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Characterization of the role of TKTL1 in Acute Monocytic Leukaemia

Inês do Carmo Viegas Baptista

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Acute Monocytic Leukaemia**

**Inês Viegas Baptista
PhD Thesis
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UNIVERSITY OF
BIRMINGHAM

Doctoral Program in Biotechnology

Department of Biochemistry and Molecular Biomedicine

Faculty of Biology

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in Acute Monocytic Leukaemia**

Doctoral Thesis submitted by Ines do Carmo Viegas Baptista to obtain the Ph.D. degree
from the University of Barcelona and University of Birmingham
in the frame of a dual degree agreement.

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*To my family,
the born and the chosen.*

Solid as dreams of a rebel,

We'll always find our way.

Frankie Chavez.

Para ser grande, sê inteiro: nada

Teu exagera ou exclui.

Sê todo em cada coisa. Põe quanto és

No mínimo que fazes.

Assim em cada lago a lua toda

Brilha, porque alta vive.

Fernando Pessoa, Odes de Ricardo Reis.

ABSTRACT

Leukaemia is one of the types of cancer where treatment resistance is prevalent. Better understanding of leukemic cells metabolism opens possibilities for new therapeutic strategies and better prognosis stratification. Leukaemia arises from many different genetic alterations, that affect distinct cellular processes, all driving leukemogenesis. Many of these result in a phenotype that grants metabolic advantages amidst the hypoxic conditions of the haematopoietic niche of the bone marrow, the point of origin of all types of this cancer. In order to understand the metabolic reprogramming that grants these advantages, we performed metabolic characterization in vitro of cell lines of acute monocytic leukaemia (THP1) and chronic myeloid leukaemia (HAP1), with focus on the role of Transketolase-like 1 (TKTL1) and ten-to-eleven Methylcytosine dioxygenase 2 (TET2), respectively, on both normoxic and hypoxic experimental settings.

We revealed that TKTL1 is a key enzyme in the metabolic reprogramming of the hypoxia adaptation, driving proliferation, higher glycolytic rates, higher glutamine consumption and subsequent glutamate production. Our results also showed the altered metabolism of many amino acids and biogenic amines, that grant more substrates for nucleotides synthesis, higher stress tolerance and manipulation of the immune response of the microenvironment. It also highlighted the vulnerabilities that arise from focusing on targeting TKTL1 for therapy and possible secondary targetable metabolic pathways for future therapies.

Additionally, we demonstrated the effects of the loss of TET2 in the leukemic cells through a tracer-based metabolomics approach, showing how its mutation primes the cells to shift their metabolism at a higher cost for their ROS homeostasis, a known hallmark of cancer which increases the risk of more mutations occurring due to genomic instability. This allowed us to create a metabolic map of the changes induced by the loss of TET2,

as a blueprint for new therapeutic venues in leukaemias that have this mutational hit.

Together, the thesis presented here contributes to the knowledge of the mechanisms underlying metabolic reprogramming of leukaemia according to specific mutational genotypes and how they open new possible therapies for patients that develop resistance.

Index

1	Introduction	8
1.1	Leukaemia: The Cancer of the Bone Marrow	8
1.1.1	Cancer Overview	8
1.1.2	Haematopoiesis and the origin of leukaemia.....	9
1.1.3	Leukaemia epidemiology	11
1.1.4	Leukaemia Classification	11
1.1.4.1	Acute Myeloid Leukaemia	12
1.1.4.2	Chronic Myeloid Leukaemia.....	14
1.1.5	Mutations in leukaemia and their implication with metabolism	14
1.1.5.1	Transketolase-like 1	16
1.1.5.2	Ten-to-Eleven Dioxygenase 2.....	19
1.2	Epigenetic Instability as a Hallmark of Leukaemia	23
1.2.1	Alterations of DNA methylation status in Leukaemia	25
1.2.2	Histone Modifications: gatekeeping access to DNA.....	27
1.2.3	Micro-RNAs, the genome “hidden agents”.....	29
1.2.4	The “symbiosis” of epigenetic events with Transcription Factors in Leukaemia	31
1.2.5	The Epigenetic machinery is dependent on metabolism ..	32
1.3	Metabolic Dysregulation as a Hallmark of Leukaemia	33
1.3.1	Glycolysis and the Warburg Effect	35
1.3.2	Pentose Phosphate Pathway	37
1.3.3	Mitochondrial Activity	40
1.3.4	Metabolism of Amino Acids	44
1.3.4.1	Glutamine: the addiction of cancer	45
1.3.4.2	Glutamate	46
1.3.4.3	Serine, Glycine and Methionine: The One-Carbon Metabolism.....	46
1.3.4.4	The amino acids of the Urea Cycle	50
1.3.4.5	Other amino acids interconnections	52

1.3.4.5.1	Branched-chain Amino Acids	53
1.3.4.5.2	Alanine	53
1.3.4.5.3	Asparagine.....	54
1.3.4.5.4	Proline	54
1.3.4.5.5	Lysine.....	55
1.3.4.5.6	Threonine	55
1.3.4.5.7	Histidine	55
1.3.4.5.8	Phenylalanine and Tyrosine	56
1.3.4.5.9	Tryptophan.....	56
1.3.5	Metabolism of Biogenic Amines.....	57
1.3.6	Redox Balance: The ROS Equilibrium Game.....	58
1.4	Hypoxia: A Hallmark of Leukaemia on his own	60
1.5	Metabolic Studies Tools.....	64
1.5.1	SIRM-tracer based Metabolomics.....	68
2	Objectives.....	73
3	Materials and Methods	76
3.1	Cell Culture	76
3.2	Cell Proliferation Rate Assays	76
3.3	Biochemical Assays for Consumption and Production by Spectrophotometry	77
3.4	Enzymatic Activity Assays	78
3.4.1	TKT and TKTL1	79
3.4.2	GAPDH.....	79
3.4.3	LDH.....	79
3.4.4	HK	80
3.4.5	G6PD and 6PGD	80
3.4.6	PK.....	80
3.5	Protein Content by Cell Number Assay	80
3.6	RNA-based Assays.....	81
3.6.1	RNA Isolation	81

3.6.2	RT-PCR.....	81
3.6.3	RNA-sequencing	82
3.7	Mitochondrial Activity Assays.....	82
3.7.1	Oxygen Consumption Rate (OCR).....	83
3.7.2	Extracellular Acidification Rate (ECAR).....	83
3.7.3	Calculations from OCR and ECAR assays	84
3.8	Reactive Oxygen Species (ROS) Assays	85
3.9	Targeted Metabolomics.....	85
3.10	Stable Isotope-Resolved Metabolomics (SIRM).....	86
3.10.1	Gas Chromatography Mass Spectrometry (GCMS).....	87
3.10.1.1	Extracellular Metabolites Extraction.....	87
3.10.1.2	Polar Intracellular Metabolites Extraction.....	89
3.10.1.3	SIRM data analysis.....	91
3.10.2	Nuclear Magnetic Resonance (NMR)	92
3.10.2.1	Intracellular Metabolite Extraction	93
3.10.2.2	Extracellular Metabolite Extraction	93
3.10.2.3	1D-NMR.....	94
3.10.2.4	¹³ C-filtered ¹ H-NMR.....	95
3.10.2.5	2D ¹ H- ¹³ C HSQC.....	95
4	Results.....	100
4.1	Chapter I – Metabolic Characterization of TKTL1 in AML..	100
4.1.1	Introduction	100
4.1.2	Results.....	101
4.1.2.1	TKTL1 contributes to cell growth in normoxia and hypoxia	101
4.1.2.2	TKTL1 is essential to trigger hypoxia-induced changes to glucose fate in THP-1 cells	106
4.1.2.3	TKTL1 is essential to trigger hypoxia-induced changes to glutamine fate in THP-1 cells	109
4.1.2.4	TKTL1 is essential to trigger hypoxia-induced changes in amino acids fate in THP-1 cells	112

4.1.3	Discussion	117
4.2	Chapter II – Metabolic Characterization of TET2 loss of function in a CML cell model	120
4.2.1	Introduction	120
4.2.2	Results	121
4.2.2.1	TET2 loss does not affect cell growth or glycolytic dependency	121
4.2.2.2	TET2 loss affects use of G6P and flux of Pentose Phosphate Pathway	126
4.2.2.3	TET2 loss affects ROS homeostasis	130
4.2.2.4	TET2 loss alters the entry point of pyruvate into TCA cycle	134
4.2.2.5	TET2 loss impairs the hypoxia-triggered switch to reductive carboxylation of glutamine	138
4.2.2.6	TET2 loss directs One-Carbon Metabolism for Glutathione production	144
4.2.2.7	TET2 loss influences metabolism of amino acids	148
4.2.3	Discussion	155
5	General Discussion	163
6	Conclusions	173
7	Bibliography	177
8	Appendix I	221
8.1	Targeted metabolomics experiments for THP-1 cell models.	221
8.2	Targeted metabolomics experiments for HAP-1 cell models.	225
9	Acknowledgments	232

1. INTRODUCTION

1 Introduction

1.1 Leukaemia: The Cancer of the Bone Marrow

1.1.1 Cancer Overview

Cancer has been described as the group of diseases that begins from an abnormal cell growth, uncontrollable and invasive of the adjoining areas from the tissue of origin¹. According to the World Health Organization (WHO) definition, cancer initiates with an accumulation of genetic mutations that can come from a hereditary background, environmental factors, chemical compounds, diet or infections. The last global report on cancer ordered by WHO in 2018 expected 18.1 million new cases of cancer and 9.6 million deaths derived from it¹. An update from 2020 was performed by the same team, with the estimations of new cases around 19.3 million and 10 million deaths². These numbers highlight how cancer has become ever more one of the top public health problems, necessary of medical advances on both screening and treatment.

Cancer can be a solid tumour, with a proper mass of cancer cells obstructing the organ or tissue of origin. Or they can be considered “liquid tumours”, which consist not of an aggregate of cancer cells but on cells being on the circulatory systems (i.e. leukaemia and lymphoma).

The biology of a tumour has been defined by Hanahan and Weinberg in 10 grouping criteria: The Hallmarks of Cancer. The initial six hallmarks are cell death resistance, evasion of growth suppressors, sustained proliferation, angiogenesis, replicative immortality and capacity for invasion and metastasis³. Further scientific breakthroughs led to the addition of 4 remaining hallmarks: inflammatory response activation, cellular energetics dysregulation, immune evasion and genome instability⁴. Different types of cancer manifest these hallmarks distinctly, creating a vast array of subtype classification within a single tumour disease.

1.1.2 Haematopoiesis and the origin of leukaemia

Leukaemia is the denomination of the type of cancer that forms from cells in the bone marrow. It is in the bone marrow that occurs the development and differentiation of the many cellular components of the blood and immune system. This process is called Haematopoiesis. It starts with the Haematopoietic Stem Cells (HSCs), which are self-renewal, multi-potent cells with extremely high proliferative capacity. They exist in the form of two subpopulations: Long-term (LT)-HSCs and Short-term (ST)-HSCs (Figure 1.1.1). The LT-HSCs are the “storage” HSCs that can repopulate the entire haematopoietic system for longer than 3-4 months⁵. Their immediate progeny are the ST-HSCs, which have a limited self-renewal and can only refill the haematopoietic system for one month⁶.

It is the ST-HSCs that differentiate into Multipotent Progenitor cells (MPP), with a limited self-renewal, lack of repopulating capacity and still not lineage-committed. The MPP cells then branches out like a tree into 2 types of progenitors: the common myeloid progenitor (CMP) and the common lymphoid progenitor (CLP). It is at this step in the haematopoietic process that lineage-restriction is truly applied, with the CMPs creating the myeloid lineage of blood cells and CLPs originating the lymphoid lineage⁷⁻⁹. Further haematological differentiation is described in Figure 1.1.1.

In recent years with the advancement in research on how the haematopoietic process happens thanks to Single-cell Assays evolution, it is now understood that the differentiation is not cut-clear into groups but more of a continuum from progenitor into final cell^{10,11}. As such, new representations of haematopoiesis are underway, using trajectory lines of differentiation for each type of cell of the haematopoietic system.

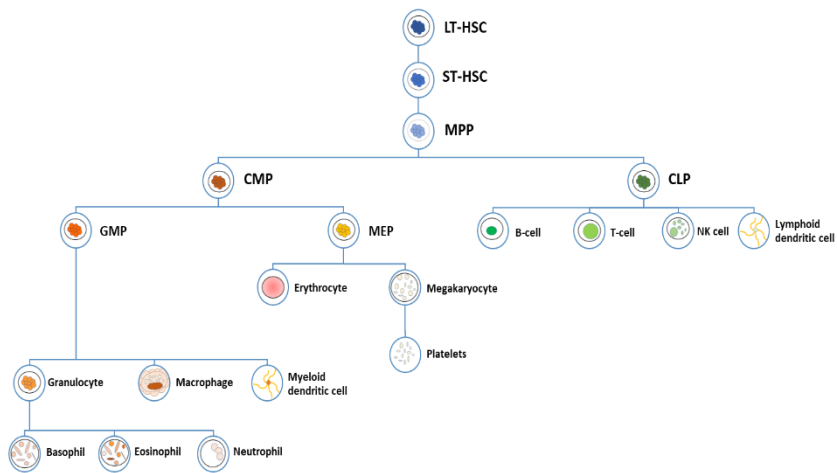


Figure 1.1.1- Schematic of Haematopoiesis. LT-HSCs differentiate into ST-HSCs and sequentially to MPPs. Then it initiates the separate lineages of Myeloid progenitors (CMPs) and Lymphoid progenitors (CLPs). The lymphoid branch generates B-cells, T-cells, Natural killer cells (NK-cells) and lymphoid dendritic cells. The myeloid progenitor differentiates in GMPs and MEPs. The MEPs create Erythrocytes and Megakaryocytes, which in turn makes platelets. The GMPs form Macrophages, Granulocytes and Myeloid dendritic cells. Lastly, granulocytes make basophils, eosinophils and neutrophils. Adapted from Laurenti et al., 2018.

Haematopoiesis is a process that needs very high levels of regulation, finely tuned to control the many different subpopulations of cells and the destiny of HSCs (quiescence, self-renewal and differentiation). This happens with the aid of a complex microenvironment within the bone marrow, denominated as niche^{12,13}. The HSC niche has been shown to be responsible for the regulation and maintenance of immune cells and blood production. It is from the niche secretions that the transcription factors and cytokines that regulate HSCs originate^{12,14}.

Any failure on HSCs regulation, homeostasis of the niche or imbalance on differentiation trajectory can lead to haematological malignancies, such as leukaemia and lymphoma.

1.1.3 Leukaemia epidemiology

As mentioned above, Leukaemia is the cancer that arises from errors during Haematopoiesis, leading to a rapid proliferation of abnormal cells that lack the proper differentiation. Leukaemia is a disease that affects all age brackets, being the 13th place by incidence and 10th by mortality, worldwide, by the end of 2020² (Figure 1.1.2).

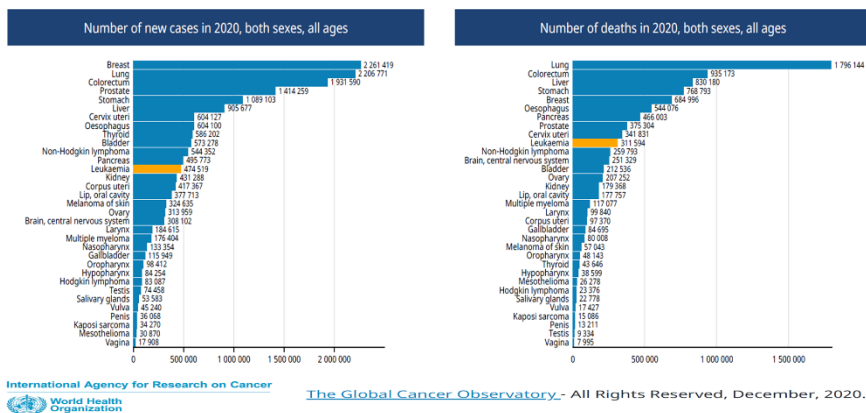


Figure 1.1.2- Cancer Statistics for the year 2020 by WHO. The number of new cases and of deaths, across all ages, for both sexes, for the year 2020 for all main types of cancers. Leukaemia is highlighted in orange. Adapted from Leukaemia fact sheet, available for free use at The Global Cancer Observatory (<https://gco.iarc.fr/today/data/factsheets>).

Leukaemia has a 5-year relative survival rate of 65%, with the estimations for 2021 of almost 62 thousand new cases and eventual 23.660 deaths¹⁵. The distribution of leukaemia per age varies according to the leukaemia subtype, with some types occurring almost entirely on children patients and other being specific of old age^{16,17}.

1.1.4 Leukaemia Classification

The WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues (4th edition, 2008) was revised in 2016 to better structure different

types of leukaemias, considering the many haematologic, morphologic, cytogenetic and molecular genetic findings¹⁸.

The classification of Leukaemia is performed using 2 principal criteria: the speed of progression and the nature of the malignant cells found. The speed of progression is defined by how long the disease evolves from acute into the chronic form. The nature of the malignant cells revolves around if it originates from the myeloid or the lymphoid subtypes. The distribution of these characteristics results in 4 major classification of leukaemia: Acute Myeloid Leukaemia (AML), Chronic Myeloid Leukaemia (CML), Acute lymphocytic Leukaemia (ALL) and Chronic lymphocytic Leukaemia (CLL). Within each of these groupings, a more detailed separation of the disease happens according to prevalent genetic events (mutations, translocations, etc.), morphology and other pathophysiological traits¹⁸.

1.1.4.1 Acute Myeloid Leukaemia

AML is the leukaemia that rises from accumulation of mutations on progenitor cells that leads to an increased proliferation of abnormal immature myeloid cells that impede the normal proceedings of Haematopoiesis. These cells infiltrate the bone marrow, bloodstream and subsequently organs. The spreading of the malignant cells leads to a variety of symptoms, such as anaemia, bleeding, infections and organ infiltration¹⁹. AML is a type of leukaemia that can result from other diseases, such as Myelodysplastic Syndrome (MDS) or Myeloproliferative Neoplasm (MPN). It can also be acquired *de novo*, due to treatments with cytotoxic therapies²⁰. AML is further structured in subtypes according to genetic

abnormalities, cytogenetic characterization, due to previous therapies or related to Down Syndrome (Table 1.1-A).

Table WHO Haematological Malignancies Classification (4th Edition)	
Acute myeloid leukemia (AML) and related neoplasms	
AML with recurrent genetic abnormalities	
	AML with t(8;21)(q22;q22.1);RUNX1-RUNX1T1
	AML with inv(16)(p13.1;q22) or t(16;16)(p13.1;q22);CBFB-MYH11
	APL with PML-RARA
	AML with t(9;11)(p21.3;q23.3);MLLT3-KMT2A
	AML with t(6;9)(p23;q34.1);DEK-NUP214
	AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); GATA2, MECOM
	AML (megakaryoblastic) with t(1;22)(p13.3;q13.3);RBM15-MKL1
	Provisional entity: AML with BCR-ABL1
	AML with mutated NPM1
	AML with biallelic mutations of CEBPA
	Provisional entity: AML with mutated RUNX1
AML with myelodysplasia-related changes	
Therapy-related myeloid neoplasms	
AML, NOS (not otherwise specified)	
	AML with minimal differentiation
	AML without maturation
	AML with maturation
	Acute myelomonocytic leukemia
	Acute monoblastic/monocytic leukemia
	Pure erythroid leukemia
	Acute megakaryoblastic leukemia
	Acute basophilic leukemia
	Acute panmyelosis with myelofibrosis
Myeloid sarcoma	
Myeloid proliferations related to Down syndrome	
	Transient abnormal myelopoiesis (TAM)
	Myeloid leukemia associated with Down syndrome

Table 1.1-A- WHO Classification of AML and related neoplasms. Classification is according to genetic abnormalities (chromosomal translocation, gene mutations), myelodysplasia characteristics, surgency due to therapy, cytogenetic profiling, tissue containment (sarcoma) and relation to Down syndrome complications (from trisomy of chromosome 21). Adapted from Arber et al., 2016.

1.1.4.2 Chronic Myeloid Leukaemia

CML is a Myeloproliferative Neoplasm, defined by a clonal disorder of aberrant myeloid blasts in the bone marrow, with an additional increase of erythrocytes and platelets in peripheral blood²¹. This disorder has its cause on the Philadelphia Chromosome, a genomic abnormality resulting from the translocation of chromosome 9 and 22 (t(9;22) (q34.1;q11.2)). This leads to the formation of the fusion gene BCR-ABL1, detected in almost all CML patients (95% from Philadelphia chromosome, remaining 5% from other translocations). Symptomologies are fatigue, anorexia and weight loss, but a portion of patients are asymptomatic²¹⁻²³.

Of the total number of new leukaemia cases, around 15% are CML, with an average age of 64 years old at time of diagnosis (American Cancer Society). The 5-year relative survival rate is at 70.6%, mostly due to the implementation of Tyrosine Kinase Inhibitors (TKIs) as a standard treatment²³.

1.1.5 Mutations in leukaemia and their implication with metabolism

As amply referenced above, Leukemogenesis originates on mutations and errors of the normal haematopoietic process. At which step the abnormality occurs or the order of accumulated errors results in many subtypes of leukaemia. AML is mostly resulting from gene mutations or translocations that disrupt the homeostasis of the cell. The main genetic abnormalities used for classification relate to transcription factors²⁴⁻²⁷, epigenetics^{26,28-31} and proliferation³² (Table 1.1-A). But these changes by themselves are not capable of converting the haematopoietic cell into a tumour cell. Other mutations contribute during the tumorigenesis. Many common mutations that happen across all AML subtypes are Fms-like tyrosine kinase 3³³ (FLT-3) (around 30%), Isocitrate Dehydrogenase 1 (IDH1) and IDH2 (15-20%),

NPM-1³¹ (around 30%) and CEBPA²⁹ (5-10%). Other recurrent mutations are NRAS, KRAS, TP53, DNA-methyltransferase 3A (DNMT3A), Ten-to-Eleven Methylcytosine Dioxygenase 2 (TET2) and Additional Sex Combs like 1 Transcriptional Regulator (ASXL1)^{17,34,35}.

On the same hand, CML with the BCR-ABL1 fusion gene results on the constitutively active tyrosine kinase that initiate the signalling pathways of proliferation (NRAS, KRAS, MAPK)^{36,37}. The BCR-ABL1 enzyme itself can also differ depending on where the break of the BCR gene during the translocation process, leading to different isoforms that translate into different CML phenotypes. CML shares some of the same common mutations of AML, such as NRAS, KRAS, TET2, ASXL and RUNX1³⁷. The overall range of mutations that happen in leukemogenesis must be detected during prognosis, for they represent separate phenotypes and correlate with different prognosis³⁸. For example, the germline mutations of MECOM (MDS1 and EVI1 complex locus protein, also known as positive regulatory domain zinc finger protein 3 (PRDM3)) lead to poor prognosis of both AML and CML adult patients³⁹.

The fact that many common mutations between AML and CML affect the cellular proliferation pathways and gene expression regulation aids the tumour cells to modulate many bioprocesses to its advantage. This creates a completely different metabolism from the progenitor cells, with high energetic demands and constant proliferation causing upregulation of Glycolysis and the Pentose Phosphate Pathway (PPP), for rapid energy gains and generation of ribonucleic building-blocks, respectively (see chapter 1.3.1). An extensive repertory of metabolic studies on distinct cancer types, including leukaemia, have identified plenty of key enzymes that cancer cells depend on for survival and to gain resistance to treatments⁴⁰⁻⁴⁶. A common example of mutations altering the metabolism in Leukaemia is IDH1 and IDH2, which mutations lead to a gain-of-function, that generates the metabolite 2-Hydroxyglutarate (2-HG), an

antagonist of α -Ketoglutarate (α KG) that competes for the same binding site on α KG-dependent enzymes but blocks their activity (such as TET2)⁴⁷⁻⁴⁹. Another case is the overexpression of transketolase-like 1 (TKTL1), an enzyme involved in cellular energetics and DNA-building blocks generation, that correlates with poor prognosis of CML patients⁵⁰. Preliminary analysis of TKTL1 capacity as a biomarker for short remission (under 6 months) in AML patients have shown promise⁵¹ but further studies of that possibility have been almost non-existent.

The tumorigenesis process relies on the synergy of the total of these mutations and its effects on the cellular homeostasis, generating a very distinct metabolic profile from its surrounding niche. Such a change can be exploited, has many research studies have already proven⁴¹. Studying these changes have helped elucidate vulnerabilities of cancer cells that can aid on the efficiency of standard treatments⁵². Further analysis of how specific metabolic genes are being altered in leukemogenesis must be a priority. As such, the focus of our work will be to elucidate on how the overexpression of TKTL1 and the loss of TET2 influence the metabolism of leukaemia cells.

1.1.5.1 Transketolase-like 1

TKTL1 is an isoform of Transketolase (TKT), a very important enzyme in cellular energetics. The TKT family is composed of TKT, TKTL1 and TKTL2⁵³. They belong to the non-oxidative branch of the PPP, that provides energy, generates ribonucleic acids for *de novo* synthesis and aids in the Nicotinamide adenine dinucleotide phosphate (NADP⁺) pool for use in redox reactions (see chapter 1.3.2). Of all the transketolases, TKTL1 is the one more commonly found overexpressed in cancer⁵⁴.

TKTL1 has a 77% homology with TKT⁵⁵ and can perform the same enzymatic activity⁵³. TKT acts through dimerization, with homodimers of

TKT or heterodimers of TKT-TKTL1⁵⁶. TKT expression is regulated positively by Nuclear factor Erythroid 2-like 2 (NRF2) and negatively by BNB and CNC Homolog 1 (BACH1)⁵⁷. The TKTL1 isoform is expressed through promoter hypomethylation^{58,59} and can be induced by hypoxia-inducible factor 1 alpha (HIF1 α)^{60,61}. Curiously, it seems that TKTL1 exists in a forward-loop with HIF1 α , the main transcription factor responsible for cellular adaptation to hypoxia (see chapter 1.4). A non-hypoxic induction of HIF1 α occurs when glucose uptake supplants the cell capacity of assimilation. HIF1 α then promotes the expression of TKT (and its isoforms) and the downregulation of glucose-6-phosphate dehydrogenase (G6PD), shifting the ribose synthesis from the oxidative to the non-oxidative branch of the PPP^{62,63}. In turn, the increased transketolases activity will feed substrates to glycolysis and lead to a positive reinforcement of HIF1 α expression and stabilization, that in turn will upregulate many glycolytic enzymes⁶⁴.

