation sequencing of the EVs' contents revealed an enrichment in mRNAs related to BCR signaling [46].

It is noteworthy that the TME's three inducers of EV release (i.e., BCR activation, CD40/IL-4 stimulation, and TLR stimulation) converge on the NF- κ B pathway.

3.3. Influence of CLL EVs on the TME

CLL–TME communication (whether mediated by direct contact or soluble molecules) is a two-way process [10–12]. When the TME influences the release of EVs by CLL cells (as detailed above), the CLL EVs can further modulate the TME (Figure 2). Indeed, EVs deliver their cargo to the various cell types in the TME and thus remotely alter cell signaling and (ultimately) tumor cell expansion and dissemination [36]. We describe three important examples of this below.

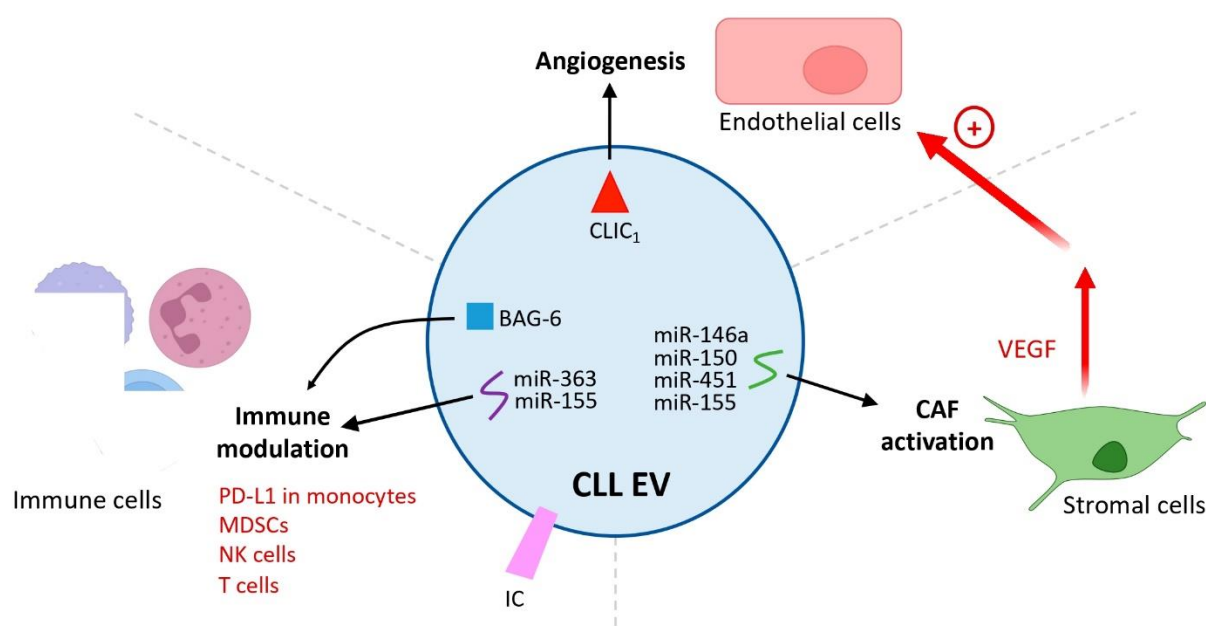


Figure 2. Influence of CLL EVs on TME. CLL EVs have the ability to influence the different cells comprising the TME through the delivery of their cargo to recipient cells, leading to a modulation of TME cell signaling in favor of the tumor. This includes the activation of stromal cells into CAFs (cancer-associated fibroblasts), increased angiogenesis in endothelial cells, and the modulation of the antitumor immune response (see text for details) (BAG6—BCL2-associated athanogene 6; CLIC1—chloride intracellular channel 1; IC—immunological checkpoint; MDSC—myeloid-derived suppressor cell; NK—natural killer; PD-L1—programmed death ligand 1; TME—tumor microenvironment; VEGF—vascular endothelial growth factor).

3.3.1. Differentiation of Stromal Cells into CAFs

A transcriptomic analysis of stromal cells exposed to CLL EXOs containing miR-202-3p showed that the delivery of their contents increased the proliferation of stromal cells and the latter's expression of c-fos and ataxia–telangiectasia mutated (ATM) [44]. Moreover, Paggetti et al. showed that CLL EXOs are internalized by BM stromal cells (MSCs and endothelial cells), which receive miRNAs (including miR-150, miR-155, miR-146a, and miR-451) and proteins that induce the inflammatory phenotype characteristic of CAFs [52] (Figure 2). Additional work demonstrated that CLL exosomal miR-146a induced the transition to CAFs via the upregulation of USP16 and the consequent induction of the CAF markers α -smooth muscle actin and fibroblast-activated protein, which drove tumor cell expansion [53].