As mentioned before, TKTL1 has been correlated with poor prognosis on CML patients⁵⁰. It has also been found across a wide range of different types of solid tumours, such as melanoma⁵⁹, nasopharyngeal carcinoma⁶⁵, uterine cervix cancer^{54,66}, glioblastoma^{67,68}, prostate cancer⁶⁹, lung cancer⁷⁰ and breast cancer⁷¹, to name a few. Across all these distinct diseases, the overexpression of TKTL1 was reported to influence cell proliferation, tumour progression, invasive capacity, treatment response and survival estimation. Of note, the ascending levels of TKTL1 correlate with tumour stage, with highest levels on cancers on the metastatic stage⁶⁹. Due to this importance, a new diagnosis test was created, the Epitope-detection in monocytes (EDIM). This blood test screens for macrophages (activated monocytes) that had phagocytosed tumour cells, carrying tumoral proteins, through specific antibodies for the biomarkers Apo10 (Apo10-epitope of Desoxyribonuclease X) and TKTL1^{72,73}.

Previous studies have also proven that the knockdown of TKTL1 decreased the proliferation rate, lowered the NADP⁺/NADPH ratio and increased Reactive Oxygen Species (ROS) levels, leading to increased apoptosis and re-sensitizing resistant cell lines back to the treatment⁷⁴.

TKTL1 has been found to work as a moonlight enzyme, which are multifunctional proteins with more than one physiologically relevant biochemical function^{75,76}. Apart from the biochemical implications due to its enzymatic activity in the non-oxidative PPP, it has been reported that TKTL1 aids on checkpoints of the cell cycle, in order to guarantee the availability of Ribose-5-Phosphate (R-5-P) to build new ribonucleic acids during S phase^{56,59}. It can also interact with the PI3K/AKT pathway, a very important signalling pathway of proliferation and cell survival, with TKTL1 overexpression correlating with higher activation of AKT^{67,71,77}. For the specific case of AML, TKTL1 was tested as a biomarker for complete remission (CR) and was found to be higher in patients that had a CR with less than 6 months⁵¹. Strangely, no follow-up studies of this potential in AML has been reported.

The fact that HIF1 α promotes the actions of the TKT family of enzymes is of most relevance for cancers of the hematopoietic niche. AML and CML both exist in hypoxia states, firstly because that is the natural condition of the bone marrow¹³ and secondly because tumours become hypoxic at the centre of their mass over time due to uncontrolled expansion^{78,79}. Since TKTL1 has already been reported as a good biomarker to predict behaviour and response to treatments in CML patients, a similar role in AML cases must be investigated more deeply.

The collective research done about TKTL1 have set it up in a position not only as prognosis biomarker but as a cross-section between metabolism, proliferation signalling and hypoxia response. These characteristics makes it a prime target for metabolic profiling. The fact that no body of work has

currently looked more in depth on the role of TKTL1 in acute myeloid leukaemia is a lack of knowledge that must be culminated most urgently.

1.1.5.2 Ten-to-Eleven Dioxygenase 2

The TET Dioxygenase family of enzymes have an important role on epigenetic maintenance, more specifically the regulation of the methylation of genes. It is composed of TET1, TET2 and TET3 enzymes⁸⁰, that participate on various steps of demethylating cytosine bases of the DNA. The TET enzymes are α -KG-dependent, needing oxygen and Fe-II to perform its function, releasing succinate and CO₂ as a result. The TET enzymes are recruited after the action of DNMT1 (responsible for methylation maintenance) or DNMT3 (responsible for *de novo* methylation), that methylate cytosines. The TET enzymes then make a sequential oxidation of 5-methylcytosine (5mC) into 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC)⁸⁰ (Figure 1.1.3). The 5fC can also be transformed to 5-hydroxymethyluracil (5hmU) by Activation-induced Deaminase (AID) and by Apolipoprotein B mRNA editing enzyme (APOBEC). All these molecules (5fC, 5caC) can then be transformed by Thymidine DNA Glycosylase (TDG) or go through Base Excision Repair (BER) mechanisms (5hmC, 5fC, 5caC and 5hmU) back into original cytosine. It is through this process that TET enzymes regulate methylation-based silencing and prevent aberrant hypermethylation⁸¹, common in cancer cells. TET2 can interact with a plethora of epigenetic agents. It can recruit O-linked β -D-N-acetylglucosamine transferase (OGT) to the chromatin, regulating gene transcription⁸². Many transcription factors influence its activity, such as Wilms' tumour suppressor 1 (WT1) recruiting TET2 to target genes⁸³ or Dvl-binding protein IDAX (Inhibition of Dvl and Axin complex) promoting its degradation by caspase activation⁸². It can also be

negatively regulated by micro-RNAs at the pre-mRNA level⁸⁴. For example, miR22 can downregulate all TET genes and was found in high levels in AML and MDS patient samples⁸⁵.

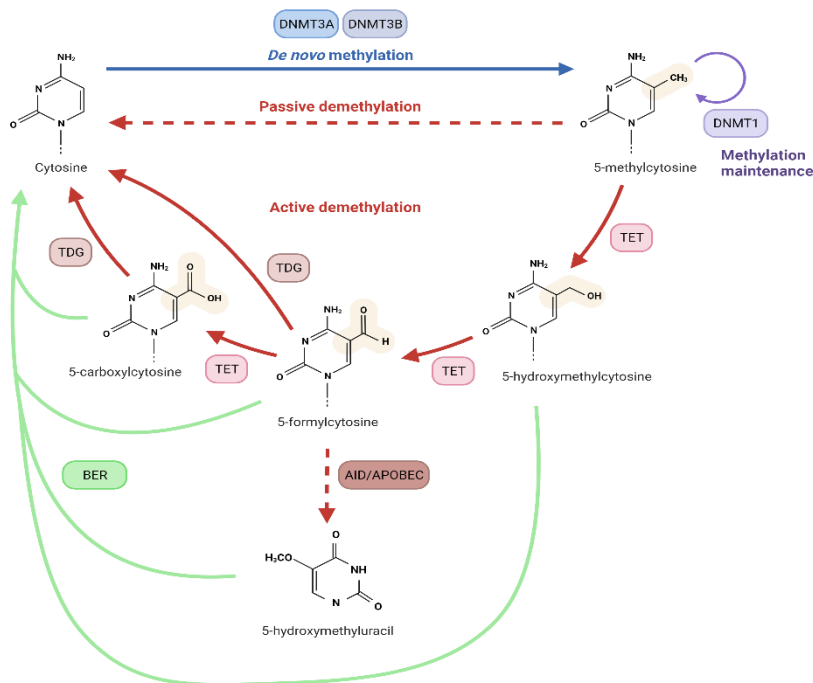


Figure 1.1.3 - Schematic of DNA methylation and demethylation processes. Cytosine base of DNA is methylated by DNMT1, DNMT3A and DNMT3B, resulting in 5-methylcytosine (5mC). Subsequent oxidation by TET enzymes (TET1, TET2 and TET3) generates 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). Additionally, AID/APOBEC converts 5fC into 5-hydroxymethyluracil (5hmU). TDG then transforms 5fC and 5caC back to cytosine. 5hmC, 5fC, 5caC and 5hmU suffer Base Excision Repair (BER) to revert back to cytosine. Adapted from Kunimoto et al., 2014. Created in Biorender.com.

TET enzymes activity can be recovered by Vitamin C (ascorbic acid)^{86,87} and by overexpression of NAD-dependent protein deacetylase Sirtuin-1 (SIRT1)^{88,89}. Additionally, TET proteins have been found to regulate telomerase shortening. The specific mechanisms of this role are still unclear, but absence of TET enzymes was followed by increased telomere-sister chromatid exchanges⁹⁰.

Studies have shown that TET2 mutations are prevalent across multiple types of leukaemia (as mentioned in chapter 1.1.5), being one of the earlier mutation hits on the leukemogenesis process. The mutations of TET2 are heterozygous, hemizygous (due to chromosomal defects) or homozygous⁹¹, although the latest is very rare. The loss of 1 allele results in partial or even total inactivation of TET, with the wildtype (WT) copy not being enough to perform its duties⁸². Of note, no distinct phenotype was identified from having a heterozygous or homozygous phenotype, with no difference of Overall Survival (OS) across different cancer patients⁸². The type of mutations that happen are mostly missense mutations, with frameshift and nonsense mutations also occurring⁹¹.

Although the other members of the TET family can perform most of the same functions, it is the loss of TET2 that generally leads to leukemogenesis. While mutations on TET1 and TET3 also occur in tumours, it is TET2 that is the preferred TET enzyme expressed in haematopoietic cells and by default the more mutated in haematological diseases⁸². The loss of TET2 cannot by itself make the full cellular transformation into cancer but it was attested to grant a growth advantage⁸². And since TET2 has such a vital function regulating epigenetic events, from methylation status to scaffolding for DNA “machinery”, its loss eventually leads to genomic instability. This creates “fertile ground” for the appearance of other mutations, that will build synergy to start tumorigenesis (Table 1.1-B). For example, TET2 mutations with additional FLT3 mutations induce AML and with ASXL1 mutations generate MPN^{80,82}.

TET2 mutation frequencies across Haematological Diseases	Frequency (%)
Acute Myeloid Leukaemia (adult)	8-32
Chronic Myeloid Leukaemia	4
B-cell Lymphoma	2-12
T-cell Lymphoma	20-83
Chronic Myelo-monocytic Leukaemia	20-58
Blastic plasmacytoid dendritic neoplasm	25-54
Systemic mastocytosis	20-29
Myelodysplastic Syndrome	6-26
Polycythemia vera / Primary Myelofibrosis/ Essential Thrombocytosis	2-20

Table 1.1-B- Frequencies of TET2 mutations across Haematological Diseases. Adapted from Scourzic et al., 2015 And Rasmussen et al, 2016.

Besides its function on DNA repair mechanisms, TET2 is also linked to immune cells regulation. It is necessary during maturation of T-cells to differentiate to T-helper (Th) cells and T-regulatory (T-reg) cells, maintaining T-cells homeostasis⁸⁹. TET2 deficiencies have been found to impair plasma cell differentiation, promoting B-cell lymphoma⁸⁹, and to have deficient-macrophages alter the microenvironment in order to reduce tumour burden in melanoma⁹².

On the metabolic front, the fact that TET2 is dependent on α KG, makes any alteration to this metabolite levels affect DNA repair. As mentioned above (see chapter 1.1.5), mutations of IDH result in the generation of 2-HG, that will compete with α KG for TET2. For these reason, IDH mutations are mutually exclusive of TET2 mutations, i.e., they target the same pathway resulting in the same phenotype⁹³. Similarly, mutations on Fumarate Hydratase (FH) and Succinate Dehydrogenase (SDH) can also inhibit TET2 activity, by scarcity of needed metabolites and are also mutually exclusive^{47,94}. Ultimately, cancer cells either have a mutated TET2 or an altered mechanism that will block its activity. Another example is Branched-chain amino acids transaminase 1 (BCAT1), with its overexpression leading to low α KG levels, DNA hypermethylation and post-translational stabilization of HIF1 α , the same phenotype as IDH and TET2 mutations⁹⁵.

The last factor to consider is the link between TET2 and hypoxia. Dioxygenases mandatorily need oxygen for their activity and hypoxic conditions put a strain on their performance. On this category of proteins is not only TET2 but also Prolyl Hydroxylase Domain (PHD) enzymes, which hydroxylate HIF1 α , marking it for degradation by ubiquitination^{96,97}. Studies have found a link between overexpression of HIF1 α and TET2 regulation, resulting in low 5hmC content, leading to gene expression that aids on aggressive phenotypes in melanoma⁸⁶. This regulation can be rescued by silencing of HIF1 α , at both the gene and protein level. If the same can be proven in leukaemia, it could be a major step in developing therapies to impede resistance or rescue pre-leukemic HSCs. At the same time, other research works have found that the reduction in activity of TET enzymes in hypoxia are only resulting from oxygen shortage, showing that 48% of hypermethylation events are hypoxia-related across multiple types of cancer and independent of mutations⁹⁸. A deeper understanding of the role of TET2 activity in hypoxia must be pursued.

Due to its many functions and the resulting array of diseases that comes from its loss, TET2 is a very interesting protein. Add to the fact that its activity relies on the availability of metabolites and oxygen, it can be used to generate diagnose-indicative metabolic profiles. In fact, it is already exploited through immunohistochemistry, by staining of 5mC and 5hmC, whose levels will increase and decrease respectively without TET2⁸². Further elucidation of the metabolic alterations due to TET2 loss on the context of leukaemia, specifically of CML, will be one of the directions of this research.

1.2 Epigenetic Instability as a Hallmark of Leukaemia

Genome Instability is one of the newer Hallmarks of Cancer⁴. It mainly consists of mutant genotypes that confer advantages to a subclone cell to

eventually dominate the tissue environment. Therefore, cancer can be described as succession of clonal expansions of a mutant genotype. But some clonal expansions can be initiated by non-mutational events such as histone modifications, reshape chromatin for gene expression. All these phenomena that affect DNA reading without altering its sequence are grouped under the term Epigenetics⁹⁹. Coined by Waddington in 1942, these events were found to create a heritable phenotype, that passes on during cell replication. Those changes can be histone modifications, DNA methylation, chromatin remodelling and changes in non-coding RNA¹⁰⁰⁻¹⁰⁴. These will govern many cellular processes, like embryonic stem cells (ESC) development^{100,102,105} and immune cells differentiation^{102,106}. The epigenetic landscape created by these modifications then works in tandem with transcription factors, to determine cell type identity^{106,107}. Different stimuli can alter this “identity”, with endogenous (genetic inheritance, cell-cell interactions, etc.) or exogenous origins (environmental factors, diet, etc.)¹⁰⁷. Proteins participating in epigenetic control of gene expression have been separated into three categories: epigenetic readers, epigenetic writers and epigenetic erasers¹⁰⁸. Simply put, the writers are the enzymes that perform the modification, that the reader recognizes and is later removed by the eraser. There is overlap of functions since many writers and erasers also possess reader domains¹⁰⁶.

Tumour cells can arise from altered epigenetic events, that can express oncogenes (genes that promote cancer) or repress tumour suppressor genes (TSG)^{101,108}. Tumorigenesis involves epigenetic aberrations, such as hypermethylation of promotor regions while having global DNA hypomethylation status and alterations to histone modifications that disrupts chromatin, affecting accessibility for transcription factors^{103,107}.

In the case of Leukemogenesis, the epigenetic landscape is highly susceptible to aberrations, because it is essential in HSCs to direct differentiation and the most common mutations of leukaemias are

epigenetic participants (DNMT3, TET2, ASXL1) (see Chapter 1.1.5)^{109,110}. For tumoral contribution, it is more likely that epigenetic dysregulation is the primary mechanism instead of just an accumulation of somatic mutations of genes of epigenetic modulation members¹⁰². Cancer Epigenetics is a fast-evolving area, with many directions of growth. Elucidation of the weight of epigenetic aberrations on the formation of tumours, across many cancer types, is a massive undertaking of many research teams across the world. Those endeavours have already resulted in therapies using chromatin-targeting drugs and hypomethylating agents^{100,108,109,111}, with high promising potential to counter-act resistance to standard therapies.

1.2.1 Alterations of DNA methylation status in Leukaemia

DNA methylation is a common epigenetic mechanism of control of gene expression. It occurs by adding methyl groups to 5'-position of cytosine, on CpG islands (Cytosine-phosphate-Guanine), considered a silencing mark¹⁰². Across the genome, CpG islands mostly occur on promoter regions of genes, with its methylation correlating with silencing transcription. This methylation is performed by DNMTs, by transferring the methyl group from S-Adenosyl-L-methionine (SAM) to create 5mC¹⁰². Different DNMTs are expressed at different times for distinct functions. DNMT1 is responsible for maintenance of methylation pattern during chromosome replication, DNMT3A and DNMT3B are the *de novo* methyltransferases that make the initial methylation (highly active during embryogenesis)^{109,112}. The demethylation process is under the care of TET enzymes, that perform a series of oxidations that eventually revert to unmodified cytosines by BER (see chapter 1.1.5.2).

As mentioned above, cancer shows aberrant methylation profile of majorly hypomethylated but pockets of hypermethylation (Figure 1.2.1). Global

hypomethylation promotes chromosomal instability (deletions, rearrangements), reactivation of transposable elements and loss of imprinting. While the hypermethylation that occurs on CpG islands corresponds to promoters of TSGs^{106,107}. This leads to silencing of many important tumour suppressors such as retinoblastoma tumour-suppressor gene (*Rb*), von Hippel-Lindau tumour-suppressor gene (*VHL*), *p16^{INK4a}*, breast-cancer susceptibility gene 1 (*BRCA1*) and *TP53*^{106,112}.

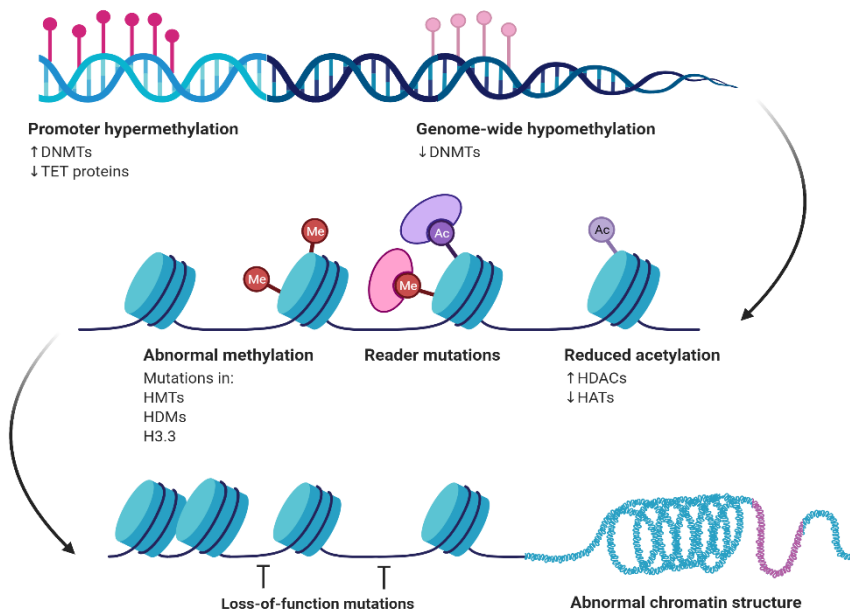


Figure 1.2.1- DNA methylation alterations and the impact on chromatin structure. Promoter hypermethylation occurs by increased DNMTs action and diminishing TET-mediated demethylation while a genome-wide hypomethylation happens. This leads to alterations to epigenetic writers (DNMTs, HATs, HMTs), readers and erasers (TETs, HDACs, HDMs), that result in mutations of nucleosome packaging and an abnormal chromatin structure. Created in Biorender.com.

In Leukaemia, mutations in participants of both methylation and demethylation have been reported (as mentioned in 2.1.5). The transcription factor CEBPA (see Table 1.1-A) is commonly mutated in AML but it can also have its expression altered by hypermethylation of its promoter¹⁰⁹.

Mutations in DNMT3 are recurrent in leukaemias and leads to a global hypomethylation profile^{111,112}. Contrary effect is the mutations in TET proteins, that result in DNA hypermethylation. And both lead to leukemogenesis, for both are equally essential for HSCs regulation and differentiation. Further elucidation of how the different genetic abnormalities of leukaemia affect the epigenetic events are underway^{101,109}. In regards for therapeutic approaches, hypomethylating agents have been developed, such as Azacitidine and Decitabine. These agents incorporate into DNA of replicant cells and irreversibly bind to DNMTs, depleting them and leading to a hypomethylated status^{100,109}. They are currently approved for treatment of AML and MDS and expansion of applications are being pursued^{101,106}.

1.2.2 Histone Modifications: gatekeeping access to DNA

Another type of epigenetic event is histone modifications. Histones are members of nucleosome, the core unit of chromatin, that consists of 147 base pairs (bp) of DNA wrapped around a histone octamer core^{102,103}. The histones are numbered into H1 (the linker), H2A/H2B (dimers) and H3/H4 (central tetramer)^{102,103}. These histones have amino termini that act as histone tails, that can suffer post-translational modifications (PTMs). These modifications can be acetylation, methylation, phosphorylation, ubiquitination, to name the most relevant and studied¹⁰³. These modifications control genomic DNA condensation and alter access for binding to DNA. Another function of histone modification and their restructuring of chromatin is regulation of alternative splicing^{106,113}. Histone marks are enriched in the borders of exons and their flanking intronic regions¹⁰⁶.

Histone acetylation relates to active transcription and deacetylated status stops it (Figure 1.2.2). This process is mediated by Histone

Acetyltransferases (HATs) and Histone Deacetylases (HDACs), many of which are mutated in leukaemia^{103,106,113}. Histone methylation can both activate or inactivate transcription, depending on the situation. Another layer to be considered is that histones can be mono-, bi- or trimethylated, with each level of methylation having different effects. These methylations are done by Histone Methyltransferases (HMTs) and removed by Histone Demethylases (HDMs)¹⁰³.

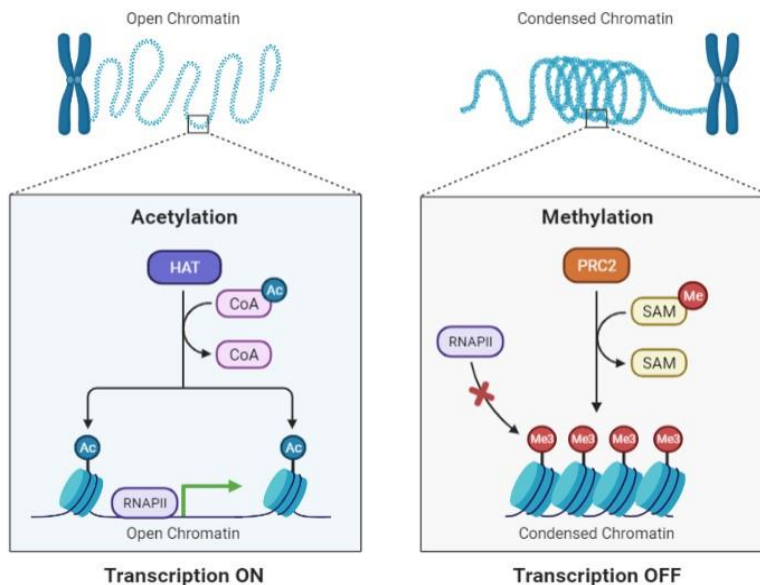


Figure 1.2.2- Histone modifications modulate chromatin condensation for transcription. Acetylation performed by HATs, by transferring the acetyl group from Coenzyme A (CoA) to the histone, opens the chromatin and allows the binding of RNA polymerase II (RNAPII) to initiate transcription. Histone methylation performed by Polycomb Repressive Complex 2 (PRC2), using methyl groups from SAM donors, condenses the chromatin and blocking RNAPII binding, impeding the initiation of transcription. Created in Biorender.com.

Most histone modifications reported and studied to date are to H3. Targeting of H3K4 and H3K36 leads to activation of transcription while H3K9/27 and H3K27me2/3 modifications induce gene silencing^{101,114}. Of great relevance is the fact that histone modifications to H3K9 are involved

in chromosome X inactivation, gene repression at promoter regions and formation of heterochromatin (condensed DNA usually found in structures such as centromeres and telomeres)^{81,82,101,102,113}.

Dysregulation of histone modifications are common in cancer. Loss of histone acetylation and increased methylation of histone H3-K9 were found in hypermethylated promoters of TSG^{103,113}. Another feature in cancer is that histone dysregulation happens downstream or upstream of DNA methylation, so both events happen in tandem during tumorigenesis^{103,113}.

In leukaemias, there are many mutations and aberration that affect histone modifications, such as PML-RARA, that induce abnormal recruitment of HDACs^{102,113}. Another common mutation in leukaemia occurs on HMTs, such as Enhancer of Zeste Homologue 2 (EZH2), a component of the Polycomb Repressive Complex 2 (PRC2), and Mixed-lineage Leukaemia (MLL) proteins^{108,113}. EZH2 loses its HMT activity after chromosomal translocation, leading to PRC2 losing its function of transcriptional silencing maintenance, while KMT2A (an MLL protein) activates gene transcription by modification of H3K4^{102,106,113}. EZH2 can also be reduced by mutations to the splicing factors U2AF1 and SRSF2, that dysregulates processing of pre-mRNA^{102,106,108,113}.

The Food and Drug Administration (FDA) regulatory body have approved several inhibitors of HDACs (HDACi), such as Belinostat and Romidepsin, for treatment of multiple myeloma and T-cell lymphoma^{101,106,108,113}. Although they show pleiotropic effects (actions beyond the specifically developed function) that blur the true mechanism of action of these drugs. Alternatives are being developed with EZH2 inhibitors and histone lysine demethylases (KDM) inhibitors, with clinical trials under way^{106,113}.

1.2.3 Micro-RNAs, the genome “hidden agents”

Of the whole human genome, only 1.2% encodes proteins but most of genome is still transcribed¹¹⁵. The elucidation of this discrepancy lead to

the discovery of short and long non-coding RNA (ncRNA) that brought to light new types of RNA to already existing ones (transporter RNA (tRNA), ribosomal RNA (rRNA), small nuclear RNA (snRNA))^{104,115}. One of these new RNAs is micro RNA (miRNA), a ncRNA of 22 nucleotides in length that is equivalent to messenger RNA (mRNA) without translating to proteins¹⁰⁴ (Figure 1.2.3 *Figure 1.2.3*). Instead these miRNAs will regulate gene expression by mRNA cleavage, translational inactivation or deadenylation^{102,116}. They participate in regulation of many important functions such as cell division, differentiation and even cardiac function regulation (cardiac rhythm, myocyte growth, etc.)^{102,104,117}. Many new miRNAs are discovered each year but there are still a large number of them unbeknownst to us.