This conversion of stromal cells into CAFs by EVs has been described in many tumor types. Conversely, once activated, CAFs can also secrete EVs that influence tumor progression. Through the transfer of a variety of molecules, including proteins, non-coding RNAs, and metabolites, CAF-EVs can influence the tumoral process at different levels by regulating the proliferation of tumor cells, their dissemination to form metastasis, and the antitumor immune response [65,66]. Importantly, CAF-derived EVs can also modulate the tumor response to therapy [67]. Hence, CLL EVs activate stromal cells and cause them to differentiate into CAFs. The EVs secreted by these activated cells then provide the CLL cells with survival signals, this vesiculation being dependent on LYN kinase [68]. MSC-derived EVs protect CLL cells from spontaneous apoptosis by inducing not only the expression of anti-apoptotic proteins but also cell migration, drug resistance, and BCR signaling activation [54]. Stromal cell EVs might therefore be the trigger in the established correlation between BCR activation and CLL EV vesicle release (see Section 3.2). Importantly, CLL cell migration and gene expression were induced more strongly by EXOs from CLL patients' MSCs than by EXOs from healthy patients' MSCs [54]. One can reasonably hypothesize that CLL EVs prime stromal cells for activation, and this creates a regulatory loop through which stromal EVs accentuate the permissive microenvironment for CLL proliferation and drug resistance.

3.3.2. Induction of a Pro-Angiogenic Phenotype

CLL EXOs increase the formation of blood vessels *in vitro* and *in vivo* [52]. In human umbilical vein endothelial cells, the transfer of chloride intracellular channel 1 (CLIC1) from CLL cell EVs is associated with greater proliferation and greater angiogenesis (Figure 2). This stimulation of angiogenesis involves the integrin- β 1-dependent regulation of VEGF [55], which is a known pro-angiogenic survival factor in CLL [69]. Furthermore, CLL EXOs activate BM stromal cells by inducing HIF-1 α signaling and thus VEGF production [50]. Interestingly, the induction of VEGF production was far more intense in CLL bone marrow stromal cells (BMSCs) than in BMSCs from healthy donors [50], suggesting that these cells had already differentiated into CAFs (mediated or not by EVs).

3.3.3. Immunomodulation by CLL EVs

EVs delivered by CLL cells can influence immune cells and thus contribute to immune suppression and tumor immune escape (Figure 2). For example, the internalization of non-coding RNA from CLL EXOs induces the expression of cytokines and the immunosuppressant molecule PD-L1 in monocytes [56]. Very recent work has shown that CLL EXOs transmit endoplasmic reticulum stress to monocytes via extracellular nicotinamide phosphoribosyltransferase, which, in turn, promotes macrophage survival, a phenotype shift, and the secretion of inflammatory cytokines [57]. Similarly, the transfer of miR-155 from CLL EXOs induces the formation of immunosuppressive myeloid-derived suppressor cells; this process is inhibited by pretreatment of the CLL cells with vitamin D [58].

EVs purified from CLL cells can also impede antitumor immune surveillance by blocking the proliferation, activation, and metabolism of T lymphocytes and promoting T cell exhaustion and the formation of regulatory T cells [59]. Böttcher et al. suggested that these processes are regulated by the detection of immunological checkpoints (ICs) on CLL EVs, although a causal relationship with T cell dysregulation was not demonstrated. These results were confirmed and extended by a study of the E μ -TCL1 CLL mouse model [60]. CLL EVs can reprogram the transcriptome, proteome, and metabolome of CD8⁺ T lymphocytes, causing exhaustion, an miRNA-dependent decrease in granzyme B, a fall in cytokine production, and tumor immune escape. Interestingly, Gargiulo et al. were able to correlate the elevated expression of genes involved in EV biology in CLL patients' cells with the presence of markers of a poor prognosis and with poor survival [70].

However, EVs can sometimes activate an immune response. For example, the presence of BCL2-associated athanogene 6 (BAG6, a ligand of the NKp30 receptor in natural killer (NK) cells) in B cell EXOs can lead to NK activation and B cell lysis [71]. Conversely, soluble

BAG6 induces the NK cells' cytotoxic activity. A dysregulated, high soluble exosomal BAG6 ratio in CLL results in the impairment of NK cytotoxicity and thus promotes tumor immune escape.

As described in Section 3.2, vesicle release by CLL cells can be modulated by a variety of factors. Restoration of the T-cell/CLL cell interaction via CD40/IL-4 stimulation resulted in an enrichment in miR-363 in EXOs; this enrichment induced the downregulation of the immunomodulatory receptor CD69, increased T cell migration and proliferation, and elevated immune synapse signaling [51].

4. Clinical Implications of EV Biology in CLL

On the clinical level, EVs can be used as biomarkers of disease progression. Since CLL EVs participate in CLL progression at several levels by modulating cell proliferation and survival, cell migration, angiogenesis, treatment resistance, and immune escape, these vesicles are promising tools for (i) the improvement of current therapies for CLL and other hematological disorders, and (ii) the design of novel therapeutic strategies [37,72–75].

4.1. The EV Profile as a Biomarker in CLL

Although the circulating EV count is higher in CLL patients than in healthy controls, it is also clearly correlated with the Rai stage and thus serves as a marker of disease progression [48,50,62]. A high EV count is correlated with advanced disease and with other markers of a poor prognosis and poor survival; it therefore constitutes an independent prognostic factor [38].

Several groups have looked for (but failed to find) a relationship between CLL EVs and the lymphocyte count [47,48,51]. At first sight, it is tempting to think that the circulating EV count does not therefore reflect the tumor burden. In fact, the count might be a better marker of tumor cells because it takes into account the EVs released by CLL cells in the circulation and those resident in the BM and secondary lymphoid organs.

The analysis of EV surface markers in CLL has shown that some are biomarkers of disease progression. Advanced disease is correlated with elevated concentrations of MVs expressing CD19, CD20, and CD37 on their surface [48]. The accumulation of CD52⁺ MVs is also correlated with disease progression in treatment-naïve CLL patients [45].

Non-coding RNAs (including miRNAs) are crucial regulators of CLL progression and constitute valuable biomarkers of disease progression and treatment response [38,76,77]. The RNAs are packaged inside circulating EXOs and are thus protected from ribonuclease degradation. Moreover, the circulating EVs' miRNA profile appears to be very different from that of the cells of origin and is therefore a very potent prognostic factor [38]. In CLL, the plasma EXOs' specific miRNA profile includes the upregulation of miR-150, miR-155, and miR-29 family members and the downregulation of miR-223 [47]. The release of exosomal miR-150 and miR-155 was further elevated by anti-IgM treatment *in vitro*, which highlighted the correlation with BCR activation. MiR-155 in plasma EVs was shown to be correlated with CLL progression, survival, and treatment response [61,78]. Conversely, the transfer of miR-155 by EXOs has not been reported. However, the observation that miR-155 increases CLL cells' responsiveness to BCR ligation/activation suggests that the BCR is transactivated by the EXO-mediated transfer of miR-155 between cells [79]. CD40/IL4 stimulation of CLL cells results in an enrichment of miR-363 in the CLL EVs [51]. It is noteworthy that the serum level of miR-150 is also a prognostic factor in CLL. However, most of the circulating miR-150 is free in plasma and not encapsulated in EVs [80]. In addition to miRNAs, the non-coding RNAs of interest in CLL include circular RNAs; for example, CLL EVs contain elevated levels of mc-COX2 (a mitochondrial genome-derived circular RNA associated with a poor prognosis) [81]. Aside from non-coding RNAs, mRNAs detected in CLL EVs also constitute great biomarkers, with a signature including some BCR specific kinases and apoptosis regulators (LYN, SYK, MAPK, and BCL2) [46].

Regarding the protein content of CLL EXOs, a large-scale proteomic analysis of samples from patients with indolent disease vs. progressive disease showed that the profile

varied with disease progression [82]. In particular, exosomal S100 calcium-binding protein A9 (S100-A9) was identified as a marker of disease progression and an activator of the nuclear factor- κ B pathway in CLL cells. CLL EVs also expressed immune checkpoint ligands (including PD-L1) at their surface [60].

A small proportion of the CLL patients who become refractory to chemotherapy will go on to develop a more aggressive lymphoma known as Richter syndrome (RS) [83,84]. Although epigenetic modifications have been described as CLL progresses into RS, the underlying mechanisms are still not completely understood, and biomarkers of this transformation are needed. In a proof-of-concept study, Jurj et al. identified exosomal miR-19b as a regulator of CLL cell proliferation and invasion and thus as a predictive biomarker of RS transformation [85].

In summary, EVs appear to be important, relatively non-invasive diagnostic, prognostic, and disease progression biomarkers in CLL.

4.2. Interference with Immunotherapy

Given the great variety of cargo molecules disseminated into the TME, CLL EVs not only contribute to disease progression but also hamper the treatment of CLL.

Two recent studies of anti-CD19 chimeric antigen receptor (CAR)-T cell therapy showed that CLL EVs can disrupt the immune synapse and contribute to the CAR-T-cell's exhaustion, metabolic quiescence [59], or even lysis [86]. These findings might explain the CAR-T-cell treatment failure observed in the clinic. The researchers also demonstrated the presence of several different ICs (including PD-L1) on the surface of CLL EVs and suggested that this might explain the failure of CAR-T-cell therapy and anti-PD-1/PD-L1 therapy in CLL. The various ICs detected on EVs might compensate for each other when a single one is targeted. Furthermore, the exposure of PD-L1 on the EV surface might compete for anti-PD-L1 antibodies with cell-surface PD-L1.

Similarly, the surface presentation of B cell surface markers on EVs can interfere with immunotherapy. Neutralization of the anti-CD20 antibody rituximab by CD20 exposed on the surface of B-cell lymphoma EXOs leads to immune evasion [52,87,88], and the blockade of ABCA3-regulated vesiculation increased the effectiveness of rituximab therapy [87]. A similar scenario might apply to other B-cell membrane antigens bound to EXOs, such as CD19, CD37, and HLA-DR [88].

4.3. Novel EV-Driven Therapeutic Strategies

Despite of their potential interference with CLL immunotherapy, EVs might also constitute a valuable curative treatment for CLL [37,74,75].

As mentioned above, exosomal BAG6 and soluble BAG6 have opposing effects on NK cell activation and antitumor immunity; hence, treatment with BAG6-containing EXOs might restore the NK cell's ability to kill CLL cells [71]. EVs are now being considered as new therapeutic vehicles, with the custom delivery of drugs or molecules packaged in engineered EVs [37]. Thus, several studies have exploited the natural affinity of Epstein-Barr virus for B cells by developing custom EXOs that specifically target CLL cells. In one study, CLL B-cells were specifically targeted by EXOs tagged with the viral envelope protein gp350, leading to the exosomal co-transfer of gp350 and CD40L to the patients' cells and thus the stimulation of an anti-CLL T-cell immune response [89]. In a second study, gp350-labelled EXOs transferred both CD40L and pp65 protein to CLL cells and thus activated a T-cell immune response [90]. Very recently, the same principle was used to deliver fludarabine specifically to CLL B cells via gp350+ EVs purified from engineered red blood cells [91].