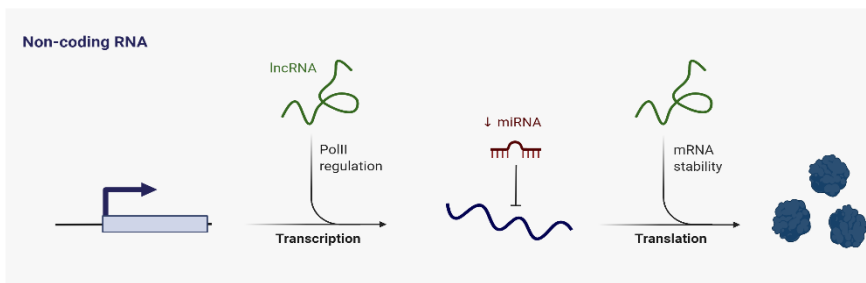


Figure 1.2.3- Epigenetic regulation by Non-coding RNAs. Long non-coding RNAs (lncRNA) can regulate with Polymerase II (PolII), modulating the transcription process. Micro-RNAs (miRNA) can bind to the resulting transcript and impede its translation by directing it for degradation processes. Created in Biorender.com.

The activity of miRNAs is by itself an epigenetic event, but they also interact with the other epigenetic events mentioned above. They can induce DNA methylation or demethylation, the many types of histone modifications or impede the events by blocking access by binding to the target area of DNA^{102,107}.

In cancer, many miRNAs are being discovered across different types of tumours and correlated with aggressive phenotypes and patient outcome¹⁰¹. It has been reported that Leukemogenesis can be triggered by aberrant

miRNAs impeding haematopoiesis. Of relevance in AML, Let-7 is a miRNA that can affect many oncogenes (KRAS, MYC, etc)^{102,118}, miR-29 can induce apoptosis¹¹⁹ and miR-24 is linked to poor survival and risk of relapse¹²⁰.

The research on miRNAs is an ongoing process, with much therapeutic applicability potential, that will revolutionize how we diagnose and treat many types of cancer.

1.2.4 The “symbiosis” of epigenetic events with Transcription Factors in Leukaemia

Epigenetic events are intertwined with the actions of transcription factors. The epigenetic events mentioned above are necessary for granting access to transcription factors for DNA binding. On the other hand, transcription factors can also initiate epigenetic events. For example, the cocktail of 4 transcription factors used to induced cells back into a pluripotency state (Oct4, Sox2, Klf4 and Nanog) happens due to their influence on changing the epigenetic landscape¹⁰⁵. Transcription factors can also make regulatory loops, that impose epigenetic regulation on that zone and most often are a positive-feedback loop^{103,107}. For example, the CTCF-binding factor (CTCF) is responsible for 3-dimensional structures in chromatin, creating the mentioned regulatory loops. These loops can both bring promoters and enhancers closer or isolate regions to avoid transcription (insulator function)¹²¹. Indirectly, transcription factors can influence epigenetic events through control of ncRNAs^{102,104}.

Across cancer types, transcription factors have been investigated for their role in tumorigenesis. Cancer cells utilize transcription factors to manipulate a multitude of pathways, from cell proliferation induction to avoiding apoptosis^{122–124}. But counteraction of this manipulation is difficult, since transcription factors are regulated by many participants and are

necessary very carefully “tailored” treatment regimens with other drugs to avoid resistance^{122,123}.

In Leukaemia, many transcription factors are implicated with the many genomic abnormalities. The chromosomal translocations that are defining of so many types of leukaemias involve transcription factors, such as PML-RARA, RUNX1, CEBPA, CBFβ and GATA2 (see Table 1.1-A). These lead to abnormal activity that can silence TSGs promoters or activate oncogenes^{106,121}. Another layer that affects transcription factors on leukaemias is the common mutations, such as ASXL1 (see chapter 1.1.5). ASXL1 is a transcriptional regulator, responsible for the recruitment of EZH2 and PRC2 stability. Although the exact effect of mutations to ASXL1 is unclear (loss or gain of function), clear correlations were established with higher risk for patients of AML and MDS, and association with RUNX1 and NPM1 mutations^{91,113}. Another example is mutations to EZH2, which involve a gain-of-function that leads to abnormal histone modification (H3K27me3) and will block B-cell development^{102,113}. Of note is also alterations to CTCF function, that can occur by hypermethylation of its binding sites, blocking its activity (correlated with loss of TET2)¹²¹. CTCF also interacts with other key transcription factors that are commonly mutated in leukaemia, such as NPM1, CEBPA and RUNX1^{121,123}.

Further research on the interplay between transcription factors and epigenetic events is needed, to discern their interactions and how to use it for therapeutic advantage. Many studies are underway to characterize the effect of epigenetic drugs on the epigenetic activity of transcription factors^{101,113,122}.

1.2.5 The Epigenetic machinery is dependent on metabolism

The epigenetic machinery is a vast network of many different components that must work in harmony to regulate gene expression of distinct cellular processes. Besides their interaction with transcription factors, there is

another cellular process that has input into the activity of epigenetic modulators: the generation of metabolites^{101,106}. Many metabolites are used as co-factors of the distinct epigenetic writers and erasers. As such, the availability of such metabolites (i.e. diet stimuli) will influence the epigenetic landscape. As mentioned above, SAM is used as a methyl donor during the DNA methylation performed by DNMTs and acetyl Coenzyme A (acetyl-CoA) is used during histone acetylation^{107,125}. Another common crosstalk of epigenome and metabolism in leukaemia is the case of mutated IDHs. The production of the oncometabolite 2-HG will interfere with the activity of α KG-dependent enzymes, such as TET enzymes (see chapter 2.1.5.2), KDM enzymes (histone lysine demethylases) and AlkB family members (nucleic acid demethylases)^{113,126}.

It has also been reported, across many types of cancer, that DNA methylation is used to induce the Warburg Effect (see chapter 1.3.1). DNA hypermethylation alters expression at gene (direct start of transcription) and protein levels (by avoidance of degradation mechanisms) of many key players of glycolysis, PPP, gluconeogenesis, redox balance and oxygen sensing pathway¹²⁷.

Further investigations of the link between DNA methylation status and Warburg effect should be done on a leukemic perspective, especially due to alterations reported to the oxygen sensing pathway (HIF accumulation)¹²⁷.

1.3 Metabolic Dysregulation as a Hallmark of Leukaemia

Since many of the molecular alterations involved in the origin of leukaemia manifest in modified phenotypes, a metabolic dysregulation is a natural occurring event arising from those changes.

In general terms, metabolism is all the reactions that occur in an organism or cell¹²⁸. These reactions can be catabolic or anabolic. The first is a destructive process that breaks molecules for energy or “building-blocks”

while the second is the reverse, involving the synthesis of compounds¹²⁸. The metabolic pathways utilised both types of reactions to generate the many metabolites needed for all cellular functions, such as energy production, proliferation, biosynthesis and homeostasis¹²⁸.

Over the many years of studying cancer, a constant factor that researchers highlight is that the tumorigenic process results in a direct or indirect dysregulation of metabolism, leading to a proliferating advantage. Within the Hallmarks of Cancer, this proliferative advantage is characterized according to the way the advantage is conferred, from deregulating cellular energetics, avoiding apoptosis or growth suppressors, sustaining proliferative signalling or induction of angiogenesis⁴.

For the case of leukaemia, many of its originating molecular events are at the epigenetic level, as covered previously (see chapter 1.1.5 and 1.2). But there are also other types of mutational events during leukemogenesis affecting metabolism, enhancing proliferation or apoptosis evasion. In fact, one of the characteristics used to identify and classify the many types of leukaemia is the abnormal proliferation of a particular type of haematopoietic cell. Another layer of adaptation that leukaemia cells must endure is the hypoxic microenvironment of the bone marrow and haematopoietic niche. Many studies have showed how cancer cells change metabolic processes to support its proliferation and survival such as glycolysis, pentose phosphate pathway, TCA cycle, lipid synthesis and mitochondrial activity^{129–132}. The metabolic reprogramming that cancer cells induce is a dynamic interplay between oncogene overexpression and inactivation of TSGs¹³³. These genes cover alterations to transcription factors, signalling pathways members, apoptosis inhibitors and inducers, cell cycle regulators, DNA repair and many other processes, resulting in the disrupted metabolism observed.

1.3.1 Glycolysis and the Warburg Effect

Glycolysis is the principal step of many energy production processes. Glucose intake to cells is facilitated by glucose transporters (GLUTs), of which there are 14 known isoforms. These isoforms expression vary according to tissue and intracellular compartment, with the GLUT 1 to 4 being the more studied given their overexpression in cancer^{134–137}.

Once inside, glucose is broken down by nine reactions, catalysed by specific enzymes at each step (*Figure 1.3.1*). The flux of this process is regulated by rate-limiting enzymes: hexokinase (HK), phosphofructokinase (PFK) and pyruvate kinase (PK)^{138,139}. The resulting pyruvate at the end of the process is normally transported to mitochondria, where it will produce acetyl-CoA, CO₂ and NADH through the oxidative decarboxylation by the Pyruvate Dehydrogenase Complex (PDC)¹³⁹. The resulting acetyl-CoA is then integrated in the Tricarboxylic Acid Cycle (TCA), also known as Krebs Cycle (see chapter 1.3.3), followed by the oxidative phosphorylation (OXPHOS) that will lead to production of Adenosine Triphosphate (ATP)¹⁴⁰. But in the unnormal ways of cancer, most of the pyruvate produced is converted to lactate by Lactate Dehydrogenase (LDH), indifferent if oxygen is present or not¹⁴¹. This aerobic glycolysis was denominated as Warburg Effect, in honour of its discovery by Otto Warburg¹⁴². Although it produces only 2 ATP molecules, this process is a common metabolic reprogramming of cancer cells for a faster energy production and avoiding production of ROS by the mitochondrial processes and subsequent apoptosis activation^{143,144}.

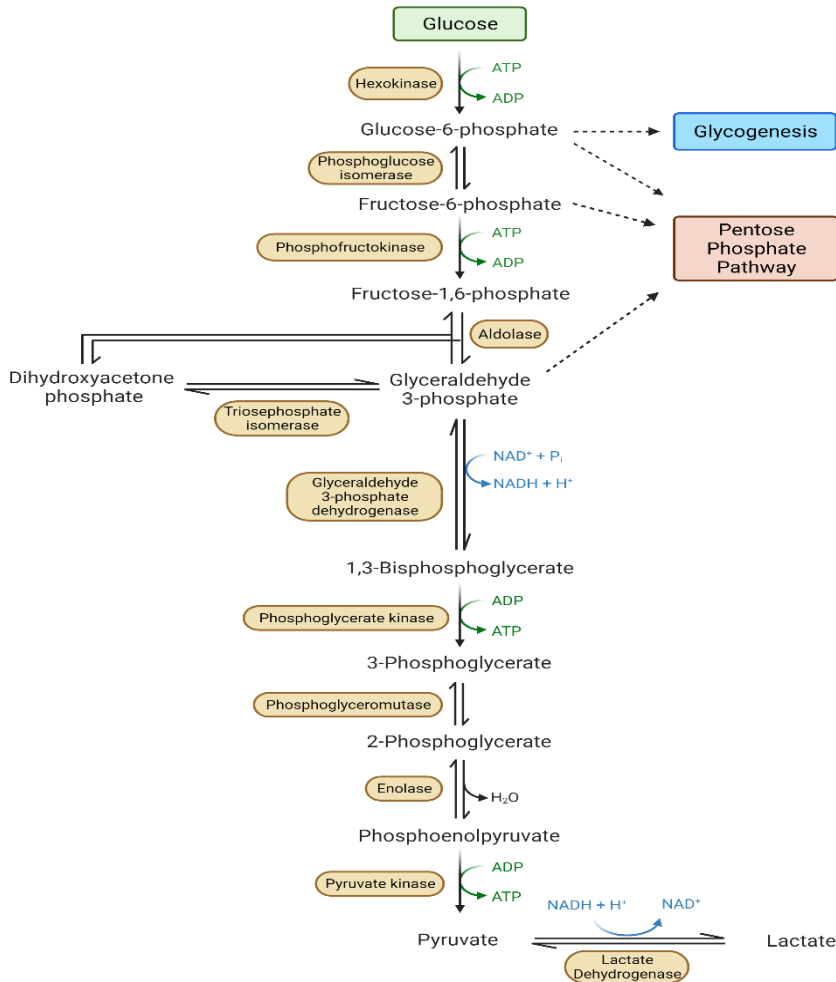


Figure 1.3.1- Schematic of the reactions of glycolysis from glucose to pyruvate and lactate. Glucose is catalyzed into Glucose-6-Phosphate (G6P) by Hexokinase (HK). G6P can be direct into other processes such as glycogenesis and Pentose Phosphate Pathway (PPP) or catalyzed by Phosphoglucose Isomerase (GPI) to form Fructose-6-Phosphate (F6P). The resulting F6P can either be directed to the PPP or catalyzed to Fructose-1,6-phosphate by Phosphofruktokinase (PFK). Subsequent reaction catalyzed by Aldolase (ALDO) generates Dihydroxyacetone Phosphate (DHAP) and Glyceraldehyde-3-Phosphate (G3P). DHAP can be converted to G3P by Triosephosphate Isomerase (TPI). G3P can also be directed into PPP or catalyzed into 1,3-Bisphosphoglycerate by Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH). Following reaction by phosphoglycerate kinase (PGK) produces 3-phosphoglycerate (3PG) and ATP. 3PG is broken down to 2-Phosphoglycerate (2PG) by Phosphoglyceromutase (PGM). Enolase then converts 2PG into Phosphoenolpyruvate (PEP), releasing H₂O. Phosphoenolpyruvate is catalyzed by Pyruvate Kinase (PK) into pyruvate, releasing ATP. Pyruvate can be transferred to mitochondria or can be converted into lactate by Lactate Dehydrogenase (LDH). Created with BioRender.com.

Many types of cancer show overexpression of many participants of glycolysis, mainly GLUTs, HK, PFK, PK and LDH¹³⁹. Another important factor is the TP53-induced glycolysis and apoptosis regulator (TIGAR), that inhibits glycolysis and redirects its flux into the PPP, for oxidative resistance and to avoid apoptosis¹⁴⁵. This regulator is usually found overexpressed in many types of cancer such as breast cancer, lung cancer, leukaemia and colon cancer¹⁴⁵.

As for the case of leukaemia, many studies have reported distinct ways that glycolysis is upregulated. In ALL, glycolysis genes are upregulated in addition to down-regulation of TCA cycle genes, driving the Warburg Effect¹⁴⁶. Leukaemias with FLT3 mutations (see Table 1.1-A) develop a glycolytic dependency, that if blocked can counteract resistance to tyrosine kinase inhibitors treatments^{147,148}. Another strong evidence of the metabolic rewiring of leukaemias is that blocking the gene expression of PKM2 and LDHA in HSCs delays the myeloid leukaemia development¹⁴⁹.

Lastly, leukaemia cells can also influence the glycolysis inside the haematopoietic niche. For example, AML cells increase the release of Insulin-like Growth Factor Binding Protein 1 (IGFBP1), that desensitizes the surrounding normal cells to insulin, conferring the cancer cells with an advantage in glucose acquisition inside the niche¹⁵⁰. Further changes have been condensed more detailed in the reviews of Wang *et al* and Rashkovan *et al*^{132,149}.

1.3.2 Pentose Phosphate Pathway

The Pentose Phosphate Pathway is a parallel pathway of glucose metabolism. After the first step of glycolysis catalysed by HK, the resulting Glucose-6-Phosphate (G6P) can be diverted to the PPP (Figure 1.3.2). The initial steps of the PPP are defined as the oxidative branch, with irreversible reactions that produce NADPH, CO₂ and protons (H⁺). The first reaction is catalysed by Glucose-6-Phosphate Dehydrogenase (G6PD), that oxidizes

G6P into 6-phosphogluconolactone, followed by hydrolyzation by Phosphogluconolactonase (6-PGL) and lastly by oxidative decarboxylation of 6-phosphogluconate into Ribulose-5-Phosphate (Ru5P) by 6-phosphogluconate Dehydrogenase (6PGD)¹⁵¹⁻¹⁵³.

The breakdown of Ru5P is then taken over by the non-oxidative branch of PPP (Figure 1.3.2). Ribulose-5-Phosphate can be interconverted both into Xylulose-5-Phosphate (X5P) by Ribulose-5-phosphate Epimerase (RPE) and Ribose-5-Phosphate (R5P) by Ribose-5-phosphate Isomerase (RPI)^{151,154}. R5P is utilized in the synthesis of RNA and DNA for cell duplication but both X5P and R5P can be used by Transketolase (TKT), that utilizes thiamine diphosphate (TDP) as a co-factor in order to rearrange 2 carbons from X5P into R5P, generating Sedoheptulose-7-Phosphate (S7P) and G3P. Both these substrates are then used by Transaldolase (TALDO), that transfers 3 carbons from S7P back into G3P, generating Erythrose-4-Phosphate (E4P) and F6P. There is a secondary reaction performed by TKT, that instead of using R5P to receive the 2 carbons from X5P, it transfers them into E4P, resulting in F6P and G3P^{151,152}. As mentioned in chapter 1.1.5.1, there are isoforms of transketolase, TKTL1 and TKTL2, with active catalytic function⁵³. Although it is TKTL1 which is found overexpressed in many types of cancer, reflecting in poor prognosis^{50,54,155}. While the oxidative branch is composed of irreversible reactions that generate NADPH for reductive reactions, the non-oxidative branch is reversible at all its steps, allowing more flexibility in adjusting to the requirements of cell. Not only the non-oxidative branch makes components that intersect with glycolysis (F6P and G3P), it produces R5P that serves as the “scaffold” for ribonucleic acids synthesis or converted into adenosine diphosphate ribose^{151,152,154}.

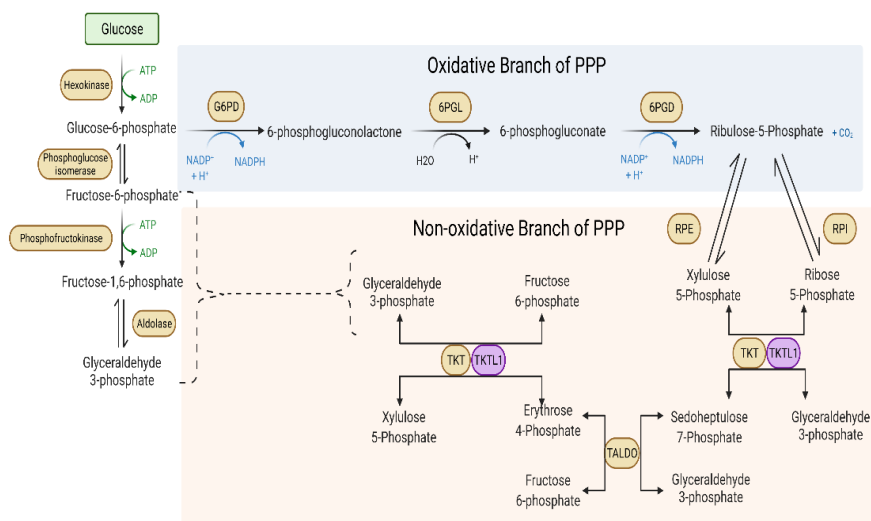


Figure 1.3.2- Interplay of Glycolysis and the branches of the Pentose Phosphate Pathway (PPP). After conversion from glucose by hexokinase (HK), Glucose-6-phosphate (G6P) enters the oxidative branch of the PPP, where it is catalyzed by Glucose-6-phosphate Dehydrogenase (G6PD) into 6-phosphogluconolactone, with side reduction of NADP to produce NADPH. Following reaction catalyzed by Phosphogluconolactonase (6PGL) generates protons (H^+) and 6-phosphogluconate, which in turn is used by 6-phosphogluconate Dehydrogenase (6PGD) to make Ribulose-5-Phosphate (Ru5P), with side byproducts of NADPH and CO_2 . Ru5P is then taken into the Non-oxidative branch of the PPP, by conversion into 2 possible substrates: Xylulose-5-Phosphate (X5P) by Ribulose-5-phosphate Epimerase (RPE) and Ribose-5-Phosphate (R5P) by Ribose-5-phosphate Isomerase (RPI). Both substrates are then used by Transketolase (TKT) and Transketolase-like 1 (TKTL1), resulting in Sedoheptulose-7-Phosphate (S7P) and glyceraldehyde-3-phosphate (G3P). A similar reaction is performed by Transaldolase (TALDO), that uses S7P and G3P to form X5P and Erythrose-4-Phosphate (E4P). These substrates are then used by TKT (also TKTL1), that generates G3P and Fructose-6-Phosphate (F6P). These last 2 substrates are a connecting point with glycolysis, flowing between both pathways depending if the cell requires more reductive power (NADPH) and ribonucleic building blocks (R5P) or energy requirements (glycolysis). Adapted from Xuemei Tong et al, 2020. Created with BioRender.com.

Cancer cells can use the interplay between PPP and glycolysis to drive their proliferation and survival, directing the flux to produce NADPH, ribonucleic acids building blocks and G3P and F6P for energy production through aerobic glycolysis¹⁵⁴. Most alterations in the oxidative branch happens to G6PD and to lesser extent to 6PGD. G6PD can have its expression influenced by transcription factor NRF2 and by mTORC1^{62,156},

by post-translational modifications for activation or dimerization^{157–160}, degradation by ubiquitination¹⁶¹ and by epigenetic regulation through H3K36me² of its promoter¹⁶². 6PGD aberrant expression is also reported in various cancers, accelerating proliferation, inducing invasiveness and resistance to treatment^{163–166}. These alterations mostly lead to increased flux from glycolysis to PPP, increasing the generation of NADPH.

Cancer manipulation of the non-oxidative branch is more varied. RPI and RPE expression can be induced by mutated KRAS, a common mutation in various leukaemias¹⁶⁷. RPI can also be regulated epigenetically, through inhibition by miR-124, resulting in slower cell growth¹⁶⁸. TALDO overexpression correlates with poor outcome and treatment resistance in breast cancer¹⁶⁹. As for TKT, both it and its isoform TKTL1 are found overexpressed across various types of cancer, but not the isoform TKTL2^{170,171}. TKT activity is promoted by HIF1 α ⁶⁰, induced by increasing fructose levels¹⁷² and modulated by signalling pathways^{57,173,174}, resulting in genomic instability¹⁷⁵. The reprogramming of these enzymes by tumour cells result in the uncoupling of the non-oxidative reactions from the oxidative branch of the PPP. This leads to an increased adaptability to obtain R5P to synthesize *de novo* RNA and DNA without producing NADPH if not necessary.

1.3.3 Mitochondrial Activity

Mitochondrial activity is vital for the cell, for it is the focal point of many processes necessary for its survival. It is the “powerhouse” of the cell, where energy production occurs with the fuel obtained from glucose, fatty acids and amino acids^{176–179}. This activity is also the source of much of the ROS produced by the cell, as a by-product of OXPHOS¹⁷⁶. Simultaneously, it is the mitochondria that regulates the intrinsic pathway of apoptosis, by modulating the release of cytochrome *c* through the formation of pores in its membrane^{180,181}.

The TCA Cycle occurs inside the mitochondrial matrix, with acetyl-CoA resulting from the PDC conversion of pyruvate being used to form citrate. This is considered the beginning of the process, that consists of 8 main reactions (Figure 1.3.3). Of these 8 reactions, the reaction of Isocitrate Dehydrogenase (IDH), α -Ketoglutarate Dehydrogenase and Malate Dehydrogenase produce NADH, that can be used for redox balance. The reaction of Succinate Dehydrogenase (SDH) transfers 2 hydrogens to Flavin Adenine Dinucleotide (FAD), producing FADH₂. The energy producing step is catalysed by Succinyl-CoA Synthetase, that converts ADP into ATP. The remaining ATP production originates from OXPHOS, with oxidation of nutrients using the previously produced FADH₂ and NADH as electron donors and O₂ as the final acceptor, generating water and ROS^{176,182}. Of all the TCA cycle reactions, only 3 are irreversible: Citrate Synthase, IDH and α -Ketoglutarate Dehydrogenase. Of note that IDH is considered irreversible due to the negative change in free energy but in certain conditions can be reversible. The remaining enzymes of the cycle can catalyse both directions. While the entirety of the cycle occurs inside the mitochondria, there are both cytosolic and mitochondrial isoforms of some of its enzymes, allowing the production of substrates in both compartments that can be transported in and out according to the needs of the cell¹⁷⁹.

The use of these TCA cycle intermediates from cytosolic production to feed the cycle is called Anaplerosis¹⁷⁶. For example, pyruvate can be converted to oxaloacetate (OAA) by Pyruvate Carboxylase, if low levels of OAA supersede the requirements of acetyl-CoA for the formation of citrate¹⁷⁶. Another case is Malic Enzyme, that transforms malate into pyruvate with NADH generation in the cytosol^{176,177}. In fact, the TCA cycle is an interconnection of energy production and the metabolism of many amino acids, both synthesis and catabolism, aiding to maintain its intermediates levels.