On the other hand, given the EVs' crucial role in CLL progression (as described above), targeting CLL vesicles (by blocking vesiculation in donor cells or uptake by recipient cells) might block tumor-TME communication [37]. For example, the inhibition of vesiculation through the targeting of Rab27a in B cells improves the post-chemotherapy antitumor response [92]. On the other hand, the pretreatment of CLL EVs with low-molecular-

weight heparin, a heparan sulfate analog, blocks the uptake of EVs by stromal cells [52]. Interestingly the authors noticed that CLL EVs were not internalized by CLL cells, and they suggested this could be linked to the difference in the exposure of heparan sulfate proteoglycans at the surface of the cells.

However, one major limitation might be a lack of specificity because the molecular drivers of vesiculation are common to many physiological processes. Hence, CLL-EV-specific mechanisms must be discovered, or EV inhibitors must be specifically delivered to CLL cells. For example, the CLL-specific delivery of Rab27a siRNA was performed by Zhang et al. through inactivated Epstein–Barr virus, limiting the inhibition of vesiculation to B cells [92]. One can also imagine that therapeutic EVs containing vesiculation inhibitors could be optimized for the delivery to CLL cells through the expression of specific tumor surface proteins and/or their production in mesenchymal stem cells that display a natural tropism toward tumor cells.

Overall, using EV communication to develop novel therapeutic strategies or to optimize current CLL treatments definitively hold some promise, but several challenges still remain [75]. To be suitable for clinics, EV-based therapies will need high-throughput technologies to produce and purify large quantities of standardized EV preparations with no off-target effects. Therapies targeting tumor EVs will also be challenging because of the shared mechanisms between physiologic and tumor vesiculation. Nevertheless, EV research has developed very rapidly during the last decade, and the numerous ongoing research works on tumor EVs will allow for the further elucidation of the modalities of EV secretion and uptake to design innovative therapeutic strategies for CLL and other tumor types.

5. Conclusions

Over the last 10 years, a large number of scientific articles on CLL EVs have been published. Several important points stand out. Firstly, the intensity of EV release and the nature of the EV contents might serve as biomarkers of CLL progression and the treatment response. Circulating EVs are particularly relevant diagnostic and prognostic biomarkers of solid tumors because a blood sample is less invasive than a tissue biopsy. However, circulating EVs are still of interest for the diagnosis and prognosis of B-cell malignancies such as CLL [37,38]. In CLL, the main prognostic factors are clinical observations, the *IGHV* mutation status, and genetic abnormalities. The EV count and the nature of the EVs' content (particularly miR-155) are correlated with CLL progression, BCR activation, and overall activation by the TME (see Section 3.2. and Section 4.1.) and thus constitute valuable biomarkers for CLL.

Secondly, EVs are actively involved in the dialog between CLL cells and the TME, which, in turn, provides the tumor cells with immune protection and a survival advantage (see Section 3.3.). The blockade of CLL EV trafficking would constitute a novel treatment option or an adjunct to current treatments for CLL.

Lastly, the rapid development of EV engineering technologies for the treatment of cancer suggests that novel, targeted approaches are possible, such as the EV encapsulation of CLL drugs and the expression of CLL-specific proteins by EVs [37,75]. It is important to bear in mind that the presence on CLL EVs of molecules that are also expressed on the surface of CLL B cells may impact CLL therapy. On one hand, this might be to the patient's disadvantage; for example, exosomal CD20 and PD-L1 neutralize anti-CD20 therapy [87] and CD19-CAR-T cells [86], respectively. More generally, the presentation of ICs at the EV surface impedes an antitumor immune response [60]. On the other hand, it should be possible to engineer EVs to express molecules also present on the surface of TME cells; the resulting competition process might inhibit tumor–TME communication and thus impede disease progression.

Several CAR-T agents (including anti-CD19 CAR-T (lisocabtagene), anti-CD20 CAR-T (C-CAR066), anti-CD19/CD20 CAR-T, and anti-CD20/CD22 CAR-T) are being tested as treatments for rituximab-resistant CLL in combination with other chemotherapeutics [6].

However, the observation of unexpected negative results and adverse events raised the question of whether CAR-T cells could be replaced by the CAR-T-EVs they generate. Very interestingly, a recent study showed that CAR-T-cell-derived EXOs carried the CAR but also had their own cytolytic activity, which led to the inhibition of tumor growth in two distinct mouse models [93]. In contrast to CAR-T therapy, CAR-T-EV therapy does not appear to be inhibited by the PD-1 pathway or induce cytokine release syndrome; it might therefore constitute a safer treatment option. Although further development is clearly needed, CD19-CAR-T-EVs have already been used to target B-cells in acute lymphocytic leukemia [94]; the transfer of this technology to CLL would be of great interest.

In summary, when considering the large number of activities exerted by CLL EVs and their roles in TME cross-talk, treatment response, and the development of resistance, targeting these vesicles might open up new therapeutic approaches. Complementary research is needed, but the combination of EV-based approaches with the currently available CLL therapies could undoubtedly help optimize treatment outcomes and improve patients' quality of life.

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Abbreviations

APRIL—a proliferation-inducing ligand; BAFF—B-cell activating factor; BAG6—BCL2-associated athanogene 6; BCL2—B-cell lymphoma/leukemia 2; BCR—B cell receptor; BM—bone marrow; BMSC—bone marrow stromal cell; BTK—Bruton's tyrosine kinase; CAF—cancer-associated fibroblast; CAR-T cell—chimeric antigen receptor T cell; CD40L—CD40 ligand; CLL—chronic lymphocytic leukemia; ECM—extracellular matrix; ESCRT—endosomal sorting complexes required for transport; EV—extracellular vesicle; EXO—exosome; IC—immunological checkpoint; IGHV—immunoglobulin heavy chain region; ISEV—international society for extracellular vesicles; LN—lymph node; LYN—Lck/Yes novel tyrosine kinase; miRNA—microRNA; MISEV—minimal information for studies of extracellular vesicles; MSC—mesenchymal stromal cell; MV—microvesicle; MVB—multivesicular body; NF- κ B—nuclear factor kappa B; NK—natural killer; PB—peripheral blood; PD-L1—programmed death ligand 1; PD-1—programmed death 1; PI3K—phosphoinositide 3-kinase; RS—Richter syndrome; SDF-1—stromal cell-derived factor 1; SYK—spleen tyrosine kinase; TLR—toll-like receptor; TME—tumor microenvironment; VEGF—vascular endothelial growth factor.

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Résumé en français

La leucémie lymphoïde chronique (LLC) est la forme la plus courante de leucémie adulte dans les pays occidentaux. Elle se caractérise par une accumulation de lymphocytes B monoclonaux CD5+ dans le sang périphérique, la moelle osseuse et les organes lymphoïdes secondaires. La LLC est considérée comme une maladie des personnes âgées avec une incidence plus élevée chez les hommes que les femmes. En ce qui concerne la pathophysiologie, et en comparant aux cellules B saines, les cellules de la LLC ont une activité plus élevée de la cascade de signalisation BCR qui est cruciale pour leur prolifération et leur survie. Le microenvironnement tumoral de la LLC est constitué de différents composants, notamment des cellules stromales mésenchymateuses, des cellules de type nurse-like cells (NLC), des cellules T et des cellules tueuses naturelles (NK), et chaque type de cellule joue un rôle dans l'activation et/ou la survie des cellules B de la LLC. L'évolution de la LLC est très variable et certains patients présentent une forme de maladie stable sans nécessité de traitement, tandis que d'autres développent une forme agressive et répondent mal au traitement. Le pronostic de la LLC dépend du stade clinique et des marqueurs biologiques, dont le statut mutationnel IGHV, les anomalies cytogénétiques caractéristiques comme la del11q et la del17p, et la complexité du caryotype. Quelques mutations génétiques sont aussi identifiées dans la LLC et certaines d'entre elles sont considérées comme des marqueurs pronostiques et prédictifs de la maladie. Cela inclut la mutation du gène TP53 dont l'identification constitue une étape importante dans le diagnostic de la LLC. Actuellement, les traitements de la LLC consistent de l'immuno-chimiothérapie Fludarabine-Cyclophosphamide-Rituximab (FCR) ou des thérapies ciblées comme les inhibiteurs de la Bruton tyrosine kinase (BTK) ou de la BCL-2. Malgré des résultats cliniques exceptionnels obtenus avec ses thérapies, la résistance aux drogues reste une cause majeure d'échec thérapeutique en raison de l'apparition de nouvelles résistances (par exemple, mutations dans la BTK, BCL-2, del8p, etc.). En effet, aucune rémission complète n'est garantie, il est donc nécessaire de poursuivre les explorations scientifiques afin de développer de nouvelles approches thérapeutiques mieux adaptées et qui assure une meilleure qualité de vie pour les patients atteints de la LLC.

Le métabolisme cellulaire correspond à l'ensemble des réactions biochimiques qui se produisent dans la cellule pour assurer sa viabilité. Il se divise en deux branches : l'anabolisme et le catabolisme. Les études sur le métabolisme des cellules cancéreuses ont connu une renaissance récente avec la reconnaissance de la « reprogrammation métabolique » comme une

composante essentielle du phénotype tumoral. Les cellules tumorales reprogramment leur métabolisme pour répondre à leur demande énergétique pour la croissance, la prolifération et la survie. L'exploration de cette reprogrammation métabolique dans le cancer permet d'explorer de nouvelles possibilités de thérapies ciblées. Une partie centrale de la recherche se concentre sur l'ATP, la monnaie énergétique de la cellule qui orchestre les fonctions cellulaires essentielles (par exemple la prolifération, la biosynthèse des macromolécules, etc.). Normalement, la génération d'ATP se passe par la phosphorylation oxydative mitochondriale (OXPHOS) qui est directement liée à la chaîne de transport d'électrons et dépend essentiellement d'une série antérieure de réactions métaboliques mitochondriales connues sous le nom du cycle de Krebs. En bref, les substrats métaboliques tels que le glucose, la glutamine et les dérivés d'acides gras produisent des intermédiaires métaboliques qui rejoignent le cycle de Krebs à l'intérieur de la matrice mitochondriale. Les produits de ce cycle, principalement le nicotinamide adénine dinucléotide (NADH) et la flavine adénine dinucléotide (FADH₂), fournissent les électrons à la chaîne de transport d'électrons pour la génération d'ATP. Une principale découverte dans le domaine de reprogrammation métabolique des cellules cancéreuses a été obtenue à partir des travaux d'Otto Warburg il y a à-peu-près cent ans. Ce scientifique a proposé que la cellule cancéreuse reprogramme son métabolisme hors de la mitochondrie pour produire l'ATP. La cellule dépend alors du glucose qu'elle transforme en lactate par un mécanisme connue sous le nom de la glycolyse aérobie ou l'Effet Warburg. Ultérieurement, les recherches sur le métabolisme du cancer ont démontré que cette reprogrammation est encore plus complexe et que certaines type de cellules tumorales restent dépendantes de la mitochondrie et utilisent des substrats autres que le glucose comme les acides gras et la glutamine.