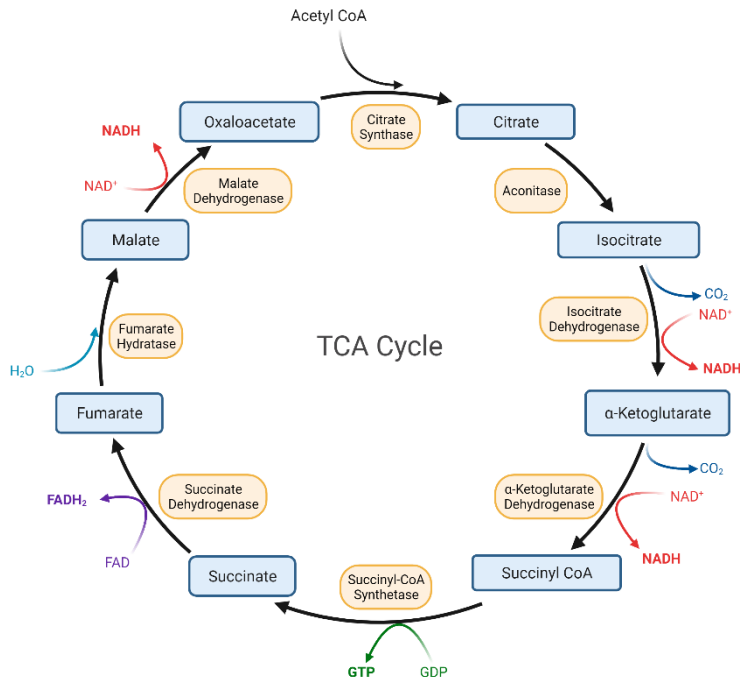


Figure 1.3.3- The reactions and products of the TCA Cycle. Acetyl-CoA is used by Citrate Synthase to generate citrate. Followed by Aconitase producing Isocitrate and subsequent production of α -Ketoglutarate (α KG) by Isocitrate Dehydrogenase (IDH), with side generation of CO_2 and NADH . Same byproducts occur on the following reaction, catalyzed by α -Ketoglutarate Dehydrogenase to produce Succinyl-CoA. The conversion of Succinyl-CoA is the step that produces GTP, by transferring the phosphate group into GDP and generating Succinate, catalyzed by Succinyl-CoA Synthetase. Succinate conversion into Fumarate is catalyzed by Succinate Dehydrogenase (SDH), generating FADH_2 . Fumarate Hydratase (FH) then hydrates fumarate into malate. Malate is then converted into Oxaloacetate by Malate dehydrogenase (MDH), also with generation of NADH . The restarting of the cycle occurs with Citrate Synthase joining Oxaloacetate (OAA) with fresh Acetyl-CoA in the first reaction described. Adapted from Sajnani et al, 2017. Created with BioRender.com.

Initially, it was theorized by Warburg that cancer cells are characterized by dysfunctional mitochondria, given their predilection for aerobic glycolysis¹⁸³. But this hypothesis did not hold true, with evidence showing that cancer cells can suppress respiration and oxidative phosphorylation in a glucose-induced manner^{184,185}. This capability is coined the “Crabtree Effect”, a reversible and short-term event that allows the cancer cells to

shift between Warburg Effect and respiration, granting adaptability to the microenvironment and the nutrient availability. In reality, mitochondrial activity in cancer cells can be impaired or enhanced, depending on the type of cancer and the specific mutations relating to mitochondrial processes such as TCA cycle¹⁷⁷, apoptotic signalling¹⁸⁶ and ROS production¹⁸².

The direct ways that TCA cycle can be altered in cancer is by mutations of its enzymes. SDH, FH and IDH are commonly mutated in renal cancer and Leukaemias^{93,177,187,188}. As mentioned in chapter 1.1.5, IDH mutations are a gain-of-function that results in the new metabolite 2HG, which impedes the flow of the TCA cycle in the direction of succinyl-CoA production and interferes on epigenetic regulation. Mutations on SDH and FH have similar effects, given that succinate and fumarate also have a role on epigenetics¹⁸⁹⁻¹⁹².

Apoptotic impairment in cancer cells is a common alteration of mitochondrial activity, given that it could be triggered by standard characteristics of tumoral environment such as hypoxia and increasing ROS levels¹⁸⁰. This alteration can occur by shifting the balance of pro- and anti-apoptotic proteins, reducing caspase functions and impairing death receptor signalling^{180,181}. Defects in p53, the gatekeeper of the genome and most important of the tumour suppressors, is one of the main reasons how cancer cells avoid apoptosis, being linked to more than half of the human cancers occurrences¹⁸¹. Manipulation of the ratio of pro- and anti-apoptotic signals is another method of survival for leukaemias. For example, CLL uses the Bcl-2 family of proteins for its survival, by increasing the levels of Bcl-2 (anti-apoptotic) and decreasing the levels of Bax (pro-apoptotic)¹⁹³. In the case of leukemogenesis, HSCs that have high levels of Inhibitor of Apoptosis Proteins (IAPs) have higher risk of developing haematological malignancies¹⁹⁴. Downregulation of CD95 (a death receptor also known as Fas) is a common feature of treatment-resistant leukaemias¹⁹⁵.

The ROS production native of the mitochondrial activity can both be a result of dysfunction and a tumorigenic factor in cancer transformation, further discussed in chapter 1.3.6.

Generally, cancer cells have a mitochondrial phenotype of high TCA Cycle activity for the generation of precursors for synthesis of lipids, amino acids and nucleotides needed for proliferation.

1.3.4 Metabolism of Amino Acids

As mentioned previously, the metabolism of amino acids is fundamental to many metabolic processes such as acquiring intermediates of the TCA Cycle, lipids and nucleotides synthesis. The existing 20 amino acids are categorized into two groups: essential amino acids (EAAs) and non-essential amino acids (NEAAs). This classification is given according to the ability of synthetization by our human cells, with essential meaning a diet requirement¹⁹⁶. EAAs consist of Histidine, Lysine, Methionine, Phenylalanine, Threonine, Tryptophan and the Branched-chain amino acids (Isoleucine, Leucine and Valine). NEAAs are all the other remaining amino acids: Alanine, Aspartate, Asparagine, Arginine, Cysteine, Glutamate, Glutamine, Glycine, Proline, Serine and Tyrosine.

The metabolism of amino acids are a web of interconnected pathways of catabolism and synthesis. As an example, glutamine generates glutamate, which in turn can be used to make alanine, aspartate, serine and proline. EAAs metabolism is mainly catabolic reactions, with their breakdown resulting in NEAAs, such as Phenylalanine producing Tyrosine¹⁹⁷.

This interconnectivity is key for cancer metabolism, allowing the tumour cells to use amino acids to produce intermediates for the TCA cycle, the urea cycle, the One-Carbon metabolism, the methionine salvage pathway and for redox homeostasis^{197,198}.

1.3.4.1 Glutamine: the addiction of cancer

Glutamine is a NEAA and one of the most consumed nutrients by cells, second only to glucose¹⁹⁹. Its importance comes from being a double source of atoms, of both carbons and nitrogens for the synthesis of other molecules¹⁹⁹. Glutamine uptake occurs through many distinct transporters of amino acids, with the most known being Alanine-Serine-Cysteine Transporter 2 (ASCT2, coded by *SLC1A5*), Sodium-coupled Neutral Amino Acid Transporter 1 (SNAT1, coded by *SLC38A1*) and SNAT2 (coded by *SLC38A2*). Once inside the cells, glutamine can be utilized for many purposes. As a nitrogen donor, glutamine aids on the synthesis of purines and pyrimidines²⁰⁰, NAD, glucosamine-6-phosphate, polyamines and other NEAAs (alanine²⁰¹, aspartate²⁰², serine²⁰³, proline²⁰⁴ and ornithine²⁰⁵). On the other end, as a carbon donor, it generates glutamate that can generate intermediates for the TCA cycle as well as intermediates for the urea cycle or for synthesis of other amino acids. Glutaminolysis begins with the deamination of glutamine into glutamate by Glutaminase (GLS)^{206,207}, which in turn can be deaminated into α KG by Glutamate Dehydrogenase (GLUD) (see chapter 1.3.4.2) or can participate in transaminase reactions transferring the amine group to other metabolites. IDH, besides the oxidative reaction during TCA cycle, can also perform reductive carboxylation, that converts α KG back to isocitrate, allowing flexibility in Anaplerosis¹⁹⁹. Glutaminolysis is also necessary for redox balance (see chapter 1.3.6), mTOR signalling, autophagy and apoptosis²⁰⁸⁻²¹⁰. Glutamine can also be produced, by the condensation of glutamate and ammonia, a reaction catalysed by Glutamine Synthetase (GS) that requires ATP²¹¹.

All these characteristics of glutamine metabolism makes it a focal point of metabolic reprogramming in cancer cells^{198,199}. In fact, many cancer types have been reported to be glutamine-addicted, with their proliferation and

survival depending on glutamine donor role for biosynthesis^{212–215} and production for ammonia detoxification and pH balancing²¹⁶. For the case of leukaemias, glutamine also plays a similar essential role for their metabolism for TCA cycle fuel, proliferation signalling and redox control²¹⁷. Another layer of importance is that hypoxia response increases expression and activity of GS^{218,219}, indicating the importance of glutamine metabolism on the microenvironment of the bone marrow¹⁹⁸.

1.3.4.2 Glutamate

Glutamate is another NEAA, that can be synthesized through many distinct pathways of metabolism. Glutamate uptake shares many transporters with other amino acids such as Excitatory Amino Acid Transporter (EAATs) and ASCTs^{215,220,221}. As mentioned above, Glutamate is transformed into α KG by GLUD, but it can also occur through transaminase reactions as a side product^{198,199}. Furthermore, glutamate can be obtained from the catabolism of BCAAs by Branched-Chain Amino Acid Transaminase 1 and 2 (BCAT1 and 2)²²².

Glutamate is used in the synthesis of glutathione²²³ (see chapter 1.3.6) and other amino acids such as proline, aspartate, alanine, serine and ornithine^{196,224}.

1.3.4.3 Serine, Glycine and Methionine: The One-Carbon Metabolism

Serine is a NEAA that is highly consumed by cancer cells²²⁵. Its uptake is done by many different transporters such as ASCT2 and SNATs^{220,221}. It can also be synthesized from 3PG, one of the intermediates of glycolysis, by Phosphoglycerate Dehydrogenase (PHGDH) with conversion of NAD⁺ into NADH^{196,226} (Figure 1.3.4). The resulting 3-phosphohydroxypyruvate (3PHP) is then used by Phosphoserine Aminotransferase (PSAT) for transamination, in a glutamate-dependent manner. This reaction results in

3-phosphoserine (3PS), with side product of α KG. Serine is then produced by the hydrolysis of 3PS by Phosphoserine Phosphatase (PSPH). Serine can then be utilized as a building block in purine and lipid synthesis, as well as other amino acids such as cysteine and glycine²²⁶. It also possesses a regulatory function over glycolysis as an allosteric activator of PKM2, an isoform of Pyruvate Kinase. This allows to block the flow of glycolysis at the phosphoenolpyruvate level and redirect it back to 3PG for serine production if its levels are low^{196,227}.

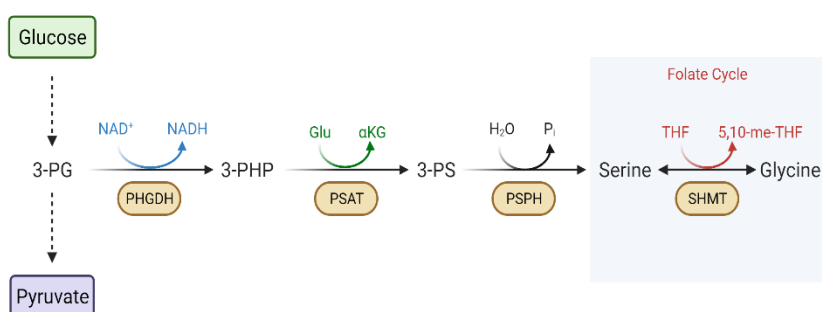


Figure 1.3.4- Schematic of the Serine Synthesis Pathway (SSP). The 3-Phosphoglycerate (3PG) produced from glycolysis is used by Phosphoglycerate Dehydrogenase (PHGDH) to produce 3-Phosphohydroxypyruvate (3PHP), with generation of NADH. Phosphoserine Aminotransferase (PSAT) converts 3PHP into 3-phosphoserine (3PS), through a Glutamate-dependent transamination. The last step that produces serine is the hydrolysis of 3PS by Phosphoserine Phosphatase (PSPH), with the release of an inorganic Phosphate (Pi). Serine is then converted to Glycine by Serine Hydromethyltransferases (SHMT), donating a one carbon unit to Tetrahydrofolate (THF) and making 5,10-methylenetetrahydrofolate (5,10-me-THF), which are members of the Folate Cycle. Adapted from Yang et al., 2016. Created with BioRender.com.

Glycine can be generated from glyoxylate aminotransferase, the Glycine Cleavage System, Threonine and Serine metabolisms¹⁹⁶. It is used as a precursor in the generation of many metabolites, such as glutathione, nucleic acids, sarcosine and uric acid¹⁹⁶. Mainly, glycine levels arise through the reaction of Serine Hydromethyltransferases (SHMT) 1 and 2, cytosolic and mitochondrial isoforms respectively^{196,226}. This conversion

between Serine and Glycine generates one-carbon units (methyl groups), which are then utilized in the cycles of Folate and Methionine. The conjugation of these pathways is denominated the One-Carbon Metabolism²²⁶.

The folate cycle is a key pathway for synthesis of nucleotides, epigenetic events substrates and reductive metabolism²²⁸. It is initiated when folate (commonly known as vitamin B9) is transformed into Tetrahydrofolate (THF) by Dihydrofolate Reductase (DHFR) in two consecutive reactions²²⁸. THF is then used on the folate cycle to generate substrates for purine and pyrimidine synthesis and for the methionine cycle (Figure 1.3.5). Although methionine is an EAA, it can be salvaged by Methionine Synthetase (MS), also known as 5-methyltetrahydrofolate-Homocysteine Methyltransferase (MTR). This enzyme uses 5-methyl-THF and homocysteine to make methionine, with the vitamin B12 (cobalamin) as a cofactor²²⁹. Methionine in turn is catalysed into S-Adenosylmethionine (SAM), a very important metabolite for methyltransferases (MATs) such as DNMTs (as mentioned in chapter 1.2.1). Not only for MATs, SAM is essential for the creation of polyamines (see chapter 1.3.5), a set of important reactions that aid in the salvage of methionine²²⁹ (Figure 1.3.5). The totality of these pathways generates many key substrates for the function of the cells, such as purine and pyrimidine synthesis, redox power in the form of NADP⁺, FAD and glutathione production (see chapter 1.3.6), deoxythymidine monophosphate (dTMP) for DNA synthesis and SAM for methylation reactions.

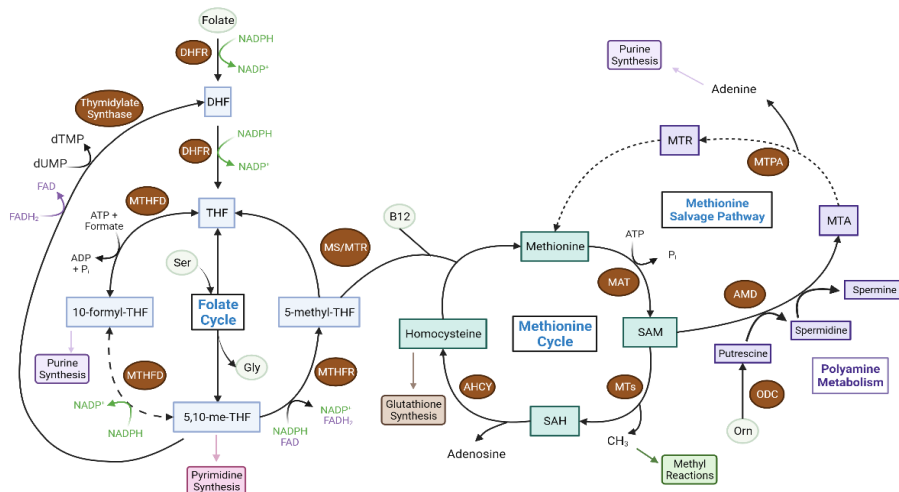


Figure 1.3.5- One Carbon Metabolism and partner metabolisms. Folate is converted to Dihydrofolate (DHF) and Tetrahydrofolate (THF) by the same enzyme, Dihydrofolate Reductase (DHFR). THF can be transformed into 5,10-methylene-THF (5,10-me-THF) by SHMT, using Serine (Ser) as carbon donor and resulting in Glycine (Gly). There are 3 reactions of the Folate Cycle performed by the enzyme Methylene-tetrahydrofolate Dehydrogenase (MTHFD). It can transform THF into 10-formyl-THF as a Formate-tetrahydrofolate Ligase, with ATP and Formate as cofactors, releasing ADP and P_i . Next, it can function as Methenyltetrahydrofolate Cyclohydrolase, that converts 10-formyl-THF into 5,10-me-THF by hydrolysis. It can also convert 10-formyl-THF and 5,10-me-THF as a Methylene-tetrahydrofolate Dehydrogenase, using $NADP^+/NADPH$. 10-formyl-THF is used for Purine Synthesis and 5,10-me-THF is used for Pyrimidine Synthesis. 5,10-me-THF is either transformed back to DHF by Thymidylate Synthase, with FAD and deoxythymidine Monophosphate (dTMP) as side products or be transformed into 5-methyl-THF by Methylene-tetrahydrofolate Reductase (MTHFR), using FAD as cofactor and NADPH as reducing agent. The resulting 5-methyl-THF is then used with Homocysteine by Methionine Synthetase (MS or MTR), with vitamin B12 as cofactor, to produce methionine and THF. Methionine is then catalyzed by Methionine Adenosyltransferase (MAT), to create S-Adenosylmethionine (SAM) in an ATP-dependent manner and generating P_i . SAM can be transformed by Methionine Transferases (MTs) into S-Adenosylhomocysteine (SAH), generating a methyl group for other reactions. SAH is then converted back to homocysteine by Adenosyl-Homocysteinase (AHCY), releasing adenosine. This homocysteine is then used for new methionine or directed for glutathione synthesis. Methionine salvage occurs through the Polyamines metabolism. Putrescine is formed from Ornithine (Orn) by action of Ornithine Decarboxylase (ODC). Then Adenosylmethionine Decarboxylase (AMD) generates Spermidine and Spermine, in a SAM-dependent manner. Both reactions result in Methylthioadenosine (MTA), that can be phosphorylated by Methylthioadenosine Phosphorylase (MTAP), releasing adenine for Purine Synthesis. The remaining Methylthioribose (MTR) is then salvaged back to methionine. Adapted from Sanderson et al., 2019. Created with BioRender.com.

Cancer cells exploit the One-Carbon Metabolism and its partnered pathways in many ways. Overexpression of SHMT2 leads to poor survival and prognosis in breast and hepatic cancer^{230,231}. The multifunctional enzyme MTHFD has a cytosolic enzyme (MTHFD1) and two mitochondrial forms (MTHFD1L and 2/L). Upregulation of these isoforms have been reported across many types of cancer, like AML and breast cancer^{232,233}. Alterations of the methionine cycle are a mutational factor in cancer, with methylation reactions for epigenetic regulation becoming compromised²²⁹, as mentioned in chapter 1.2.1. Lastly, another point of One-Carbon Metabolism in cancer is its connection with the polyamines metabolism, with over expression of Ornithine Decarboxylase (OCD) and Adenosylmethionine Decarboxylase (AMD), aiding tumoral proliferation²³⁴.

Another point of overlap between pathways with One-Carbon Metabolism is the NADP⁺/NADPH production. MTHFD1 complements the NADPH production in the cytosol and the mitochondrial isoforms assist the generation done by the TCA cycle^{228,229}. These interactions aid to control the negative effects of ROS production, allowing to avoid apoptosis. Cancer cells take advantage of these to avoid the negative effects of their rapid proliferation²³⁵.

1.3.4.4 The amino acids of the Urea Cycle

The Urea Cycle is an important detoxification process in cells. It requires many NEAAs such as arginine, ornithine, citrulline and aspartate. Ornithine is used by Ornithine Transcarbamylase (OTC) to form citrulline, using Carbamoyl Phosphate (Figure 1.3.6). This molecule is very important because it originates from toxic products of respiration (HCO_3^-) and glutaminolysis (NH_4^+), through a reaction catalysed by Carbamoyl Phosphate Synthase 1 (CPS1). CPS1 function is ATP-dependent and requires use of N-Acetyl-Glutamate (NAG), which is produced by N-

Acetyl-Glutamate Synthase (NGAS) through joining an Acetyl-CoA to glutamate²³⁶. The resulting citrulline is transported out of the mitochondria to cytosol by Ornithine Transporter 1 (ORNT1), where it is used by Argininosuccinate Synthetase 1 (ASS1) to make Argininosuccinate, spending ATP and Aspartate. Argininosuccinate is then broken down by Argininosuccinate Lyase (ASL) into arginine, releasing fumarate which is integrated back to the TCA cycle. The reaction of Arginase (ARG1 and ARG2) converts arginine into ornithine²³⁶, producing urea for release (Figure 1.3.6). This cycle of reactions allows for the expulsion of toxic ammonia from the cells in the form of urea, which is normally excreted in urine²³⁶. A point of note is that there is one reaction in the cycle that actually produces ROS, in the form of Nitric Oxide (NO).

Although the main amino acids used in the Urea Cycle are arginine, ornithine, citrulline and aspartate, in reality its function relies on other amino acid metabolism, such as TCA cycle and Glutaminolysis. In addition, many resulting substrates of the cycle can be fuel for pyrimidine and polyamines synthesis²³⁶.

Cancer cells can interfere on the urea cycle depending on their necessities, with the majority of tumours shunting the intermediates of this cycle into anabolic routes²³⁶. Studies have reported overexpression of CPS1 correlates with oncogenic KRAS in lung cancer^{237,238} and poor prognosis in colon cancer²³⁹. ASS1 expression has both been reported to be lowered to increase pyrimidine synthesis²⁴⁰ or overexpressed to support proliferation²⁴¹ and migration²⁴².

Leukaemias have been reported to alter their urea cycle by lowering expression of OTC and ORNT1, redirecting ornithine for other uses such as the polyamine synthesis^{243–246}.

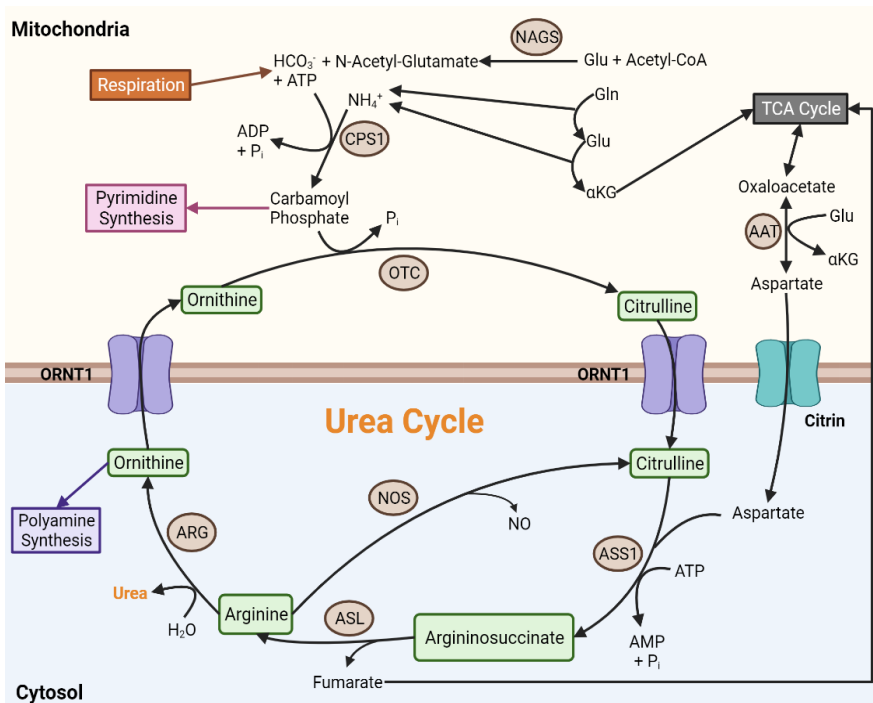


Figure 1.3.6- Urea Cycle mechanisms in cytosol and mitochondria. Urea is formed by hydrolysis of Arginine by Arginase (ARG), resulting in ornithine. Ornithine can be used in polyamine synthesis or transported to mitochondria by Ornithine Transporter 1(ORNT1). There it is used by Ornithine Transcarbamylase (OTC), in conjunction with Carbamoyl Phosphate, producing Citrulline and P_i . Citrulline is then transported by ORNT1 back to cytosol, which is converted to Argininosuccinate by Argininosuccinate Synthetase 1 (ASS1), in an ATP-manner and using aspartate. Argininosuccinate is then broken down by Argininosuccinate Lyase (ASL) into arginine and fumarate. Fumarate can be reutilized by the TCA cycle and Arginine is re-used either to generate new urea or transformed to citrulline by Nitric Oxide Synthase (NOS), with side production of Nitric Oxide (NO). Production of Carbamoyl Phosphate is done by Carbamoyl Phosphate Synthase 1(CPS1), in an ATP-manner using HCO_3^- and NH_4^+ , products of respiration and glutaminolysis, respectively. It also requires N-acetyl-glutamate, that results from joining Acetyl-CoA with Glutamate (Glu), catalyzed by N-Acetyl Glutamate Synthase (NAGS). Carbamoyl Phosphate is also used for Pyrimidine Synthesis. Aspartate can be obtained from Oxaloacetate through Aspartate Aminotransferase (AAT), using Glu and producing α -Ketoglutarate (α KG). Aspartate is then transported by Citrin (SLC25A13) to the cytosol, to be used by ASS1. Adapted from Keshet et al. Created in BioRender.com.

1.3.4.5 Other amino acids interconnections

Of the many pathways and metabolisms explained previously, there were some amino acids mentioned but only explained in the context of their relationship with the main amino acid under focus. In truth, these amino acids also have their own role to play in the cellular metabolism and on the tumoral development.

1.3.4.5.1 Branched-chain Amino Acids

As mentioned in glutamate production, BCAAs are an important source of nitrogen for its production through BCATs but it also contributes to the TCA cycle²²². Inside the mitochondria, Valine generates succinyl-CoA, Leucine generates acetyl-CoA and Isoleucine is able to make both²⁴⁷. They can also be used in the amination of Pyruvate, resulting in alanine²⁴⁸.