Récemment, plusieurs études sur le métabolisme des cellules de la LLC ont démontré que ce métabolisme semble être hétérogène, tout comme la maladie elle-même. Les préférences métaboliques des cellules de la LLC peuvent changer en fonction des variations cytogénétiques ou de la progression de la maladie. En plus, un changement métabolique peut survenir en réponse à l'infiltration de cellules dans le tissu lymphoïde, voire en réponse à un traitement. Les préférences de substrats métaboliques dépendent également de leur utilisation, que ça soit pour servir des réactions anaboliques ou cataboliques spécifiques, tout au profit des cellules tumorales. Ainsi, mon projet de thèse vise à identifier une spécificité métabolique caractérisant les lymphocytes B de la LLC en se concentrant sur une voie métabolique qui pourrait être cruciale pour la génération d'ATP dans ces cellules tumorales. L'identification d'adaptation métabolique peut révéler des vulnérabilités propres aux cellules tumorales. Ainsi, la

perturbation sélective de ce métabolisme pourrait être une façon efficace pour l'élimination sélective des cellules de la LLC. En outre, cibler cette vulnérabilité métabolique en combinaison avec les molécules thérapeutiques existantes apparaît comme une stratégie convaincante pour améliorer l'efficacité du traitement.

Ainsi, j'ai démontré une augmentation de l'OXPPOS dans les cellules B CD5+ de LLC par rapport aux cellules B CD5-B saines, malgré une masse mitochondriale identique, suggérant une prédominance de la génération d'ATP par la mitochondrie. L'utilisation du glucose comme intermédiaire métabolique pour l'OXPPOS a été exclue par trois approches différentes : i) une comparaison du niveau de métabolites qui sont impliqués dans le métabolisme du glucose (mesuré par spectrométrie de masse), ii) une mesure du niveau d'absorption du glucose par les cellules iii) et un glucose stress test (mesuré par un analyseur de seahorse). La première approche (l'étude métabolomique) a aussi confirmé l'exclusion de la dépendance des cellules de la LLC sur l'effet Warburg car elle a montré des niveaux significativement plus bas du lactate dans ces cellules malignes en comparant aux cellules normales. Une analyse d'enrichissement d'ensembles de gènes (GSEA) d'une donnée publique d'ARN-Seq comparant les cellules B de donneurs sains aux cellules B de patients de LLC a révélé en premier lieu la surexpression de gènes impliqués dans la β -oxydation des acides gras (FAO). Cette surexpression, qui concerne des gènes impliqués dans la FAO mitochondriale et peroxysomale a été confirmée par une approche qPCR. En particulier, ACOX1, une enzyme clé de la FAO peroxysomale (pFAO), est surexprimée dans les cellules B de LLC. Une approche métabolomique complémentaire a dévoilé un niveau élevé de la carnitine, une molécule essentielle pour le transport des acides gras à travers la membrane mitochondriale. En plus, la bétaine, une molécule impliquée dans la régulation de l'expression des gènes codant pour des enzymes clés de la voie de la bêta-oxydation des acides gras, est significativement augmentée dans les cellules B de la LLC par rapport aux cellules B de donneurs sains. En outre, une augmentation des C2-C6 acylcarnitines, les produits métaboliques finaux de la pFAO ont été aussi observés.

Les peroxysomes sont de petits organites présents dans les cellules eucaryotes. Ils contiennent une variété d'enzymes essentielles pour différentes fonctions métaboliques, notamment le métabolisme des acides gras et des acides aminés, et la réduction des dérivés réactifs de l'oxygène. En effet, les peroxysomes sont appelés ainsi en raison de leur capacité à générer et à dégrader le peroxyde d'hydrogène. En fait, environ 30 % des niveaux de H₂O₂ dans les cellules proviennent de réactions peroxysomales. Ces organites sont cruciaux pour la santé et

le développement humain car les défauts du métabolisme ou de la biogenèse peroxysomale sont impliqués dans des troubles peroxysomaux graves et multiples. L'une des fonctions principales et essentielles des peroxysomes est l'oxydation des acides gras. En effet, en raison de leur structure et de leur configuration, certains acides gras ne peuvent pas être pris directement par les mitochondries pour y être catabolisés, et doivent d'abord subir une première transformation dans les peroxysomes. Les peroxysomes peuvent décomposer principalement les acides gras à très longue chaîne, à chaîne ramifiée et les acides dicarboxyliques. Comme la mitochondrie, la pFAO implique quatre réactions successives. Dans les deux organites, les réactions sont catalysées par des enzymes qui ont la même fonctionnalité sauf la première réaction chimique qui est catalysée par deux enzymes différentes : une oxydase dans les peroxysomes et une déshydrogénase dans les mitochondries. ACOX1 est l'enzyme clé de la de la première réaction de la pFAO. Après une succession de réactions, la chaîne des acides gras qui sont oxydés dans les peroxysomes est réduite et les produits finaux doit donc être exporté de l'organite pour être métabolisé davantage. Principalement, ces produits finaux sont transportés vers les mitochondries pour être traités générer du NADH et du FADH₂, utilisés par la chaîne de transport d'électrons mitochondriale pour produire de l'ATP. Pour y être transporté des peroxysomes aux mitochondries, ces acides gras à chaînes réduits doit être conjugués à la carnitine, par des réactions catalysées par la carnitine acetyltransferase (CRAT) ou la carnitine octanoyltransferase (CROT).

L'implication des peroxysomes dans le développement et la progression des cancers n'est pas bien reconnue et pas prise en compte au même titre que les mitochondries. Pour cette raison, j'étais intéressé à étudier plus en détail l'implication de la bêta-oxydation des acides gras peroxysomales comme potentiel caractéristique métabolique des cellules B de la LLC. Afin d'étudier en détail cette voie métabolique, je me suis concentré sur ACOX1. J'ai choisi cette enzyme peroxysomale pour plusieurs raisons principales : (i) La surexpression de son niveau d'ARNm et de protéine (ii) Cette enzyme est spécifique aux peroxysomes et est limitante dans la pFAO. (iii) Elle peut être inhibée pharmacologiquement, ce qui peut nous aider à déterminer l'importance de cette voie dans le métabolisme et la survie des cellules LLC. En commençant par une approche shRNA visant à diminuer l'expression du gène codant pour ACOX1, on a pu démontrer la capacité des cellules de la lignée OSU-CLL à reprogrammer leur métabolisme vers la glycolyse. Ceci a été démontré par une augmentation significative des niveaux de glucose, pyruvate et lactate. En plus, une augmentation de la glutamine a été observée. De ce fait, la bêta-oxydation des acides gras peroxysomale dirigé par ACOX1 semble être une voie métabolique centrale qui influence l'ensemble du métabolisme des cellules primaires de la

LLC. Afin d'étudier l'importance de cette voie métabolique dans les cellules primaires de la LLC, on a testé un inhibiteur spécifique de ACOX1 nommée 10,12-tricosadiynoic acid (TRCDA). D'abord, on a pu démontrer l'efficacité du TRCA à causer l'inhibition d'ACOX1. Pour ce faire, on a fait recours à trois approches. La première c'est la mesure du niveau de H₂O₂ après avoir traité les cellules B primaires de LLC par TRCDA. On a pu démontrer une diminution rapide du H₂O₂ dans les cellules traitées. A noter que le H₂O₂ est un produit de la première réaction de la bêta-oxydation qui est catalysée par ACOX1. Une deuxième approche nous a permis de démontrer la diminution des produits finaux de la pFAO après 24h de traitement avec TCRDA et de façon doses dépendantes. Notamment, La diminution significative des taux d'acétyl-carnitine (C2-carn) renforce l'hypothèse d'une activité de beta-oxydation peroxysomale puisque chaque cycle de beta-oxydation d'acide gras normalement génère un acétyl-CoA, donc ce produit est transporté vers les mitochondries pour rejoindre le cycle du TCA sous forme de C2-carn. Enfin, une accumulation des acides gras a été observée sous formes de gouttelettes lipidiques (LD) par deux approches : cytométrie de flux et microscopie électronique. Ceci suggère une interaction entre les peroxysomes et les gouttelettes lipidiques. Nous émettons l'hypothèse que ces gouttelettes sont capables de séquestrer les acides non oxydés par les peroxysomes. Deux explications sont possibles: i) les acides gras non métabolisés sont transférés de l'intérieur des peroxysomes vers les LD par une interaction directe entre les deux organites. ii) l'inhibition de la bêta-oxydation peroxysomal médiée par ACOX1 bloque indirectement l'entrée des acides gras dans cet organite, et des LD se forment pour séquestrer les acides gras accumulés dans le cytosol afin d'éviter la lipotoxicité. En continuant avec l'effet de l'inhibition d'ACOX1 sur le métabolisme de la LLC, on a observé une diminution importante et significative des niveaux d'ATP après 24h du traitement avec TRCDA. Cette diminution d'ATP est indépendante du status mutationnel d'IGHV ou de la complexité du caryotype. Cette observation confirme une connexion peroxysomes-mitochondries, dans laquelle les peroxysomes fournissent leurs produits finaux pour alimenter le cycle de Krebs et l'O₂PHOS. Aucune chute d'ATP n'a été observée en traitant les cellules B de donneurs sains (qui dépendent très probablement du glucose comme substrat pour la génération d'ATP) par TRCDA ce qui confirme la spécificité de cette voie métabolique à la LLC. Donc on peut conclure que la pFAO joue un rôle central à alimenter la génération d'ATP mitochondriale dans la LLC. Ensuite, l'inhibition pharmacologique d'ACOX1 *in vitro* pour 24h induit la mort cellulaire de façon dose dépendante. Cette inhibition était inefficace et n'a montré aucun effet sur les cellules B de personnes saines ainsi que sur d'autres cellules immunitaires comme les cellules T et les cellules NK. Cela donne un intérêt supplémentaire à l'inhibition de