Leucine is also an allosteric regulator of GLUD and mTORC1^{249,250}. In a leukemic setting, alterations of BCAT1 are common, both as overexpression that drives transformation²⁵¹⁻²⁵³ or through its blocking by the oncometabolite 2-HG²⁵⁴. The MLL1 fusion protein, known to initiate leukemogenesis, can induce overexpression of BCAT1 by chromatin hyperacetylation²⁵⁵. An important point of investigation is that inducing the knockdown of BCAT1 in LSCs results in rising levels of α KG and decreased levels of HIF1 α and impaired cell growth⁹⁵.

1.3.4.5.2 Alanine

Alanine metabolism is another crossroad between other metabolisms²⁵⁶. Transamination using alanine is done mainly by Glutamate-Pyruvate Transaminase (GPT) (also known as alanine aminotransferase (ALT)) and Alanine-Glyoxylate and Serine-Pyruvate Aminotransferase (AGXT)^{256,257}. GPT or ALT can use glutamate, together with pyruvate to produce alanine and α KG²⁵⁶. On the other hand, AGXT is responsible for 3 reactions, the first uses alanine and glyoxylate to make pyruvate and glycine (EC 2.6.1.44)^{256,257}. The second reaction uses glyoxylate with serine to produce

glycine and 3-hydroxypyruvate (EC 2.6.1.45) and the third reaction uses serine and pyruvate to obtain also 3-hydroxypyruvate and alanine (EC 2.6.1.51)^{256,257}. All these reactions are bidirectional, depending on the chemical balance of substrates. Together, these reactions make alanine a focal point between glutamine metabolism, serine-glycine metabolism and generation of pyruvate for use on the TCA cycle. And that many of these enzymes have alterations reported across many types of cancer²⁵⁶⁻²⁵⁹.

1.3.4.5.3 Asparagine

Asparagine is mainly utilized for protein synthesis and as an exchange factor in the transport of glutamine, serine, histidine and arginine^{260,261}. Its synthesis is done by Asparagine Synthase (ASNS), through an ATP-dependent reaction that requires glutamine and aspartate^{262,263}. Asparagine levels must be tightly regulated, or it will activate the Unfolded Protein response (UPR), that results in cell death²⁶⁴. Of note is the use of asparaginase that can force starvation and block growth of leukemic cells^{265,266}.

1.3.4.5.4 Proline

Proline metabolism is linked to the metabolism of glutamate. Proline catabolism is done by Proline Dehydrogenase (PRODH) and Pyrroline-5-Carboxylate Dehydrogenase (P5CDH), producing glutamate with the reduction of FAD into FADH₂²⁶⁷. The reverse reactions for proline production are done by Pyrroline-5-Carboxylate Synthase (P5CS, coded by the gene ALDH18A1) and Pyrroline-5-Carboxylate Reductase (PYCR), utilising NADH/NADPH²⁶⁷. PYCR has 2 isoforms, PYCR1 is cytosolic and PYCR2 is mitochondrial^{267,268}.

Proline metabolism can both be anti- or pro-tumour. PRODH is up-regulated by p-53 and found downregulated in many tumours²⁶⁹⁻²⁷². Its overexpression can diminish DNA synthesis, block cell cycle²⁶⁸ and

suppress HIF-signalling by increasing levels of α KG^{273,274}. On the other hand, PRODH aids the survival of breast cancer after HDACs inhibitors treatment²⁷⁵ and induces migration and metastasis in lung cancer²⁷⁶. Proline synthesis also aids tumoral progression, with upregulation of PYCR1 and ALDH18A1 across many cancers^{232,277}. Of note is that upregulation of proline synthesis can modulate an increase in glycolysis and PPP through the ratio of $\text{NAD}^+/\text{NADP}^+$ ²⁷⁸.

1.3.4.5.5 Lysine

Lysine is an EAA, which catabolism requires α KG and NADPH, producing saccharopine, α -amino adipic acid (α AAA)²⁷⁹ and acetyl-CoA^{280,281}. This catabolism involves various transamination reactions, resulting in a side production of glutamate. Lysine is necessary for protein synthesis and for a positive nitrogen balance inside the cell²⁸². In a cancer setup, lysine is one of the main amino acids that suffers PTMs, especially in epigenetic events regarding histones^{283,284}.

1.3.4.5.6 Threonine

Threonine is an EAA that can only be catabolised by Threonine Dehydratase in humans, for the genetic loss of the other enzymes^{285–287}. Threonine catabolism through threonine dehydratase results in NH_3 and α -Ketobutyrate, with the latter being further transformed into succinyl-CoA, that then feeds the TCA cycle²⁸⁸. Furthermore, as it happens with other amino acids, threonine is also an amino acid which relevance in cancer arises from the PTMs that suffers as part of kinases (such as mTOR and AKT).

1.3.4.5.7 Histidine

Histidine is also an EAA, that can function as a pH buffer^{289,290}. It has also antioxidant activity, as a scavenger of ROS and Reactive Nitrogen Species

(RNS) ^{291–294}. Histidine catabolism intersects the Folate Cycle, utilizing THF for the conversion of Formiminoglutamate (FIGLU) into glutamate, so it exerts influence on the One-Carbon Metabolism^{295,296}. Histidine is required for the synthesis of metabolites such as Histamine through Histidine Decarboxylase (HDC) and Carnosine through Carnosine Synthase 1 (CARNS1)^{290,297}. HDC can be induced by cytokines, low pH and hypoxia-inducible factors^{298–300}. Histidine is also very important for protein synthesis, being an abundant part of the composition of haemoglobins, cytochromes, Nitric Oxide Synthase (NOS) and catalases^{290,301}. Another factor of histidine metabolism is that despite being an EAA, cells can compensate the lack of histidine intake by catabolising haemoglobin and carnosine^{302–305}.

1.3.4.5.8 Phenylalanine and Tyrosine

Phenylalanine is an EAA that is used in protein synthesis and can be hydroxylated into the NEAA Tyrosine by Phenylalanine Hydroxylase (PAH)³⁰⁶. Tyrosine is also a necessary component of many important proteins, such as kinases. Tyrosine catabolism is done by aminotransferases and dioxygenases, with the final products of fumarate and acetoacetate³⁰⁶, that can fuel the TCA cycle and fatty acids synthesis. In cancer, phenylalanine metabolism has been linked with suppression of T-cell immune response³⁰⁷ and PAH is found altered in various malignancies^{308,309}. Tyrosine is mostly reported in cancer as a phosphorylation site of many important kinases of signalling pathways³¹⁰. The genes of the catabolic reactions of tyrosine have been reported to be downregulated and to suffer epigenetic regulation through miRNAs, aiding the development of hepatocellular carcinoma³¹¹. Investigations of tyrosine catabolism in cancer appear to be on the rise^{308,312,313}.

1.3.4.5.9 Tryptophan

Tryptophan is an EAA, used in the synthesis of metabolites such as serotonin and Niacin (vitamin B3) and its catabolism results in the biogenic amine Kynurenine^{198,312}. This reaction can be done both by Tryptophan-2,3-dioxygenase (TDO) or Indoleamine-2,3-dioxygenase (IDO). The resulting kynurenine is then further catabolised to generate precursors for NAD⁺ production³¹⁴. Tryptophan and Kynurenine play a role on suppressing immune response³¹⁵ and some of the enzymes of this pathway have been reported to correlate with CML progression³¹⁶.

1.3.5 Metabolism of Biogenic Amines

Biogenic amines are a vast group of metabolites that arise from the decarboxylation of amino acids, reductive aminations and transamination of aldehydes and ketones^{317,318}. They are divided into 3 groups: Monoamines, diamines and polyamines. Among them are neurotransmitters such as dopamine, serotonin and histamine³¹⁷⁻³¹⁹.

Biogenic amines participate in many basic cellular functions such as the synthesis of proteins, hormones and nucleic acids. They also have a supportive role in proliferation, immune response, response to stress and senescence. Additionally, they easily interact with negatively charged molecules (DNA, RNA, phospholipids, etc.), playing a role in their stabilization^{320,321}. But their excess is considered carcinogenic, with effects over apoptosis (like pore formation of mitochondria)^{322,323} and their catabolism resulting in cytotoxicity (formation of H₂O₂)³²⁴⁻³²⁶. Of note is the metabolism of polyamines, specially putrescine, spermine and spermidine. Their synthesis is dependent on ornithine and SAM (Figure 1.3.5) and it can be upregulated by C-MYC, cyclin D1, HRAS and KRAS (through upregulation of ODC and AMD1)³²⁷⁻³²⁹. They have a role on cell cycle, on the transition from G₁ to S phase³³⁰. Their uptake and synthesis increase have been reported in breast, colon, skin and prostate cancers^{331,332}.

1.3.6 Redox Balance: The ROS Equilibrium Game

The Redox control inside a cell is a multifaceted process, combining many metabolic pathways in order to achieve a balance between reductive factors and reactive species. These reactive species are grouped in four designations: ROS, RNS, reactive sulphur species (RSS) and reactive chloride species (RCS), with the first being the more commonly by-product of cellular metabolism^{182,333}.

ROS can be produced primarily from OXPHOS but also inside other organelles such as peroxisomes³³⁴. It can also arise of the activity of enzymes such as NADPH oxidases (NOXs) and Xanthine Oxidases. This production can be activated by external stimuli such as TNF α , EGF, Interleucin-1 β (IL-1 β), hypoxia and radiation^{335–339}.

A tight regulation of ROS levels is required in order to use them for signalling, as evident on haematopoiesis, that uses varying levels of oxidative stress to drive the differentiation of HSCs³⁴⁰ (see chapter 1.1.2). But ROS can cause damage to DNA leading to genomic instability, with emergence of mutations^{341,342}. It can also inactivate important TSGs such as PTEN and activate transcription factors like HIF1 α and NRF2, reprogramming the metabolism³⁴³.

And is for the regulation of ROS that the other players of this balance game enter, the ROS scavengers. They are the Glutathione Peroxidases (GPX), peroxiredoxins, catalases (CATs) and Superoxide Dismutase (SODs)^{182,333,344,345}. These enzymes convert free radicals (the highly reactive forms of ROS) into less toxic and stable molecules for excretion. Complementary to their action is the Glutathione Cycle. Glutathione is an antioxidant and one of the most important detoxifying agents for cellular homeostasis^{345,346}. Glutathione exists in two forms: the reduced glutathione (GSH) and the oxidised form Glutathione Disulphide (GSSG) (Figure 1.3.7). GSH is used by GPXs to detoxify ROS, resulting in GSSG^{345,347}.

The glutathione cycle is another interconnection of many paths of metabolism. For the generation of GSH there is the involvement of substrates from glutaminolysis, One-Carbon metabolism and ATP. And during the salvage of GSSG back to GSH, it requires the NADPH generated by the PPP and TCA cycle.

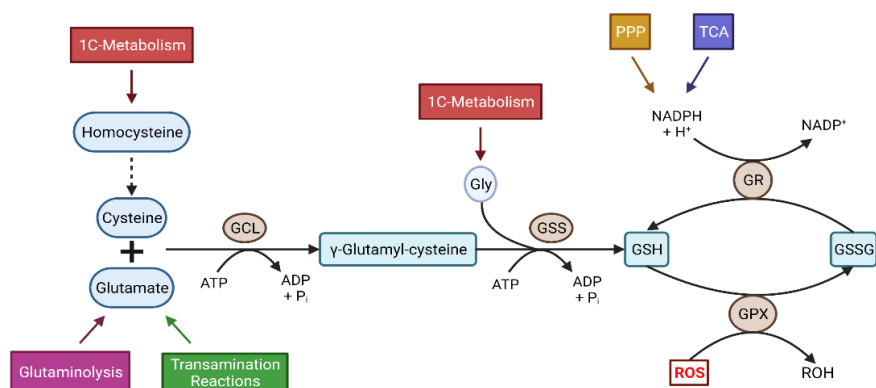


Figure 1.3.7- Glutathione Cycle and its interactions with metabolism. Glutathione (GSH) production starts with the joining of glutamate and cysteine, obtained from glutaminolysis and One-carbon (1C) Metabolism, by Glutamyl-cysteine Ligase (GCL), in an ATP-dependent reaction. The resulting γ -Glutamyl-cysteine is then joined with glycine (Gly) by Glutathione Synthetase (GSS), also requiring ATP. GSH (reduced form) is then used by Glutathione Peroxidases (GPX) to detoxify reactive oxygen species (ROS), resulting in Glutathione disulphide (GSSG). The oxidized form GSSG can then be rescued by Glutathione Reductase (GR) back to GSH, using NADPH generated by other metabolisms, mainly the Pentose Phosphate Pathway (PPP) and Tricarboxylic Acid (TCA) Cycle. Adapted from Bansal et al, 2018. Created with BioRender.com.

Cancer cells utilize ROS to drive their tumorigenesis but also its regulation mechanisms in order to avoid apoptosis. They reduce the ROS production from respiration by reducing the entrance of pyruvate to the TCA cycle, through overexpressing PDK1³⁴⁸. On the other hand, they increase the expression of SODs, catalases, GPXs and ramping up the production of GSH^{345,349}. This is one of the main reasons why there is increased glutaminolysis (overexpression of GLS and GLUD), granting more glutamate and also glycine and cysteine (through its nitrogen donor

function)³⁵⁰. The glutathione cycle in cancer cells is one of the main driving forces to increase the flux of glucose into the PPP. The oxidative branch of the PPP is essential to produce NADPH for the reaction that recovers GSH³⁵¹ (see chapter 1.3.2). Other sources of NADPH are the reactions of IDH, Malic Enzyme and MDH of the TCA cycle^{352–354} (see chapter 1.3.3) and the reactions of MTHFD of the Folate Cycle^{355,356} (see chapter 1.3.4.3). Lastly, cancer cells can exploit the glutathione mechanisms as a tool for chemotherapeutic resistance, given that GSH is also capable of detoxifying xenobiotics^{357–359}.

1.4 Hypoxia: A Hallmark of Leukaemia on his own

Hypoxia is the low O₂ state that naturally occurs in living tissues such as the bone marrow and the kidney^{360,361}. It is also a regulating factor during embryogenesis³⁶⁰ and can modulate the cellular metabolism³⁶². In the case of cancer, solid tumours usually possess hypoxic regions within their mass, due to a mismatch between rapid proliferation and aberrant vascularization, resulting in bad supply of oxygen^{363–365}.

As mentioned previously, the main effectors of the oxygen sensing response are the transcription factors HIFs. This group is comprised of HIF1, HIF2 and HIF3, with each dimerizing an α -unit with a β -unit^{366,367}. The β -units are constitutively expressed, while the α -units are regulated by hydroxylation from PHDs, that marks it for ubiquitination by VHL and subsequent degradation^{96,368}. This hydroxylation occurs in the presence of oxygen, so in normoxia the α -units are very short-lived (τ). But in the hypoxic setting, the lack of O₂ impedes the degradation of α -units, allowing the joining with β -units and translocation into the nucleus. There is promiscuity between the 3 α -units and HIF1 β (also known as Aryl Hydrocarbon Receptor Nuclear Translocator (ARNT))³⁶⁶. In the nucleus, the dimers bind to hypoxia-response elements (HREs) in the promoters and enhancers to activate transcription of target genes^{367,369}.

Of these numerous targets, they range through many cellular functions, from proliferation (growth factors, signalling pathways), angiogenesis (blood vessels formation), metabolic adaptation and avoiding apoptosis^{362,370}. All these processes have a great overlap between genes that are regulated by HIF (directly or indirectly) and tumorigenesis. This makes hypoxia as a master regulator of various functions defined as Hallmarks of Cancer^{4,364}.

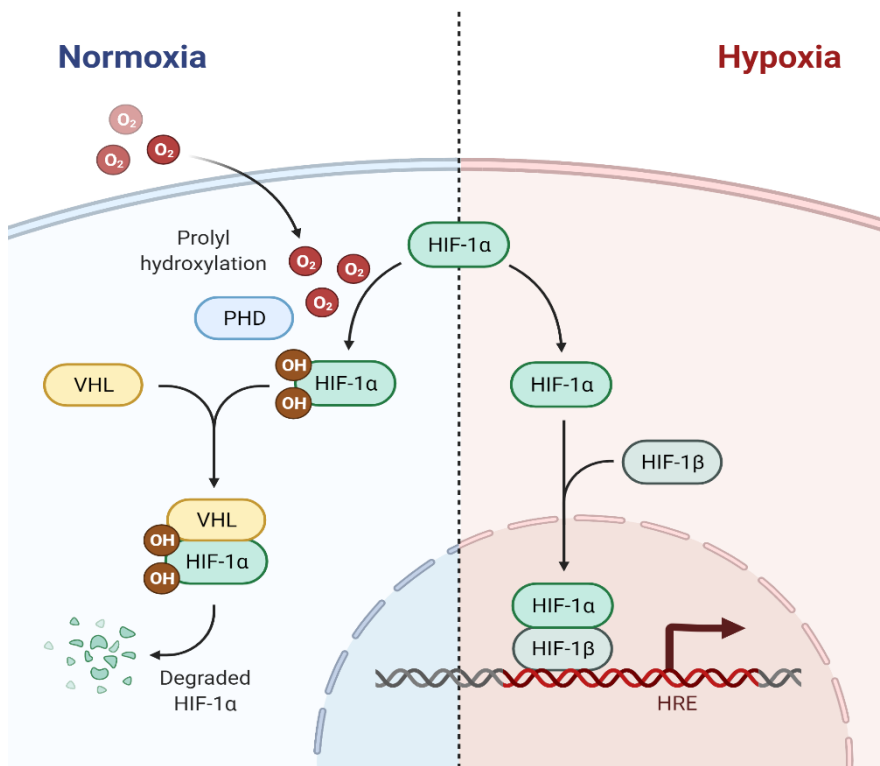


Figure 1.4.1- Destiny of Hypoxia-inducible Factors (HIFs) according to Oxygen presence. In Normoxia, HIF1α is hydroxylated by Prolyl Hydroxylase Domain-containing (PHDs), tagging it for Von Hippel-Lindau (VHL), that binds HIFs to ubiquitination complexes, resulting in their degradation. In hypoxia, lack of O₂ impedes PHDs, leaving HIF 1α free to form heterodimers with HIF 1β, that will translocate into the nucleus and bind to Hypoxia-response Elements (HREs) of various genes promoters and enhancers. Created with BioRender.com.

Hypoxia can induce tumour cells to make their own growth factors (EGF, IGF1, EPO, etc.), increasing their proliferation and growing independent of exogenous mitogenic growth signals³⁷¹. It can also induce the expression of VEGFs, to initiate angiogenesis and increase the access to nutrients^{79,372}. Many signalling pathways of proliferation interact with HIFs, such as MAPK and PI3K/AKT, like the AKT phosphorylation of HIF1 α that allows the binding with HIF1 β ^{363,365,373–378}.

Hypoxia induces anti-apoptotic proteins³⁷⁶ and can exert a selective pressure against cells with wildtype p53, aiding on the clonal expansion of cancer cells with p53 mutated³⁷⁹. Hypoxic conditions can also favour cell immortalization, by increasing the Human Telomerase (hTERT) activity through MAPK activation³⁸⁰ and affecting the PHD2-mediated downregulation³⁸¹.

Hypoxia modulates the microenvironment by signalling tumour-associated macrophages to secrete mitogenic factors, proangiogenic cytokines and immunosuppressive agents through the HIF1 and HIF2 transcription programming^{371,372}. This allows tumour cells to escape immune detection and response^{371,382}. Hypoxic settings or an overexpression of HIF1 α can promote the Epithelial Mesenchymal Transition (EMT), leading to more invasive phenotypes and formation of metastasis^{383,384}.

In the deregulating of cell energetics, hypoxia induces the Warburg Effect by increasing the expression of GLUT1, PDK1 and many glycolytic enzymes (HKII, GAPDH, ENO, etc). This leads to increased glucose uptake and PDH inhibition to redirect energy production from the TCA cycle^{365,373}. The control of ROS levels is one of the main reasons that HSCs have a metabolic profile of low TCA cycle activity and favour anaerobic glycolysis. Studies have proven that an increase in ROS production (from OXPHOS or NOXs) will induce proliferation, differentiation and maturation of the haematopoietic cells in a spectrum, depending on the lineage commitment^{374,378,385–388}. Another key factor on this hypoxic

regulation is that the changes in metabolism is affecting PHDs. As a dioxygenase, not only are they being directly impeded by the absence of O₂ but also being inhibited by increased ROS, phosphorylation by Protein Kinase C (PKC), Nitric Oxide (NO) and the availability of αKG^{96,368} (just like the TET enzymes).

The pathophysiological importance of available O₂ would be counterintuitive for haematological malignancies, given that “blood tumours” is assumed to have direct access to the circulating oxygen. But inside the bone marrow it is a different story. Within the stem niche, the O₂ presence is below 1%. During the hematopoietic differentiation, the cells face a gradient of O₂, from around 1% inside the niche up to 12% when nearing the vascular system³⁸⁹. HSCs are in chronic hypoxia for the majority of their lifetime until differentiation³⁹⁰. Many studies have showed that hypoxia is a functional component of the haematopoietic niche but the role of HIFs in stem cells regulation is not so clear. While the impact of HIFs overexpression in solid tumours have been established, with correlations to poor prognosis³⁹¹ and therapeutic resistance³⁹², investigations on their function on leukemic cells are contradictory^{377,393,394}. Further investigations are necessary to discern these discrepancies, but it is clear that HIFs play a role on the quiescence of HSCs and the differentiation initiation^{368,375,395}.

One key factor of interest of leukemic stem cells is their quiescent metabolic phenotype, how they balance the signals of the niche, use anaerobic glycolysis for energy, maintain low levels of ROS without inducing apoptosis and are able of resisting therapy³⁹⁶, leading to the eventual relapse of many patients. Investigating how leukemic cell lines vary their metabolism from normoxia into chronic hypoxia can elucidate how metabolic reprogramming due to O₂ levels can be hijacked during leukemogenesis at the origin point, the bone marrow. And light a path to breaking the cruel cycle of relapse that persists in many leukaemias

nowadays and free leukaemia survivors of that pending doom becoming reality.

1.5 Metabolic Studies Tools

The advancements of science in modern history have allowed a deeper understanding of cancer down to the molecular level. The deciphering of the human genome was a marking event, helping to understand how mutations of genes led to many types of diseases³⁹⁷. But it also brought to light that alterations of the genotype do not account directly to the phenotype observed. As such, the research efforts shifted their focus from studying a singular gene, molecule or pathway isolated into an englobing view of the cellular biological system with its many components and their interactions. Thus, the Multi-Omics studies emerged. These “Omics” are composed of genomics, epigenomics, transcriptomics, proteomics, metabolomics and other emerging omics (lipidomics, microbiomics, foodomics, etc.)^{398,399}.

The main goal of a Multi-Omics approach is to combine the many different layers of knowledge, with high-dimensional datasets of multiple levels of a cell biological system to try to correlate genotype to phenotype (Figure 1.5.1)⁴⁰⁰. But a smooth integration of all these different areas is not yet a reality, with development in each Omics-field at uneven stages, speeds and costs. Starting with genomics, it is the study of all genetic information, with its many variants and abnormalities (mutations, chromosomal rearrangements, etc)⁴⁰¹. The advances in high-throughput techniques have improved to allow Whole Genome Sequencing (WGS) studies, that have aided in more accurately classifications of cancer subtypes^{402,403}. It also created a “repertoire” of sequence variants and mutations that are associated with higher cancer risk and have become part of the routine-testing during tumour-staging and treatment decisions⁴⁰⁴. Despite the many improvements to genomic studies, it is still insufficient in predictive capabilities if used

without other Omics for cancer risk assessment, treatment response or recurrence risk.

The second-most advanced Omics study to date is Transcriptomics, that englobes all the gene transcripts (coding and non-coding) being generated in the cell. It complements the genomics information, by revealing which genes are actively being transcribed and if any alternative splicing events is occurring⁴⁰³. The development of RNA-sequencing (RNA-Seq) has proven a great tool, that can measure the amount of gene transcripts and their sequence across the genome⁴⁰⁵. Through its datasets, differential expression analysis can be performed and highlight different gene expression signatures, that can be used to understand signalling networks and pathways during different stages of cancer or during treatments⁴⁰⁶. Transcriptomics analysis has many utilities such as genomic interpretation, identification of biomarkers and discerning new therapeutic avenues from gene interactions^{407,408}. For these capabilities, RNA-Seq was chosen for analysis of the alterations induced by TKTL1. A downside of transcriptomics is that to this day it is a high-cost technique, with a very high requirement for data storage and computing processing power^{398,409,410}. Another difficulty is when integrating with protein levels, there is only around 40% correlation of transcript-protein⁴¹¹.