cette voie métabolique comme un potentiel traitement dans la LLC car en épargnant les cellules immunitaires, on évite l'impact de cette modulation métabolique sur d'autre fonction immunitaire. En outre, j'ai montré que l'inhibition de cette voie métabolique peroxysomale est efficace pour induire la mort cellulaire des cellules indépendamment du statut mutationnel IGHV, de la présence d'altérations cytogénétiques liées à un pronostic défavorable (del11q, del17p) ou la complexité du caryotype. Par des approches supplémentaires, on a appris que cette mort cellulaire programmée est caspase-dépendante, et est accompagné par une perte du potentiel mitochondrial membranaire et une surproduction des espèces réactives de l'oxygène. *In vivo*, les cellules de la LLC reçoivent des signaux de survie et des signaux prolifératifs de leur microenvironnement. Ces signaux peuvent avoir un impact sur le métabolisme de ces cellules. Pour cette raison, nous avons testé l'effet du TRCDA sur la viabilité des cellules B de la LLC en activant en priori le BCR par des anti-IgM solubles, en les co-cultivant avec des cellules stromales ou en les stimulant en priori par CD40L et CpG. Nos résultats ont indiqué que l'inhibition d'ACOX1/pFAO par TRCDA restait un moyen efficace pour induire la mort aux cellules recevant des signaux pro-survi et pro-prolifération. Enfin, les thérapies combinées ont un grand intérêt dans la recherche car elle permette d'obtenir de meilleurs résultats thérapeutiques, tout en réduisant la résistance aux thérapies ainsi que les effets toxiques indésirables. Pour cette raison, nous avons testé l'effet de la combinaison du TRCDA avec des drogues utilisé en clinique pour le traitement de la LLC : la fludarabine (un analogue de la purine), l'ibrutinib et l'acalabrutinib (des inhibiteurs de BTK) et le venetoclax (un inhibiteur de BCL-2). De façon intéressante, la combinaison des inhibiteurs d'ACOX1 et de BTK à faibles doses induit l'apoptose de manière synergique dans les cellules primaires de la LLC, y compris les cellules avec del17p, del11q ou de caryotype complexe. De façon intéressante, l'effet cytotoxique synergique de la combinaison TRCDA/ibrutinib persiste lorsque les cellules sont activées du BCR pour 24h avec de l'anti-IgM soluble.

En résumé, cette thèse met en évidence le rôle potentiel de l'oxydation des acides gras peroxysomale médiée par ACOX1 en tant que spécificité métabolique des cellules LLC. Le métabolisme peroxysomal et son implication dans le cancer est encore un domaine sous-exploré, avec seulement une poignée d'études portant sur la contribution de cet organelle et ses voies métaboliques à la survie des cellules tumorales. Nous fournissons ici des preuves de la contribution du pFAO médié par ACOX1 à la génération d'ATP mitochondrial dans la LLC. L'un des points forts de cette thèse est que la pFAO médiée par ACOX1 est une vulnérabilité métabolique caractéristique de la LLC, quel que soit le stade de la maladie, le statut mutationnel de l'IGHV ou le profil génétique et la complexité du caryotype. Dans une maladie caractérisée

par son hétérogénéité, je propose la voie de la bêta-oxydation peroxysomale dirigée par ACOX1 comme une caractéristique métabolique commune de la LLC. Par conséquent, cibler cette voie métabolique peut constituer une approche thérapeutique efficace contre cette leucémie. Alors que nos études ont été réalisées *in vitro* sur des cellules primaires de LLC, il serait intéressant de tester l'effet du TRCDA (ou même de nouveaux inhibiteurs développés contre ACOX1), sur la croissance et la progression tumorale *in vivo*, par exemple sur le modèle murin E μ -TCL1.

D'un point de vue métabolique, nous avons pu détecter les produits finaux de pFAO médié par ACOX1 en abondance dans la LLC par rapport aux cellules B saines. Ce qui manque dans notre étude, c'est la détection du substrat qui alimente cette voie métabolique. Plusieurs acides gras à très longue chaîne peuvent être oxydés par les peroxysomes, donc identifier lequel(s) est/sont préférentiellement catalysé(s) par les cellules LLC reste une question à élucider.

Notre étude introduit un rôle des peroxysomes et plus spécifiquement le métabolisme peroxysomal dans la LLC. Des études plus vastes restent nécessaires pour élucider le rôle général de cet organite dans la maladie. Plusieurs aspects peuvent être étudiés pour examiner les peroxysomes en tant qu'organites pro-tumoraux dans la LLC. Celles-ci incluent une comparaison de l'abondance des peroxysomes entre la LLC et les cellules B saines. De plus, l'identification d'un ou plusieurs facteurs de biogenèse des peroxysomes surexprimés dans la LLC pourrait fournir des preuves supplémentaires d'une éventuelle augmentation de la biogenèse des peroxysomes pouvant être associée à la progression de la maladie.

En conclusion, la plupart des objectifs de ce travail doctoral ont été atteints. J'ai pu identifier une caractéristique métabolique spécifique dans les cellules LLC qui peut être utilisée comme une potentielle cible thérapeutique. En effet, priver la cellule maligne de sa source d'énergie en ciblant la voie métabolique pFAO, seule ou en combinaison avec des thérapies ciblés utilisés actuellement en clinique, pourrait représenter une stratégie thérapeutique métabolique innovante contre la LLC.