The advancement of Epigenomics is a fast-evolving Omics, younger than Transcriptomics but quickly catching up in developing techniques and clinical translation. The study of how the epigenetic landscape influences gene expression is necessary as complementary knowledge to interlink genomics and transcriptomics⁴¹². The current techniques used for epigenomic studies are Whole-genome Bisulphite Sequencing (WGBS), Chromatin Immunoprecipitation Sequencing (ChIP-Seq) and Assay for Transposase-Accessible Chromatin Sequencing (ATAC-Seq). The complexity of the many modifications that happens to DNA and histones is a layer of difficulty for the evolution of epigenomics (see chapter 1.2). In

addition, the epigenetic landscape is influenced by time and spatial factors, with a very tissue-specific response to external stimuli³⁹⁸. As such,

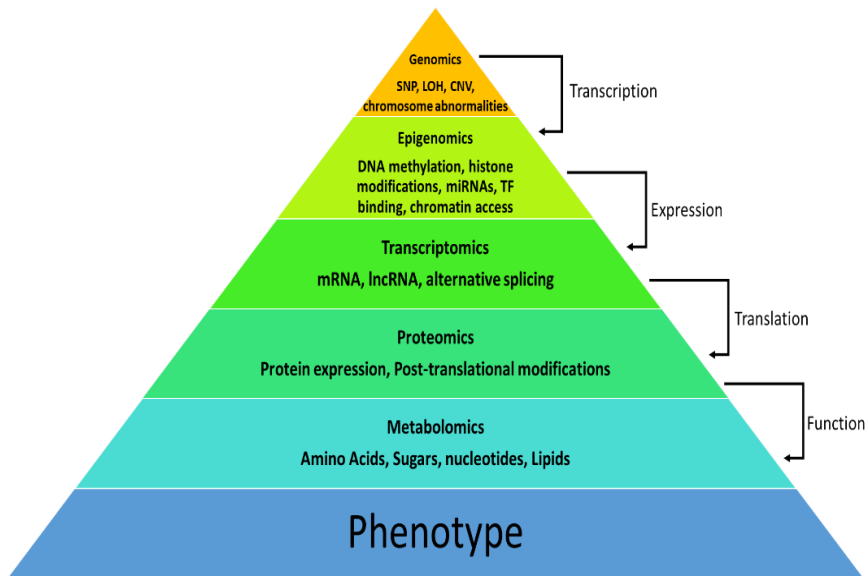


Figure 1.5.1- Multi-Omics Studies Hierarchy. Genomics can identify single nucleotide polymorphisms (SNP), loss of heterogeneity mutations (LOH), copy number variations (CNV) and chromosome abnormalities (translocations, fusions, etc.). Epigenomics can check the status of chromatin access, DNA methylation, histone modifications, activity of micro-RNAs (miRNA) and binding of Transcription factors (TF). Transcriptomics evaluate small RNAs (mRNA), long noncoding RNAs (lncRNA) and if alternative splicing is occurring. Proteomics englobes all the proteins being translated and the many distinct post-translational modifications that they suffer. Metabolomics studies small molecules such as amino acids, sugars, nucleotides and lipids. All the omics combined can inform on the final phenotype expressed. Adapted from Momeni et al.

epigenomics techniques need to be bolstered by transcriptomics performed under the same conditions, to cover these caveats.

The other remaining Omics areas are more recent and require more development, in both techniques and translation to clinical application. Proteomics embodies all the proteins that are translated from the genome, with the many post-translational modifications they suffer^{403,413}. Due to these variations, no single technique can assess the whole “proteome” in all its facets. Most proteomic methodologies are focused to identify and

quantify specific proteins, mostly through Mass Spectrometry (MS) or affinity-based protein arrays^{414,415}. Although proteomics studies have aided in identifying drug target protein pools⁴¹⁶ and established molecular signatures for cancer subtypes⁴¹⁴, showing their promised potential to personalise cancer therapies, the techniques are still demanding in time and resources⁴¹⁵.

Lastly, Metabolomics is the Omics study that quantifies small molecules such as sugars, lipids, amino acids and nucleotides. It can be applied to all types of cells, tissues and fluids^{41,417,418}. It evaluates the levels of metabolites since they are the product of many cellular processes and their changes are reflective of metabolic function. Metabolomics falls in the last bracket of the Omics approach (Figure 1.5.1), which will be the closest to expression of phenotype. This makes it more susceptible to external stimuli, from environmental factors, cell-cell interactions or drug targeting effects⁴¹⁰. In fact, Metabolomics have shown much effective in identifying metabolic biomarkers and to discern metabolic changes during cancer progression and response to treatment⁴¹⁹. The Achilles' heel of metabolomics lies that it encompasses an enormous amount of metabolites, many still unidentified, with great diversity in concentration levels and chemical composition, complicating its analysis and reproducibility^{398,420}. There are many approaches for metabolomic studies that can be separated into 2 categories: targeted and nontargeted studies. The first is a quantitative measure of selected metabolites of a specific pathway or biological process, requiring *a priori* knowledge of the target compounds⁴²¹. The second relates to a global coverage and profiling of the metabolome, with no pre-selection applied in order to obtain a more unbiased analysis^{422,423}. Both methodologies utilize MS and Nuclear Magnetic Resonance (NMR) techniques for the metabolite readouts, with MS being less costly and possessing higher sensitivity but NMR being more effective in metabolite identification⁴²⁴. At the end, both techniques require specialized

bioinformatics software for accurate statistics and analysis of very complex datasets (due to the many species of metabolites amalgamated). Another approach to consider is tracer-based metabolomics, a targeted methodology that it will be explained in more detail in chapter 1.5.1.

Overall, Multi-Omics studies have aided in a more pathophysiological stratification of cancer subtypes and are a great step in the direction of personalized medicine. There are still limitations to be overcome. The overlap mismatch between the different Omics makes difficult to correlate the data of different methods, making the decision of which omics to perform and how to integrate them a complex one. This could be improved if the generation of the datasets could come from the same biological sample but the sample processing for a distinct Omics makes it unsuitable for the others. On the software front, improvements to analytical and annotation tools are always a must. There are many reviews that summarize the areas of future improvement for Multi-Omics approaches and their contributions for precision medicine^{398,399,418}.

1.5.1 SIRM-tracer based Metabolomics

Stable Isotope Resolved Metabolomics (SIRM) is a targeted metabolomics technique that relies in the quantification of metabolites through the distribution of ^{13}C in metabolites generated from an enriched precursor, mapping the biological system where the labelled intermediates are present⁴²⁵⁻⁴²⁷. These stable isotopes are non-radioactive and functionally identical to the natural occurring isotopes. Incorporation of ^2H , ^{13}C and ^{15}N into biological precursors, such as glutamine and glucose, are a proven method to trace their metabolism in living systems⁴²⁸. The measurements of these isotopes are done through MS and NMR spectroscopy. Following the isotope labelling, the metabolites are extracted and determined by number of heavy atoms (isotopologues) and the position of those heavy atoms (isotopomers)⁴²⁹ (Figure 1.5.2).

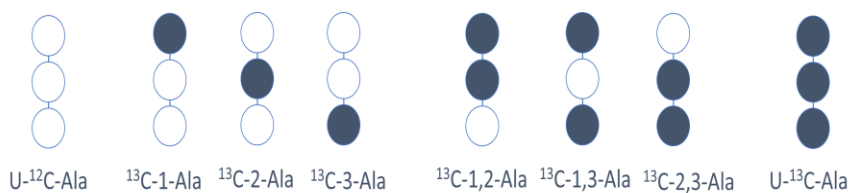


Figure 1.5.2- Permutations of ^{13}C -labelling positions of Alanine. Isotopologues are distinguished by the number of atoms labelled (from 1 up to 3 maximum). Isotopomers distinguish which of the atoms in the molecule is labelled (the 1st, 2nd or 3rd carbon in alanine).

The difference in nominal mass of the heavy atoms from the natural occurring ones allows MS to determine the number of atoms incorporated. MS is coupled with gas and liquid chromatography (GC and LC) methods in order to resolve large amounts of metabolites from different sample types⁴²⁹. On the other hand, NMR techniques are more suited for determining the positional labelling, due to the magnetic properties of the atomic nuclei, through direct detection or indirectly by their attached protons^{426,429}. NMR 1D and 2D methods enable isotopomer analysis capable of discerning distinct patterns in complex biological systems. The joining of both NMR and MS methods can enable a more faithful reconstruction of the pathways and networks of the metabolism under study^{425–427,429–432}.

The main goal of SIRM is to analyse the different utilizations of the labelled substrate and the subsequent metabolic transformations between two conditions or samples, such as cells, biofluids and tissues. SIRM studies have aided in multiple cancer biological models, such as 2D and 3D cell cultures, *in vivo* or *ex vivo* tumour models⁴²⁷. SIRM studies have proven again Warburg's findings (see chapter 1.3.1) and disproven the bias that tumour cells have dysfunctional mitochondria¹⁸³. Thus, SIRM is excellent in profiling metabolic changes and highlighting distinctions between normal tissue and cancer.

2. OBJECTIVES

2 Objectives

Leukaemia is the 10th cancer cause of death worldwide². Although the overall survival rate of Leukaemia has increased across the board due to modernized treatments, there are still extremely aggressive cases that fail to remise. This is due to the capacity to gain resistance to therapy.

Since leukemogenesis is based on various genetic alterations that lead to different subtypes with distinct phenotypes and metabolic profiles, understanding these events can elucidate on new therapeutic targets. Many investigative works have established the important role of studying the metabolism of cancer cells, to improve patient prognosis, risk stratification and counteract eventual resistance to therapy⁴¹.

The present work aims to characterize metabolic adaptations granted by specific enzymes to leukaemia, in both the normal oxygen conditions and a hypoxia state, for closer representation of the haematopoietic niche. The enzymes chosen are TKTL1 and TET2, due to their many peculiar properties listed and to better shed light on their role on leukemogenesis, in order to ultimately aid in help patients with these mutations.

Therefore, the main objectives of the present thesis are the following:

1. Characterization of the role of TKTL1 in the metabolic reprogramming of AML in normoxia and hypoxia, using THP1 Control and THP1 TKTL1-Knockdown as a cell model.
2. Characterization of the role of TET2 in the metabolic reprogramming of CML in normoxia and hypoxia, using HAP1 Parental and HAP1 TET2 Knockout as a cell model.

3. MATERIALS AND METHODS

3 Materials and Methods

3.1 Cell Culture

Human THP-1 acute monocytic leukaemia were obtained from the American Type Culture Collection (Manassas, VA, US). THP-1 WT and THP-1 TKTL1^{KD} cells were generated by Sirion Biotech GmbH (Munich, Germany). The THP-1 cell lines are suspension cells, grown in RPMI 1640 culture medium of 10mM Glucose, 2mM glutamine and supplemented with 10% heat-inactivated Fetal Calf Serum (FCS) and antibiotics: 100 U/ml penicillin, 100 ug/ml streptomycin and 50 ug/ml gentamicin.

Human HAP1 chronic myeloid leukaemia were obtained from Horizon Discovery© using CRISPR/Cas9 technology to delete 19 base pairs in exon 3 of the *TET2* gene (HZGHC001091c010). These cell lines are adherent cells, grown in Iscove's Modified Dulbecco's Medium (IMDM) culture medium with 25 mM glucose, 4mM glutamine and supplemented with 10% heat-inactivated FCS and antibiotics: 100 U/ml penicillin and 100 ug/ml streptomycin. In hypoxia settings, cells were cultured for 5 days for chronic hypoxic adaptation in Hypoxystation H35 (Don Whitley Scientific Limited, United Kingdom) before any experiment was performed.

3.2 Cell Proliferation Rate Assays

Cell proliferation curves were performed by cell counting with Scepter™ Handheld Automated Cell Counter (Merck Millipore, USA) and Countess II Automated Cell Counter (Thermo Fisher Scientific, Waltham, MA, USA), for both THP1 and HAP1 cell lines, respectively. THP1 cell lines were seeded at 3×10^5 cells per 25 cm² flasks in normoxia and seeded at 4×10^5 cells in hypoxia. HAP1 cell lines were seeded at 7.5×10^4 cells per p6 well (9.6 cm²) in normoxia. In hypoxia, HAP1 Parental was seeded at 7.5×10^4 cells per p6 well and HAP1 TET2 KO was seeded at 1.1×10^5 cells per p6 well. Cell counting was done at 24 hours timepoints up until a total of

72h or 96h. THP1 counting samples were collected in eppendorfs from seeding flasks, with volumes of 500 ul, centrifuged at 1500 rpm (rotations per minute) for 5 min, removed supernatant and resuspended in 1 ml PBS solution (Phosphate Buffered Saline). HAP1 counting samples were collected from sacrificing replicate wells, through trypsinization with 1ml trypsin, incubating at 37°C for 5 min, inactivating trypsin with 3ml media (1:3) and centrifuged at 1200 rpm for 5 min. Pellets were then collected from supernatant and resuspended in 1ml PBS for counting.

3.3 Biochemical Assays for Consumption and Production by Spectrophotometry

In order to measure the concentration of each metabolite, media samples were collected at all timepoints of growth curves experiments, and frozen until being analysed. Glucose, lactate, glutamine and glutamate concentrations were determined by using spectrophotometer (COBAS Mira Plus, Horiba ABX, Japan) from frozen cell culture medium as previously described above.

Glucose concentrations for each time point was measured by calculating NAD(P)H concentration decrease (measured at 340 nm) after the conversion of total glucose by hexokinase and conversion of resulting glucose-6-phosphate into D-gluconate-6-phosphate by G6PDH using coupled enzymatic reactions (kit ABX Pentra Glucose HK CP, HORIBA ABX, Japan).

Lactate measurements were performed by mixing media samples with LDH enzyme buffer (LDH at 87.7 U/ml, 28.6 mM $(\text{NH}_4)_2\text{SO}_4$, 1.55 mg/ml NAD^+ in 0.2 M hydrazine, 12 mM EDTA pH 9 at 37°C) and NADH release readouts at 340nm.

Before glutamate measurements, the media samples were previously deproteinated, by boiling at 80°C for 15 min in a heating block. After

cooling for 5 min in ice, samples were centrifuged at 13 200 rpm at 4°C for 10 min. Collected supernatant was frozen at -20°C for future readings.

Glutamate was quantified by mixing the deproteinated samples with a glutamate dehydrogenase mix buffer (GLUD1 at 39 U/ml, 2.41 mM ADP, 3.9 mM NAD⁺ in 0.5 M hydrazine, 0.5 M glycine pH 9 at 37°C) and NADH release measured at 340 nm.

The remaining volume of deproteinated samples was then used in a glutaminase reaction, to convert glutamine into glutamate for an indirect measurement. The samples were mixed with a GLS buffer (125 mU/ml of GLS, 125 mM acetate pH 5 at 37°C) for 30 min in constant agitation. The converted glutamate was then measured by the same procedure described before.

The consumptions and productions estimation of each metabolite was done with the measurement variation between the final and initial point of the exponential growth window, taking into account the cell number of those timepoints. Ratios between metabolites were also calculated.

3.4 Enzymatic Activity Assays

For HAP1 cell lines, fresh cell culture plates were rinsed with PBS and lysed with lysis buffer (20mM Tris-HCL, pH 7.5, 1 mM dithiothreitol, 1mM EDTA, 0.02% (v/v) Triton X-100, 0.02% (v/v) sodium deoxycholate) supplemented with protease and phosphatase inhibitor cocktails (Sigma and Thermo Scientific, respectively) for 30 minutes at 4°C. The cells were scrapped and collected in eppendorf tubes and the cell lysate was disrupted by sonification using titanium probe (Vibracell, Sonics & Materials Inc., Tune 50, Output 20, 3 cycles of 5 seconds each). For THP1 cell lines, pellets were collected, washed with PBS before adding same lysis buffer and being sonicated. The tubes were centrifuged at 13 400rpm at 4°C for 20 minutes. The supernatant was separated and immediately used for the determination of specific enzyme activities using spectrophotometer

(COBAS Mira Plus, Horiba ABX, Japan). All enzymatic activities were determined by monitoring NAD(P)H increment or decrement at 340 nm wavelength. The enzyme activity for each sample was then normalized to the total protein content of the samples quantified by BCA Assay at 550 nm wavelength (Pierce, Thermo Scientific).

3.4.1 TKT and TKTL1

All transketolase activity (both TKT and TKTL1) was determined adding sample to cuvettes containing 5 mM MgCl₂, 0.2 U/ml triose phosphate isomerase, 0.2 mM NADH, 0.1 mM thiamine pyrophosphate in 50 mM Tris-HCl, pH 7.6, at 37°C. Reaction starts by addition of prepared mixture of dissolved 50 mM R5P in 50 mM Tris-HCl, pH 7.6, in the presence of 0.1 U/ml ribulose-5-phosphate-3-epimerase and 1.7 mU/ml phosphoriboisomerase. This mixture is constantly stirred and at 37°C for 1h during preparation and stored at -20°C until utilization.

3.4.2 GAPDH

GAPDH activity is obtained by coupling the activities of GAPDH and PGK. Measurements are obtained by adding samples to a cuvette containing 1.1 mM ATP, 0.9 mM EDTA, 1.7 mM MgSO₄, 0.2 mM NADH, 14.8 U/ml of PGK in 82.5 mM Triethanolamine- HCl pH 7.6 at 37°C. Reaction starts with the addition of 50 mM G3P up to a final concentration of 6 mM.

3.4.3 LDH

Lactate Dehydrogenase activity was measured by adding samples to a cuvette containing 0.2 mM NADH in 100 mM KH₂PO₄/K₂HPO₄ pH 7.4 at 37°C. Reaction initiated by the addition of 10 mM pyruvate up to a final concentration of 0.2 mM.

3.4.4 HK

HK activity was determined by coupling HK and G6PD reactions in the following conditions: 3.3 mM NADP⁺, 14.8 mM ATP, 14.8mM MgCl₂, 2.8 U/ml of G6PD and 50mM Tris-HCl, pH 7.6, at 37°C. Reactions were initiated by the addition of glucose up to a final concentration of 10 mM.

3.4.5 G6PD and 6PGD

6-Phosphogluconate dehydrogenase specific activity was measured adding samples to a cuvette containing 0.5 mM NADP⁺ in 50 mM Tris-HCL pH 7.6, at 37°C. Reaction was initiated by the addition of 6-phosphogluconate (6PG) and glucose-6-phosphate (G6P), respectively, up to a final concentration of 2 mM.

3.4.6 PK

PK activity was determined by coupling PK and LDH reactions in the following conditions: 0.8 mM NADH, 1.6 mM ADP, 12.1 mM MgCl₂, 36.8 mM KCl and 5.2 U/ml LDH in 20 mM KH₂PO₄/K₂HPO₄ buffer pH 7.2 at 37°C. Reactions were initiated by the addition of phosphoenolpyruvate (PEP) up to a final concentration of 3.5 mM.

3.5 Protein Content by Cell Number Assay

Cells were cultured and then collected with different concentrations, starting from 2.5 x 10⁵ cells/ml up to 2 x 10⁶ cells/ml, for THP1 and HAP1 cell lines, in both normoxia and hypoxia. After cell counting, protein extraction was performed with lysis buffer utilized in enzymatic assays (see chapter 3.4) and protein content was measured through BCA Assay at 550 nm wavelength (Pierce, Thermo Scientific). Values were then plotted by cell concentration and protein by cell factors were obtained by statistical analysis of linear regression.

3.6 RNA-based Assays

3.6.1 RNA Isolation

RNA was extracted from THP1 cells, both fresh and frozen extracts using Trizol (Sigma) according to manufacturer's protocol. Then chloroform was added to the mixture and centrifuged to generate aqueous and organic phases. The aqueous phase was collected, added cold isopropanol and incubated overnight at 4°C to precipitate the RNA. The samples were then centrifuged at 14 000 rpm at 4°C for 15 min. RNA was purified with several washing steps with 75% ethanol and resuspended in RNase-free water. Purified RNA was quantified using Nanodrop Spectrophotometer (ND 1000 V3.1.0, Thermo Fisher Scientific Inc.).

3.6.2 RT-PCR

The Reverse Transcription reaction (convert RNA into DNA) was performed at 37°C using 1 µg of RNA added to mixture containing 5x Buffer (Invitrogen), 0.1 M dithiothreitol (DTT) (Invitrogen), Random Hexamers (Roche), 40 U µL⁻¹ RNase inhibitor (Promega, Fitchburg, WI, USA), 40 mM dNTPs (Bioline, London, UK), 200 U µL⁻¹ M-MLV-RT (Invitrogen).

Gene Expression analysis was performed by RT-PCR system (Applied Biosystems® 7500 Real Time PCR) standard manufacturer's protocol employing TaqMan® (Applied Biosystems) gene specific probes for TKT (Hs00169074_m1), TKTL1 (Hs00202061_m1), GLS (Hs01014019_m1). Reactions were performed in a volume of 20 µl containing 9 µl of cDNA and 11 µl of TaqMan Master Mix (Applied Biosystems). RT-PCR program set-up parameters were: 1) initial incubation at 50°C for 2 minutes, 2) denaturalization at 95°C for 10 minutes, 3) amplification of 40 cycles alternating between 95°C for 15 seconds and 60°C for 1 minute. The house-

keeping gene used as reference was PP1A (Hs99999904_m1, Applied Biosystems) and expression levels were quantified using $\Delta\Delta\text{Ct}$ method.

3.6.3 RNA-sequencing

The transcriptomics methodology chosen was RNA-sequencing, to detect differentially expressed genes. Total RNA was extracted from lysates of the THP1 parental and THP1 TKTL1 knock-down cell lines, with 10×10^6 cells, using the RNeasy Kit (Qiagen, Hilden, Germany), with a DNase digestion step. Resulting samples were then sent to the Genomic Platform of the CNIC (National Centre for Cardiovascular Diseases) Institute in Madrid. Data alignment was performed using the Homo sapiens high coverage GRCh37.75.dna.primary_assembly by Juanjo Lozano of the Bioinformatics Platform of CIBEREHD, Institute of Health Carlos III, Madrid. Data processing and analysis was done by Effrosyni Karakitsou, fellow colleague of the HaemMetabolome ITN research initiative. The differentially expressed genes were identified using the Differential gene expression analysis based on the negative binomial distribution 2 (DESeq2) package from Bioconductor⁴³³. Throughout the analyses, a threshold was set for differentially expressed transcripts to be considered as statistically significant if their adjusted p-value or false discovery rate (padj) was lower than 0.05.

3.7 Mitochondrial Activity Assays

Mitochondrial activity assays were performed on a Seahorse XFe24 Flux Analyzer (Seahorse Bioscience, USA), following the Mitostress Test and Glycolytic Test protocols. The first measures the Oxygen Consumption Rate (OCR) and the second measures the Extracellular Acidification Rate (ECAR). XFe24 sensor cartridges were hydrated overnight using 1ml per well of XF Calibrant Solution (Seahorse Bioscience, USA), at 37°C in a non-CO₂ incubator. Both measurements were carried out together, with

each plate performing both assays on the same oxygen condition. For data correction, the cells were extracted after measurements, using RIPA buffer and protein content quantified by BCA assay.

3.7.1 Oxygen Consumption Rate (OCR)

HAP1 cell lines were seeded at 2.7×10^5 cells per ml in normoxia and 2.85×10^5 cells per ml in hypoxia, in 24-well plates (Seahorse Biosciences). Plating was done with 100 μ L seeding of cell suspension and 150 μ L extra addition of medium 2h later once cells had attached to the surface.

After overnight growth, the medium was replaced with Seahorse medium (buffer-free DMEM supplemented with 22 mM glucose, 4 mM glutamine, 1mM pyruvate and antibiotics). The plates were equilibrated in a 37°C incubator without CO₂ for 60 min. The plate was then joined with the sensor cartridge, previously hydrated and calibrated as described above, and loaded into Analyzer. The Mito Stress Assay protocol was altered for baseline conditions (25 mM glucose, 4 mM glutamine, 1% streptomycin and penicillin), with injections of 5 μ M oligomycin (ATP synthetase inhibitor), 600 nM CCCP (Carbonyl Cyanide m-Chlorophenyl hydrazone, uncoupling agent), and 2 μ M rotenone (inhibitor of complex I) together with 2 μ M antimycin A (inhibitor of complex III), prepared and loaded into injection ports of cartridge, in order to calculate de different respiratory parameters (see chapter 3.7.3).

3.7.2 Extracellular Acidification Rate (ECAR)

The Glycolytic Stress Test was performed at the same cell concentrations per well as the Mito Stress Test. Assay media was buffer-free DMEM supplemented with 4 mM glutamine and antibiotics. The cell plate went under the same prepping procedure before entering the Analyzer. The protocol of injections was defined as 22 mM glucose, to initiate glycolysis; 5 μ M oligomycin; and 250 mM 2-deoxy-D-glucose (2-DG), to inhibit

glycolysis. Glycolytic parameters were then calculated from ECAR measures.

3.7.3 Calculations from OCR and ECAR assays

From each assay data, calculations of each parameter are required. All calculations were done after correction by protein values of each corresponding well.

For the Mito Stress Test Assay:

- Basal mitochondrial respiration is obtained from the subtraction of the non-mitochondrial respiration (OCR value after the injection of rotenone and antimycin A) to the baseline OCR.
- ATP production is calculated from the subtraction of baseline OCR from OCR rate following oligomycin injection.
- Proton leak is the subtraction of non-mitochondrial respiration from the OCR rate after oligomycin injection.
- Maximal respiration results from the subtraction of non-mitochondrial respiration to the OCR rate after CCCP injection.
- Spare capacity is calculated by removing the baseline OCR from the OCR rate after CCCP injection.

For the Glycolytic Stress Test Assay:

- Basal acidification without Glucose is the baseline acidification of the medium before any injection.
- Glycolysis rate is obtained from the subtraction of non-glycolytic acidification (ECAR rates after 2-DG injection) to ECAR rates after glucose injection.
- Glycolysis capacity is calculated from removing non-glycolytic acidification values from the ECAR rates after oligomycin injection.
- Glycolytic Reserve is the subtraction of ECAR rates after glucose injection to rates after oligomycin injection.

Crabtree Effect is calculated from OCR levels measured during the Glycolytic Stress Test. It is the subtraction of the baseline OCR to the OCR rate after the glucose injection divided by the baseline OCR. Next, the logarithm base 2-fold change(\log_2) of the resulting value was calculated.

3.8 Reactive Oxygen Species (ROS) Assays

Intracellular ROS levels were determined by flow cytometry using the H₂DCFDA reactive (Invitrogen, Thermo Fisher Scientific, USA). HAP1 cell lines were seeded at 1×10^5 cells per p6 well, for both normoxia and hypoxia. After 48h incubation, the medium was removed, and cells were incubated with 5 mM glucose and 2 mM glutamine in PBS solution containing 5 μ M of H₂DCFDA at 37°C for 30 min in the cell culture incubator. After probe incorporation in cells, the solution was removed and replaced by standard culture medium, for another incubation at 37°C for 45 min. This incubation step allows the probe activation, through intracellular esterases. Lastly, cells were washed with PBS, trypsinized and resuspended in PBS with 50 μ M of H₂DCFDA and 20 μ M of Propidium Iodate (PI), followed immediately by analysis at the Gallios™ Flow Cytometer (Beckman Coulter, USA), which detected the fluorescent probe that was excited at 492 nm and emitted at 520 nm, as well as PI fluorescence.

3.9 Targeted Metabolomics

Intracellular metabolite profiling and determination of uptake and secretion rates were performed using the Biocrates Absolute IDQ™ p180 kit (Biocrates Life Sciences AG, Austria). THP1 cell lines were seeded at 4×10^5 cells/ml for both normoxia and hypoxia. After 48h incubation, extracts were collected from 5×10^6 cells each. HAP1 cell lines were seeded at high confluence conditions in order to obtain the maximum quantity of cell pellet (in a range of 10 to 15×10^6 cells) in 100 mm plates. After 48h incubation,

cells were trypsinized and centrifuged at 1200 rpm for 5 min to obtain the cell pellet and cell medium. All pellets were resuspended in 70 μ L of 85:15 ethanol:PBS buffer and sonicated 3 times for 5 seconds each, then submerged in liquid nitrogen for 30 seconds and thawed at 95°C. Centrifuged at 13 400 rpm, at 4°C for 5 min, collected the supernatant and protein content was measured. For the determination of metabolites uptake and production, metabolites were extracted from cell media taken from beginning and end of incubation in the same conditions as intracellular measurements. Both extracts from pellets and media were processed simultaneously in the same Biocrates plate together with the calibration standards, and derivatized to be ready for UHPLCMS reading (for amino acids and biogenic amines) and FIA-MS/MS (for lipids, sugars and acylcarnitines) according to the manufacturer's instructions. Metabolites analysed comprise of 21 amino acids, 19 biogenic amines, 90 glycerophospholipids, 15 sphingolipids, 40 acylcarnitines and hexose sugars. The data were normalized by protein (intracellular) and by cell number (extracellular medium). In order to determine the metabolite uptake and secretion rates, used the same data processing specified in chapter 3.3.

3.10 Stable Isotope-Resolved Metabolomics (SIRM)

HAP1 cell lines were seeded at 3.8×10^6 cells in normoxia and 5.5×10^6 cells in hypoxia, in 100 mm plates. After 24h incubation, media was changed for 22 mM glucose 100% enriched in U- 13 C-glucose, 4 mM glutamine 100% enriched in U- 13 C-glutamine, 4 mM glutamine 100% enriched in 3- 13 C-glutamine (for NMR only) or standard media. Cells and media were obtained and frozen at -80°C for metabolite extractions at 0h, 6h and 24h after the labelled substrates were added. Extracellular metabolite measurements were also performed by spectrophotometry for these samples according to the methods in 3.3.

3.10.1 Gas Chromatography Mass Spectrometry (GCMS)

The analyses of ¹³C-labelled intracellular and extracellular metabolites for isotopologue distribution were performed using an Agilent 7890A gas chromatograph (Agilent Technologies, USA) with an HP-5 capillary column coupled to an Agilent 5975C mass spectrometer. For analysis, 1 µl of each sample was injected together with helium gas as a carrier at a 1 ml/min of flow rate.

3.10.1.1 Extracellular Metabolites Extraction

For intracellular ribose analysis, RNA was isolated from cell pellets using Trizol reagent, mixing it with chloroform. The aqueous phase was obtained, and cold isopropanol was added and centrifuged 13 400 rpm, at 4°C for 15 min. The samples were washed several times using cold 75% ethanol and isolated RNA was quantified using a Nanodrop spectrophotometer (ND 1000 V3.1.0, Thermo Fisher Scientific). The samples of purified RNA were hydrolysed in 2 ml of 2 M HCl at 100°C for 2 hours, dried under airflow and resulting ribose was derivatized into its aldonitrile acetate derivative using 100 µl of 2% hydroxylamine hydrochloride in pyridine at 100°C for 30 min and 75 µl of acetic anhydride at 100°C for 1 hour. Then, samples were dried under N₂ flow and resuspended in ethyl acetate before GCMS analysis using chemical ionization mode. The oven temperature variations were programmed as follows: 150°C for 1 minute, then increased to 275°C at 15°C/min, to 300°C at 40°C/min, and hold for 2 min. The detector was run at single ion monitoring (SIM) mode. Ribose complete fragment C1-C5 was detected at 5.3 minutes of retention time monitoring the cluster 257 m/z from the range 256-261 m/z.

For extracellular lactate analysis, lactate from cell culture media was isolated using HCl and ethyl acetate previous to airflow drying. Lactate was derivatized to lactate acid n-propylamide-heptafluorobutyric ester by

incubation with 200 μ l of 2,2-dimethoxypropane and 50 μ l of 0.5 N methanolic HCl at 75°C for 1 hour and then adding 60 μ l of n-propylamine at 100°C for 1 hour more. After drying under N₂ flow, samples were filtered using glass wool through a Pasteur pipette and dried again under N₂ flow. Then, samples were resuspended and incubated with 200 μ l of dichloromethane and 15 μ l of heptafluorobutyric anhydride at room temperature for 10 min, dried under N₂ and resuspended under ethyl acetate before GCMS analysis under chemical ionization mode. The oven temperature variations were programmed as follows: 100°C for 3 min, then increased to 160°C at 20°C/min, and hold for 2 min. The detector was run at single ion monitoring (SIM) mode. Lactate complete fragment C1-C3 was detected at 5.4 minutes of retention time by monitoring the cluster 328 m/z from the range 327-332 m/z.

For polar amino acids analysis, media samples were mixed with milliQ water, added 5 μ l of norvaline 1 mg/ml and cold methanol. Then, the samples were vortexed for 60 seconds in cold room and cold chloroform was added. After gentle shaking (30 min at 4°C) the samples were centrifuged (13 400 rpm, at 4°C for 15 min) and aqueous phase was collected. This phase was then dried under airflow and mixed with dichloromethane (CH₂Cl₂), to completely dehydrate the samples. Dried again using N₂ gas flow and derivatized by adding 50 μ l of 2% methoxamine hydrochloride in pyridine for 90 minutes at 37°C and (N-methyl-N-tert-butyldimethylsilyl) trifluoroacetamide + 1% tertbutyldimethylchlorosilate) for 60 min at 55°C before GCMS analysis using electron impact mode. The oven temperature variations were programmed as follows: 100°C for 3 min, then increased to 165°C at 10°C/min, to 225°C at 2.5°C/min, to 265°C at 25°C/min and finally to 300°C at 7.5°C/min. The detector was run at single ion monitoring (SIM) mode. Following table lists the extracellular metabolites that were analysed and their retention times (Table 3.10-A).

Compound	Fragment	Retention Time (min)	m/z Cluster	m/z Cluster Range	m/z Control
Pyruvate	C1-C3	7.7	174.1	173.1-179.1	115.1
Lactate	C1-C3	10.8	261.1	260.1-266.1	303.1
Alanine	C1-C3	11.4	260.1	259.1-265.1	232.1
Glycine	C1-C2	11.7	246.1	245.1-250.1	218.1
Valine	C1-C5	12.8	260.2	259.2-267.2	288.1
Norvaline	C1-C5	12.9	260.2	259.2-267.2	288.1
Leucine	C1-C6	13.2	274.2	273.2-282.2	302.1
Isoleucine	C1-C6	13.6	302.2	301.2-310.2	274.1
Proline	C1-C5	14.0	286.1	285.1-293.1	258.2
Glutamine	C1-C6	15.9	300.1	299.1- 308.1	272.1
Methionine	C1-C5	16.0	320.2	319.2-327.2	292.2
Serine	C1-C3	16.1	390.2	389.2-395.2	362.2
Phenylalanine	C1-C9	16.9	336.2	335.2-347.2	308.2
Glutamate	C1-C5	17.9	432.3	431.3-439.3	474.3

Table 3.10-A- Extracellular metabolites quantified by GC-MS. Extracellular metabolites and corresponding fragments that were quantified with retention time, monitored clusters and respective ranges. Previously optimized for maximum detection. Extra cluster detected for control purposes.

3.10.1.2 Polar Intracellular Metabolites Extraction

For polar intracellular metabolites analysis, cells were washed with ice-cold PBS and scrapped with 1:1 metanol:water (adding first 1 ml of methanol, waiting for 1 minute and adding 1 ml of milliQ water afterwards). At that point, 5 μ l of norvaline 1 mg/ml was added for quantification purposes to minimize the differences that could be associated with efficiency of extraction. Then, the samples were sonicated (3 cycles of 5 seconds) and 2 ml of cold chloroform was added. After gentle shaking (30 min at 4°C) the samples were centrifuged (13 400 rpm, at 4°C for 15 min) and the supernatant was completely dried under airflow. The extracted metabolites were derivatized by adding 50 μ l of 2% methoxamine hydrochloride in pyridine for 90 minutes at 37°C and (N-methyl-N-tert-butyldimethylsilyl)

trifluoroacetamide + 1% tertbutyldimethylchlorosilate) for 60 min at 55°C before GCMS analysis using electron impact mode. The oven temperature variations were programmed as follows: 100°C for 3 min, then increased to 165°C at 10°C/min, to 225°C at 2.5°C/min, to 265°C at 25°C/min and finally to 300°C at 7.5°C/min. The detector was run at single ion monitoring (SIM) mode. Following table lists the intracellular metabolites that were analysed and their retention times (Table 3.10-B).

Compound	Fragment	Retention Time (min)	m/z Cluster	m/Z Cluster Range	m/z Control
Lactate	C1-C3	11.0	261.1	260.1-266.1	303.1
Alanine	C1-C3	11.8	260.1	259.1-265.1	232.1
Alanine	C2-C3	11.8	232.1	231.1-237.1	260.1
Glycine	C1-C2	12.2	246.1	245.1-250.1	218.1
Glycine	C2	12.2	218.1	217.1-221.1	246.1
Valine	C1-C5	13.9	260.2	259.2-267.2	288.1
Norvaline	C1-C5	14.2	260.2	259.2-267.2	288.1
Leucine	C1-C6	14.8	274.2	273.2-282.2	302.1
Isoleucine	C1-C6	15.5	302.2	301.2-310.2	274.1
Proline	C1-C5	16.4	286.1	285.1-293.1	258.2
Proline	C2-C5	16.4	258.2	257.2-264.2	286.1
Methionine	C1-C5	21.7	320.2	319.2-327.2	292.2
Methionine	C2-C5	21.7	292.2	291.2-298.2	320.2
Serine	C1-C3	22.2	390.2	389.2-395.2	362.2
Serine	C2-C3	22.2	362.2	361.2-366.2	390.2
Threonine	C1-C4	23.1	404.2	403.2-410.2	376.2
Phenylalanine	C1-C9	25.2	336.2	335.2-347.2	308.2
Malate	C1-C4	25.8	419.2	418.2-425.2	461.3
Aspartate	C1-C4	27.1	418.2	417.2-424.2	390.2
Aspartate	C2-C4	27.1	390.2	389.2-396.2	418.2
Cysteine	C1-C3	28.6	406.2	405.2-411.2	378.2
Cysteine	C2-C3	28.6	378.2	377.2-382.2	406.2
Glutamate	C1-C5	30.7	432.3	431.3-439.3	474.3
Citrate	C1-C6	36.9	591.4	590.4-599.4	459.2
Tryptophan	C1-C11	37.6	375.2	374.2-388.2	302.2

Table 3.10-B- Intracellular metabolites quantified by GC-MS. Intracellular metabolites and corresponding fragments that were quantified with retention time, monitored clusters and respective ranges. Previously optimized for maximum detection. Extra cluster detected for control purposes.

3.10.1.3 SIRM data analysis

Raw MS data was examined using MSD5975C Data Analysis (Agilent Technologies). The data obtained by mass spectrometry represents a spectral distribution of ions with its molecular weights (m/z) at each

retention time. The ion clusters, which were specified for each case, were used to determine the distribution of ^{13}C at each carbon of the fragment. Manual integration of peak areas was done to quantify the distribution of each compound, using the data of the present work, a software called “Ramid” for automated integration was being performed and optimized by the computational team of our research group⁴³⁴. The software, based in “R”, allows automatic extraction of the NetCDF files containing the m/z raw time course to isotopologue distributions. However, the values of peak areas, which are proportional to the ^{13}C incorporation to each molecule of the compound, needed to be corrected for the presence of natural abundance of ^{13}C as well as Si isotopes that are present from the derivatization reagents and contribute to the mass isotopologue distribution. The correction was performed from isotopologue distributions extracted before, using the “Midcor” software package^{434,435}. Afterwards, data was analysed and represented as direct substrate contribution of to specific isotopologues (m1, m2, m3, etc. according to the number of ^{13}C that were labelled). For malate and citrate, ratios of labelled carbons were calculated to discern glucose and glutamine contributions.

3.10.2 Nuclear Magnetic Resonance (NMR)

NMR experiments were performed at the University of Birmingham, as part of a secondment under the tutelage of Prof Ulrich Guenther at the Henry Wellcome Building for Nuclear Magnetic Resonance (HWB-NMR). Data processing and analysis was performed by Nuria Vilaplana, as a collaboration within the HaemMetabolome ITN research initiative.

Samples used for NMR analysis were generated in tandem with GCMS samples, as detailed in chapter 3.10, media samples from the three different ^{13}C -tracers used for GCMS were collected and analysed by NMR. An additional set of samples labelled with U- ^{13}C -glucose and 3- ^{13}C -glutamine were also analysed using NMR for intracellular metabolite studies.

All NMR data was acquired at Bruker 600 MHz spectrometers equipped with Avance-III consoles using a cooled Bruker SampleJet autosampler. For cell extracts, a triple resonance cryoprobe (TCI) 1.7mm z-axis pulsed field gradient (PFG) cryogenic probe was used, and for media samples, a 5mm TCI z-PFG cryogenic probe was used. Probes were equipped with a cooled SampleJet autosampler (Bruker) and automated tuning and matching.

3.10.2.1 Intracellular Metabolite Extraction

For intracellular metabolite extraction, cells were washed with 5 ml cold PBS, scraped in 600 μ l cold Methanol (-20°C) and collected into glass vials (2 ml). 200 μ l HPLC grade chloroform (Merck) and 300 μ l Milli-Q water were added with 1 min vortex steps between them. Samples were incubated for 10 min on ice and then centrifuged at 4000 rpm for 15 min at 4°C. 400 μ l of the polar phase (upper phase) were collected into in a new tube. Polar phase samples were dried with vacuum and stored at -80°C.

Dried polar phases samples were reconstituted in 50 μ l of 0.1 M phosphate buffer including 3 mM NaN₃, 0.5 mM 3-(Trimethylsilyl) propionic-2,2,3,3-d₄-acid sodium salt (TMSP) and 10% (v/v) D₂O (all from Sigma-Aldrich). Samples were sonicated for 10 min and transferred to 1.7 mm NMR tubes (CortecNet) using the Micro Pipet System 1.7 (New Era Enterprises). Sample preparation was done within 24 hours before the acquisition and kept at 4°C.

3.10.2.2 Extracellular Metabolite Extraction

For the extracellular metabolite extraction, media samples were collected from the adherent cells plates (1 ml per plate) and stored at -80°C. The day prior to acquisition, media samples were diluted with a 10% (v/v) 10X phosphate buffer (1 M phosphate buffer with 3 mM NaN₃ and 5 mM TMSP in 100% D₂O) to a final concentration equal to the one used with dried polar

phases described in the previous section. Buffered samples were transferred to 3 mm NMR tubes (CortecNet) using glass pipettes and stored at 4°C.

3.10.2.3 1D-NMR

Spectra acquisition at 300K using 1D ¹H-NOESY (Nuclear Overhauser effect spectroscopy) pulse sequence, with pre-saturated water suppression (noesygppr1d, standard pulse sequence from Bruker). Data point numbers was TD 32,768, the spectral width was 12 parts per million (ppm), with interscan delay of d1 4 seconds and NOE mixing of d8 10 milliseconds. The ¹H carrier was set on water frequency, with ¹H 90° pulse calibrated at 0.256 W and length of ca 7-8 μs. For medium samples from HAP1 cell lines, 64 transients and 8 steady state transients were acquired. The experimental time for media samples was 7.5 min.

Data processing: 1D 1H-NMR

Spectra processed with *MetaboLab* software⁴³⁶ within the MATLAB environment (MathWorks). The FID signals were multiplied by 0.3 Hz exponential window function, with zero-filling up to 131,072 data points applied prior to Fourier transformation. Chemical shift was calibrated using the TMSP signal as reference for 0 ppm and spectra were phase corrected manually. The baseline correction was done by applying a spline baseline correction. The TMSP, water and edge regions of the spectra were excluded before scaling, using a probabilistic quotient normalization (PQN). For metabolite identification, utilized Chenomx 7.0 software (Chenomx Inc.) and the Human Metabolome Database (HMDB). Peak intensities were obtained directly from the spectra. Relative metabolite changes were calculated by obtaining the peak intensities of each metabolite at 0 and 24h, the initial intensity subtracted from the final intensity and the result divided by the initial intensity.

3.10.2.4 ^{13}C -filtered ^1H -NMR

Medium samples labelled with $\text{U-}^{13}\text{C}$ -glucose, $\text{U-}^{13}\text{C}$ -glutamine and $3\text{-}^{13}\text{C}$ -glutamine were analysed with ^{13}C -filtered ^1H -NMR spectroscopy as described in Reed et al., 2019. Sample preparation is described in 4.1.1.2. Sample acquisition was done in the Bruker 600 MHz with a 5mm TCI z-PFG cryogenic probe. Spectra acquired at 300 K using a double gradient BIRD filter pulse sequence developed in-house⁴³⁷. Used pulse program that combines the $^1\text{H}[^{12}\text{C}]$ and the all- ^1H experiments in scan-interleaved mode. The FIDs difference results in the $^1\text{H}[^{13}\text{C}]$ signal. Spectral width was 12 ppm, data points numbers were 16,384 in each dimension and relaxation delay was 5.3 sec. Acquired 256 transients with 64 steady state transients and the experimental time was 15 min.

Data processing: ^{13}C -filtered ^1H -NMR

The ^{13}C -filtered ^1H -NMR spectra were processed in *Topspin 4.0.5* (Bruker). Spectra were zero filled to 32,768 data points before Fourier transformation. Phase correction was applied to all the spectra ($^1\text{H}[^{-12}\text{C}]$ and all- ^1H spectra) and the difference $^1\text{H}[^{13}\text{C}]$ spectrum was obtained through chemical shift alignment. Metabolites were picked in the $^1\text{H}[^{13}\text{C}]$ spectrum and integrated in all the spectra ($^1\text{H}[^{12}\text{C}]$, $^1\text{H}[^{13}\text{C}]$ and all- ^1H) using the *Topspin 4.0.5* (Bruker) integral tool. For each metabolite label incorporation, we divided the corresponding peak area in the $^1\text{H}[^{13}\text{C}]$ spectrum by the peak area in the all- ^1H spectrum.

3.10.2.5 2D ^1H - ^{13}C HSQC

Sample acquisition was done in Bruker 600 MHz with a 5mm TCI z-PFG cryogenic probe at 300K using a modification of the HSQCGPHRSP from Bruker, with additional gradient pulses during the insensitive nuclei enhanced by polarisation transfer (INEPT) echo periods and using soft 180° pulses for ^{13}C . For the 1H dimension: spectral width of 13.018 ppm with

1024 complex points. For the ^{13}C dimension: spectral width of 160 ppm with 2048 complex points. 2 transients were acquired per spectra, with an interscan delay of 1.5 seconds. Generated a non-uniform sampling (NUS) of 25% sampling schedule using the Wagner's schedule generator (Gerhard Wagner Lab, Harvard Medical School), with a tolerance of 0.01 and default values for the other parameters. The total experimental time was 4 hours.

Data processing: 2D ^1H - ^{13}C HSQC

For the ^1H - ^{13}C HSQC spectra, processed and phase corrected with *NMRPipe* (National Institute of Standards and Technology of the U.S.)⁴³⁸. *MetaboLab* was used to reference using the signal for the methyl group of L-lactic acid, at 1.31/22.9 ppm in the ^1H and ^{13}C dimensions, respectively. For the 2D spectra, metabolite identification was carried out using the *MetaboLab* software which includes a chemical shift library for ca. 200 metabolites. Intensities were obtained from signals in the spectra. For normalization, a ^1H -NMR spectrum was acquired per each sample and the total area was obtained from *MetaboLab* to normalize the 2D data for differences in cell number. To calculate the % of ^{13}C per metabolite, the normalized intensity of a certain carbon in the labelled sample was divided by the normalized intensity of the same carbon in the unlabelled sample and was multiplied by the natural abundance of ^{13}C (1.1%).

4. RESULTS

4 Results

4.1 Chapter I – Metabolic Characterization of TKTL1 in AML

4.1.1 Introduction

Synthesis of R5P is crucial to ensure the production of nucleotide building blocks for DNA duplication. As explained before, R5P can be synthesised through both branches of the PPP, mediated by G6PD on the oxidative side with the additional production of NADPH and controlled by TKT non-oxidative side, that links glycolysis to the production of R5P^{439,440}.

Increased expression of TKT and its isoform TKTL1 has been reported in different types of cancers^{54,69,70,441} and is related to malignant transformation⁴⁴² and poor prognosis^{50,443–445}. As described in chapter 1.1.5.1, the TKTL1 isoenzyme is missing 38 amino acids in the active site compared to TKT and but retains its enzymatic activity⁵³. The contribution of TKTL1 to the overall TKT activity has been proven by silencing TKTL1 in different cell lines⁴⁴². Evidence across several cancer tissues up until now have highlighted that TKTL1 is tightly linked to adaptation to hypoxia and its overexpression has been correlated with resistance to ionizing radiation and chemotherapy⁴⁴⁶ as well as to the “Warburg effect”⁴⁴⁷. For example, in glioma cells it has been demonstrated that hypoxia lead to TKTL1 induction and that its knockdown decreased flux through the PPP and resulted in cell death⁴⁴⁶. Induction of TKTL1 under hypoxia has also been observed in different cell lines of colorectal cancer (CRC) and upregulation has been demonstrated for a subset of CRC patients⁴⁴⁴. It has also been reported that hypoxia induced dramatic metabolic changes in AML cell lines, including adaptation of both lipid and glycolytic metabolism⁴⁴⁸. In addition, increased levels of TKTL1 has been reported in response to decitabine (DAC) treatment in primary acute myelogenous leukaemia (AML) samples⁴⁴⁹ and

correlated to the acquisition of tyrosine kinase inhibitor resistance in chronic myeloid leukemia⁴⁵⁰.

However, to date, there has been less exploration about the role of TKTL1 in the metabolic response triggered by hypoxia in leukaemia and detailed studies on its role during metabolic reprogramming of AML cells to hypoxia are lacking.

One of the objectives of this body of work is to untangle the functional relationship between TKTL1 expression and metabolic adaptation of AML cells to hypoxia. For this purpose, we performed a comparative analysis of hypoxia-induced changes using the THP1 cell line, an monocytic leukemic cell line established in 1980 from the blood of an AML child patient, with an *MLL* fusion⁴⁵¹. Through the use of THP1 transduced with a lentiviral vector expressing a TKTL1-specific shRNA (TKTL1^{KD}) and a wildtype control transduced with an empty vector (THP-1^{WT}), we attested the differences between them both at the transcriptional and metabolic levels. Together, our findings highlight that in response to hypoxia, THP-1 cells deeply rewire central metabolism at the transcriptomic level to sustain an enhanced consumption of glucose and glutamine and that this response is severely impaired in the cells lacking TKTL1.

4.1.2 Results

4.1.2.1 TKTL1 contributes to cell growth in normoxia and hypoxia

To characterize the role of TKTL1 in growth properties and metabolic response to hypoxia of AML cells, we initially evaluated the effect of the shRNA-mediated silencing of TKTL1 on the expression of TKTL1 and TKT genes by RT-PCR. We obtained a knockdown efficiency of $63.3\% \pm 0.03$ for TKTL1 expression whereas the TKT expression has not been significantly affected (Figure 4.1.1).

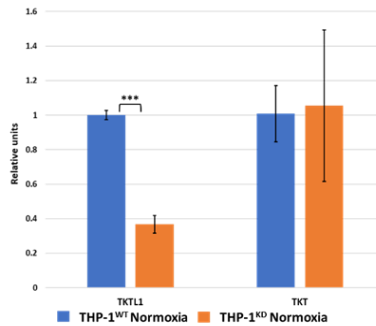


Figure 4.1.1- Effects of TKTL1 silencing on TKTL1 and TKT expression. RT-PCR performed using PPA1 as a housekeeping gene for data normalization. Decrease of TKTL1 expression determined as $63.3\% \pm 0.03$. Data showed is mean \pm SD of normalized values of $n=2$ reads of $n=3$ replicates of a single experiment. Statistically significant differences were determined by two-tailed independent sample Student's t-test: $p < 0.001$ (***).

Next, we evaluated the impact of TKTL1 knockdown on cell proliferation during hypoxia. At 96h of cell incubation, in either normoxia (O_2 at 21%) or hypoxia (O_2 at 1%), an increase in THP-1^{KD} cells duplication time with respect to THP-1^{WT} of around 25 % and 29% respectively was observed (Figure 4.1.2A-B). When compared to normoxia, hypoxia induced a 3-fold increase in duplication time of both THP-1^{wt} and THP-1^{KD} cells.

The protein content per cell was also quantified, to attest that TKTL1 silencing did not affect total cell protein content. The obtained results showed that the protein content of both cell lines in normoxia was not statistically different, which permits us to conclude that the silencing of TKTL1 is not affecting the total cell protein content (Figure 4.1.2C). Same quantification has not been possible in hypoxia due to too much dispersion (data not shown). Altogether, these results reinforce the knowledge that TKTL1 is a necessary enzyme for proliferation as reported in many previous studies, highlighting that its function is essential under normal oxygen conditions, as well as during a hypoxic state.

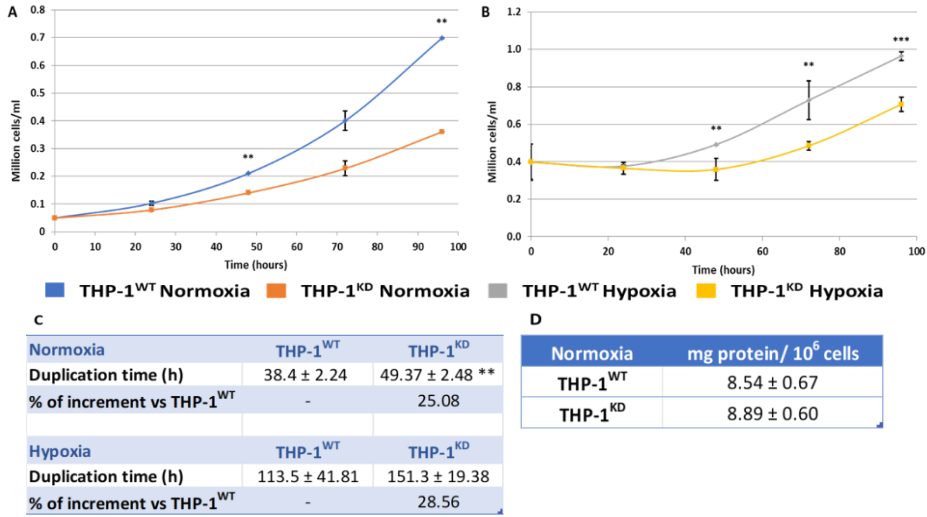


Figure 4.1.2- Effect of TKTL1 Knockdown on Proliferation. Growth curve from 0 to 96h incubation in (A) normal oxygen conditions and (B) in hypoxic conditions (1% O₂). Data shown as representative experiments, with mean ± SD for n=2 reads of n= 3 independent experiments for normoxia condition and n=2 reads of n=2 independent experiments for hypoxia condition. (C) Duplication rates calculations in both conditions. (D) Cell protein content of THP1 Control and THP1 TKTL1 Knockdown under normoxia. Statistically significant differences in all panels was determined by two-tailed independent sample Student's t-test: p<0.01 (**), p<0.001 (***).

Since it is through the synthesis of R5P and other pentoses that both branches of the PPP mainly contribute to proliferation, we determined the effect of the TKTL1 knockdown on the total enzymatic activity of TKT, G6PD and 6PGD. TKTL1 knockdown resulted in a decrease of all TKTs activity of around 45% in normoxia and 49% in the hypoxic condition and of total G6PD activity of around 65% (normoxia) and around 75% (hypoxia) (Figure 4.1.3A-B). By contrast, the knockdown did not alter significantly 6PGD activity, neither in normoxia or hypoxia (Figure 4.1.3C).

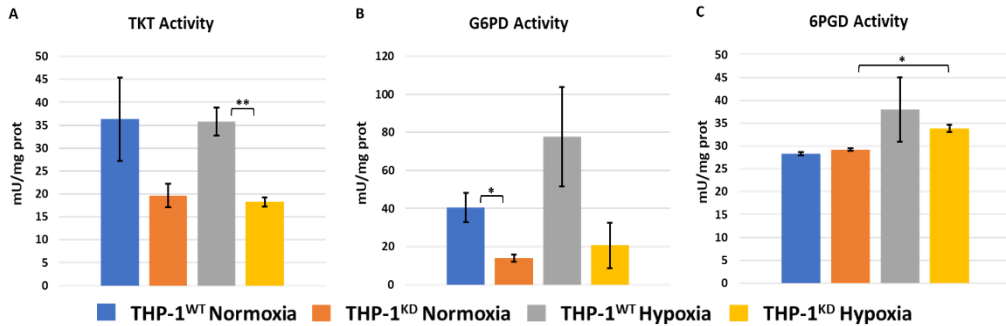


Figure 4.1.3- Changes in total enzyme activity capacity of key enzymes in pentose-phosphate pathway triggered by hypoxia. Enzymatic Activity Assays performed through spectrophotometry in normoxia and hypoxia for (A) Transketolase (TKT), (B) Glucose-6-Phosphate dehydrogenase (G6PD) and (C) 6-phospho-gluconate dehydrogenase (6PGD). Data represented is mean \pm SD for n=2 reads of n=2 replicates of n=2 independent experiments. Statistically significant differences in all panels was determined by two-tailed independent sample Student's t-test: p<0.05 (*), p<0.01 (**), p<0.001 (***). The Transketolase activity assay measures both TKT and TKTL1 activity combined, cannot disassociate.

Through transcriptomic analysis we attested if these differences in activity originated from differential gene expression. Consistent with the hypoxia-induced decrease in proliferation rates of these cells, we observed that the G6PD gene expression (quantified as Log₂ Fold-Change of hypoxia *versus* normoxia transcript levels (Log₂FC)) in hypoxia is decreased significantly for THP-1^{WT} (Log₂FC= -0,72) as well as for THP-1^{KD} (Log₂FC= -1,24) (Figure 4.4). Taking into account that G6PD is regulated by PTMs, the fact that total G6PD activity did not change could be due to post-transcriptional activation of the enzyme. In fact, it has been described that G6PD is activated by SIRT5-catalyzed deglutarylation⁴⁵². The transcriptomic results show that under hypoxic conditions, SIRT5 levels increase in both THP-1^{WT} (Log₂FC= 0,78) and THP-1^{KD} (Log₂FC= 1,08) (Figure 4.1.4). In regard to the TKT and TKTL1 genes, there was a very slight increase in gene expression induced by hypoxia which however did not result in effective activity increase, demonstrating that post-translation regulation of the PPP enzymes is occurring and dependent on TKTL1 activity.

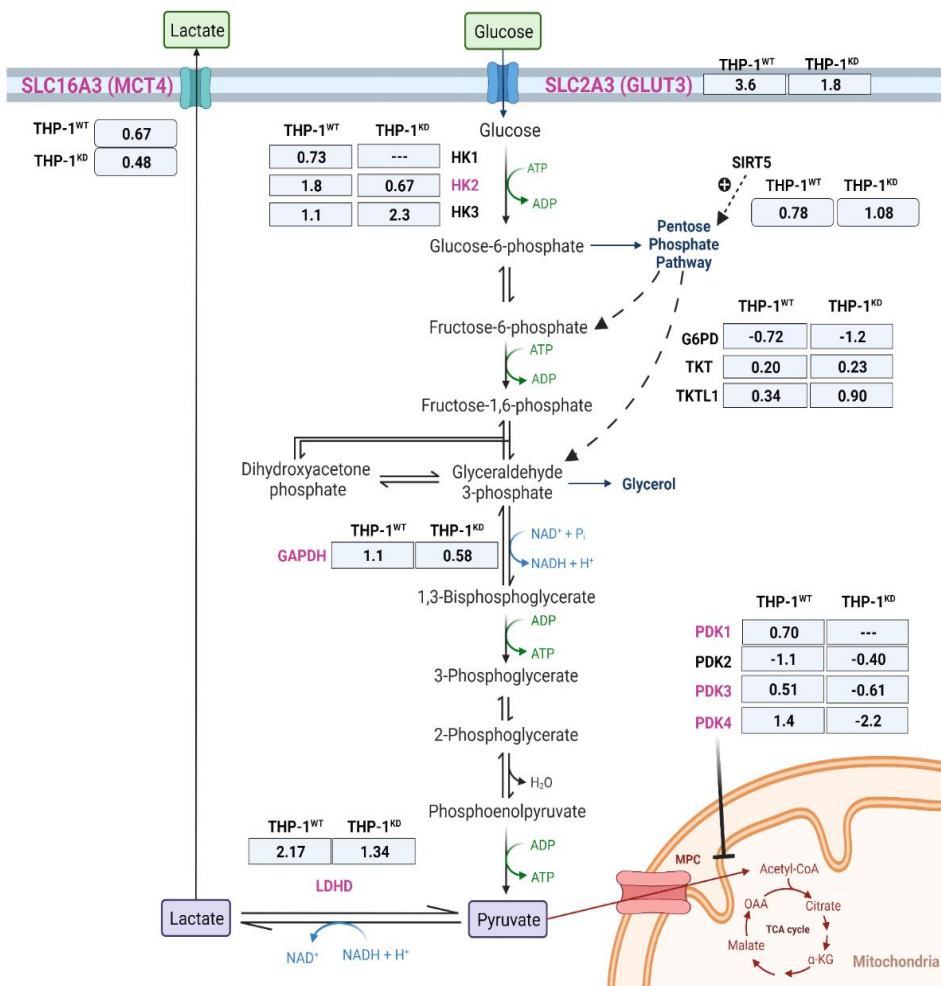


Figure 4.1.4- Gene expression changes of key enzymes and transporters between THP-1^{WT} and THP-1^{KD} in hypoxia. Significant changing genes due to TKTL1 highlighted in pink. Expression changes of normoxia versus hypoxia, indicated in log₂-Fold-change of n=3 samples per condition. Abbreviations: G6PD (glucose-6-phosphate dehydrogenase), GAPDH (glyceraldehyde-3-phosphate dehydrogenase), GLUT3 (glucose transporter 3), HK1-3 (hexokinase 1 to 3), LDHD (lactate dehydrogenase D), MCT4 (monocarboxylate transporter 4), PDK1-4 (pyruvate dehydrogenase kinase 1 to 4), SIRT5 (sirtuin 5), SLC (solute carrier family), TKT (transketolase), TKTL1 (transketolase-like 1). Created with BioRender.com.

4.1.2.2 TKTL1 is essential to trigger hypoxia-induced changes to glucose fate in THP-1 cells

A common behaviour of most cancer cells under hypoxic conditions is the increase of the glycolytic pathway flux as a metabolic adaptation to their environment. To see how this feature is dependent on TKTL1, we characterized glucose consumption and lactate production rates, under normoxia and hypoxia conditions in THP-1^{WT} and THP-1^{KD} cells. Results obtained (Figure 4.1.5A-C) showed that in normoxia, THP-1^{WT} glycolytic rates (glucose consumption and lactate production) were around 2-fold higher than in THP-1^{KD} cells. The ratio of lactate production per glucose consumption (Figure 4.1.5C) was significantly different in WT and KD cells in normoxia but not in hypoxia. Nevertheless, the increased values of around 2 for both cell lines in hypoxia indicate that under O₂ restriction, glucose is readily oxidized to lactate.

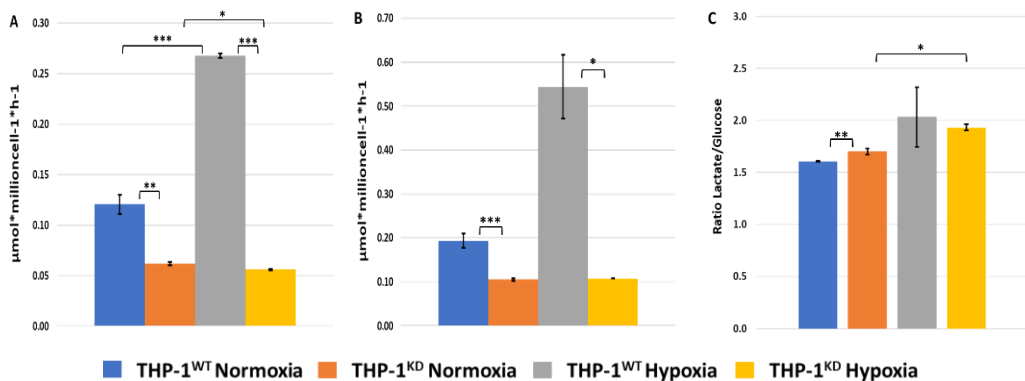


Figure 4.1.5- Loss of TKTL1 impairs hypoxia response. Consumptions and production rates measurements through spectrophotometry from cell culture media after 48h incubation, in both normoxia and hypoxia conditions. (A) Glucose consumption. (B) Lactate production. (Data represented by mean \pm SD of n=3 reads of a representative experiment per normoxia and hypoxia. (C) Ratios of lactate produced per glucose consumed. Statistically significant differences in all panels was determined by two-tailed independent sample Student's t-test: p<0.05 (*), p<0.01 (**), p<0.001 (***)).

Hypoxia triggered a metabolic switch of around 2.2-fold increase in glucose consumption and lactate production in THP-1^{WT} cells, whereas in THP-1^{KD} the glycolytic rates were not statistically different in hypoxia with respect to the normoxia measurements (Figure 4.1.5A-B). These results unveil a key role of TKTL1 in the adaptive metabolic switch to glycolysis that THP-1^{WT} cells undergo under hypoxia.

It has been well documented that the hypoxia response induction of glycolysis occurs due to increased gene expression of enzymes, transporters and growth factor receptors (identified in Figure 4.1.4)⁴⁵³⁻⁴⁶¹. We investigated if the impaired enhancement of glycolysis in response to hypoxia observed in THP-1^{KD} was due to a failing of upregulation of gene expression.

Results showed that the expression of the isoforms of hexokinase that bind to mitochondria (HK1 and HK2) and the pyruvate dehydrogenase kinase isoenzymes (PDK1, 3 and 4) were strongly up-regulated in THP-1^{WT} in response to hypoxia (see Figure 4.1.4). In the contrary, in THP-1^{KD}, hypoxic conditions induced a decrease of the expression of PDK3 and PDK4 but did not alter the expression of neither HK1 nor PDK1. Worth to note, that the hypoxia-induced increase in gene expression of the hexokinase isoenzyme HK3, that binds to the nucleus, is 2.5-fold higher in THP-1^{KD} than in THP-1^{WT}, which suggests a TKTL1-independent mechanism of regulation of the expression of this gene in hypoxia. In fact, HK3 has been described to have a role in decreasing ROS levels and has been associated with higher levels of transcription factors that regulate mitochondrial DNA content and mitochondrial biogenesis⁴⁶².

Other glycolytic enzymes and transporters that are reported to be overexpressed in hypoxia, such as GAPDH, glucose transporter SLC2A3 (GLUT3), lactate dehydrogenase D (LDHD) and lactate transporter SLC16A3 (MCT4), were found to be upregulated under hypoxic conditions in both THP-1^{WT} and in THP-1^{KD} (see Figure 4.1.4).

Measurement of total HK and GAPDH activity in THP-1^{WT} and THP-1^{KD} cells (Figure 4.1.6A-B) showed that activity was higher in THP-1^{WT} than in THP-1^{KD} in both normoxia and hypoxia conditions. In the contrary, we observed that the knock-down of TKTL1 did not affect total LDH activity and that LDH activity was increased in both THP-1^{WT} and THP-1^{KD} cells under hypoxia (Figure 4.1.6C). The results obtained are consistent with the lower K_m for glucose reported for HK3 isoenzyme, when compared to HK1 and HK2⁴⁶³. LDHD has been reported to function as a “metabolite repair enzyme” that controls metabolic damage in glycolysis by contributing to the elimination of methylglyoxal formed from another glycolytic enzyme, namely the triose-phosphate isomerase (TPI)⁴⁶⁴. Total GAPDH activity (Figure 4.1.6B) as well as GAPDH gene expression (Figure 4.1.4) increased in both cell lines, in response to hypoxia, favouring the THP-1^{WT}.

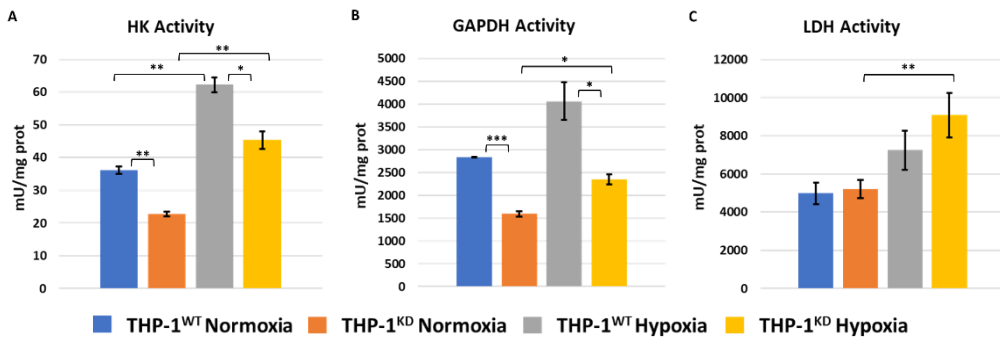


Figure 4.1.6- Changes in total enzyme activity capacity of key glycolytic enzymes triggered by hypoxia. Enzymatic Activity Assays performed through spectrophotometry in normoxia and hypoxia for enzymes (A) Hexokinase (HK), (B) Glyceraldehyde-3-Phosphate dehydrogenase (GAPDH) and (C) Lactate Dehydrogenase (LDH). Data represented is mean \pm SD for n=2 reads of n=2 replicates of n=2 independent experiments. Statistically significant differences in all panels was determined by two-tailed independent sample Student’s t-test: p<0.05 (*), p<0.01 (**), p<0.001 (***).

Altogether the gene expression profile observed provided strong evidence that the knockdown of TKTL1 impairs the metabolic switch to glycolysis in response to hypoxia, through the downregulation of PDK3 and PDK4

and the increase in the gene expression of HK3. Another factor in the hypoxic response is the use of growth factors and the increase of their receptors. We observed that hypoxia triggered a dramatic increase in Estrogen-Related Receptor b (ESRRb) ($\log_2FC= 4,6$) in THP-1^{WT} whereas ESRRb levels remain constant in THP-1^{KD}. The family of Estrogen-Related Receptors (ESRRs) has been described to mediate PDKs gene overexpression in hypoxia^{458,465}, leading to the hypothesis that TKTL1 plays a key role in the switch from OXPHOS to glycolysis, by induction of PDKs that result in PDH inhibition, in an ESRRb-mediated way.

4.1.2.3 TKTL1 is essential to trigger hypoxia-induced changes to glutamine fate in THP-1 cells

Second to glucose, glutamine is a major carbon source for energy production and anabolic processes (see chapter 1.3.4.1). It has been described that hypoxia enhances cancer cells glutamine synthesis and uptake, by increasing glutamine synthetase (GS, also known as GLUL) and the number of glutamine transporters such as SLC38A2⁴⁶⁶. To attest how TKTL1 could impact glutamine metabolism, we characterised glutamine consumption and glutamate production rates, under normoxia and hypoxia conditions in THP-1^{WT} and THP-1^{KD} cells. The results obtained showed that TKTL1 knockdown resulted in a 20-25% decrease in glutamine consumption and glutamate production under normoxia and in a 70-75% decrease under hypoxia (Figure 4.1.7A-B). When comparing the effect of this knockdown on the ratio of glucose consumption versus glutamine consumption (Figure 4.1.7C), we observed a decrease of around 20% in normoxia and of 27% in hypoxia, which indicates that the overall impact of silencing TKTL1 is more pronounced in hypoxia. In fact, the hypoxic metabolic switch triggered an increase of around 20% of glutamine consumption and glutamate production in THP-1^{WT} cells, while the THP-

1^{KD} cells in hypoxia had around 50-60 % decrease of these metabolites levels (Figure 4.1.7A-B).

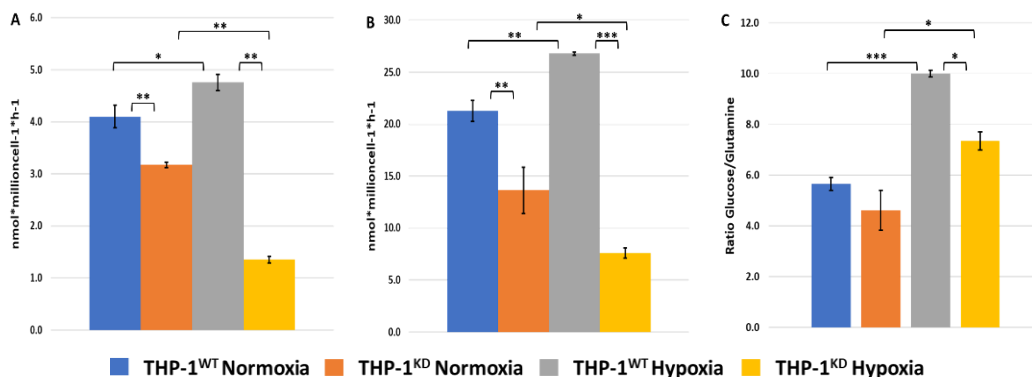


Figure 4.1.7- Loss of TKTL1 impairs hypoxia response. Consumptions and production rates measurements through spectrophotometry from cell culture media after 48h incubation, in both normoxia and hypoxia conditions. (A) Glutamate production. (B) Glutamine consumption. Data represented by mean \pm SD of n=3 reads of a representative experiment per normoxia and hypoxia. (C) Ratios of glucose consumed (depicted in Figure 4.1.5A) versus glutamine consumed. Statistically significant differences in all panels was determined by two-tailed independent sample Student's t-test: p<0.05 (*), p<0.01 (**), p<0.001 (***)

Another key characteristic of hypoxia adaptation in cancer cells is changes to expression of key glutamine metabolism genes and transporters⁴⁶⁶. We investigated if the contrary response seen in hypoxia in our results was due to a failure in the upregulation of gene expression (Figure 4.1.8). We observed that *GS* gene expression was strongly upregulated (log2FC= 1.3) and also *SLC38A2* was slightly upregulated in THP-1^{WT} whereas glutaminase (*GLS*) expression did not change. By contrast, *GLS* and *SLC38A2* were downregulated in THP-1^{KD} and the observed overexpression of *GS* (log2FC= 0.60) was 50% lower than the observed in the control cells. In addition, *SLCIA3* (glutamate-aspartate transporter) and *SLC17A7* (glutamate transporter) were strongly overexpressed under hypoxia conditions in THP-1^{WT} whereas in THP-1^{KD} were downregulated or unaltered. Altogether, the observed changes at transcriptomic level

relating to glutamine/glutamate intake and metabolism correlate with an impairment of the metabolic response induced by hypoxia due to TKTL1 knockdown.

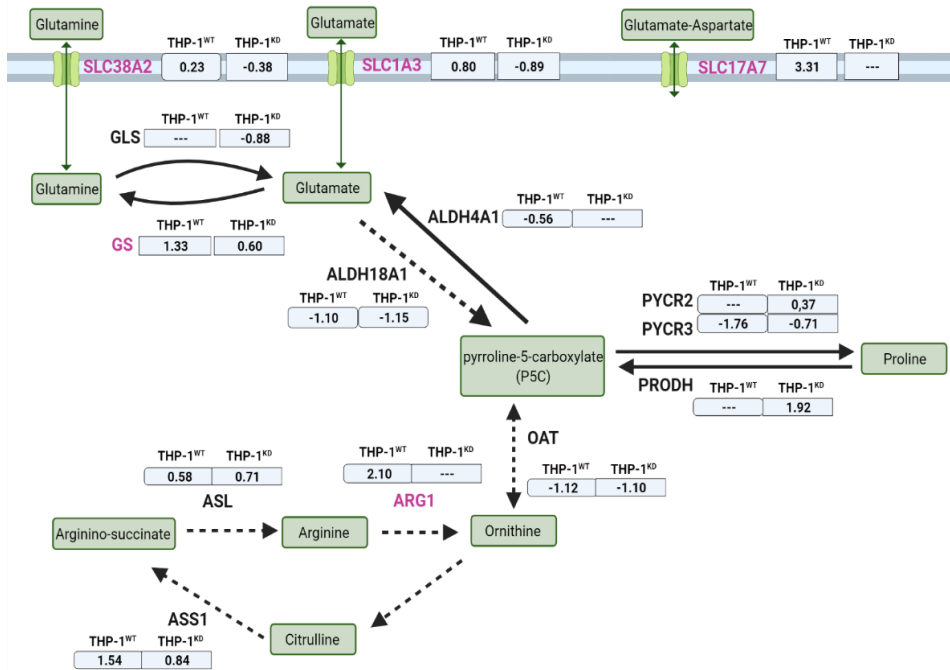


Figure 4.1.8- Gene expression alterations of key players in glutamine and glutamate metabolism and transport between THP-1^{WT} and THP-1^{KD} in hypoxia. Significant changing genes due to TKTL1 highlighted in pink. Expression changes of normoxia versus hypoxia, indicated in log₂-Fold-change of n=3 samples per condition. Abbreviations: ALDH (aldehyde dehydrogenase family), ALDH4A1 (pyrroline-5-carboxylate dehydrogenase, mitochondrial), ALDH18A1 (pyrroline-5-carboxylate synthase), ARG1 (arginase 1), ASL (Argininosuccinate lyase), ASS1 (Argininosuccinate synthase 1), GLS (glutaminase), GS (glutamine synthetase), OAT (ornithine aminotransferase), PRODH (proline dehydrogenase), PYCR2 and 3 (pyrroline-5-carboxylate reductase 2 and 3), SLC (solute carrier family). Created with BioRender.com.

4.1.2.4 TKTL1 is essential to trigger hypoxia-induced changes in amino acids fate in THP-1 cells

Besides glutamine and glutamate, we also analysed and compared THP-1^{WT} and THP-1^{KD} consumption/production of additional amino acids in normoxic and hypoxic conditions. THP-1^{WT} produced more proline and ornithine in response to hypoxia than THP-1^{KD} (Figure 4.1.9). The observed dramatic increase in gene expression of PRODH (THP-1^{KD} Log2FC=1.92), that converts proline in pyrroline-5-carboxylate (5PC), and of ARG1 (THP-1^{WT} Log2FC= 2.10), that converts arginine into ornithine, is in accordance with the observed metabolite production differences between THP-1^{WT} and THP-1^{KD}. (Figure 4.1.8). Moreover, in THP-1^{WT} we observed hypoxia inducing a slight decrease in the production of acetylorntithine (Figure 4.1.10), an intermediate metabolite between glutamate and ornithine²²⁴, which is consistent with a more active production of ornithine in the wildtype cells in hypoxia conditions. Interestingly we also observed a strong increase in the excretion of total asymmetric dimethylarginine (ADMA) in THP-1^{KD} cells (Figure 4.1.10) which is consistent with a strong overexpression of Arginine Methyltransferase 8 (PRMT8) (log2FC= 5.7). Worth to note that PRMP8 has been recently described as a key player in maintaining stress tolerance by ensuring proper ADMA levels⁴⁶⁷.

Regarding BCAAs metabolism, we observed that hypoxia significantly reduced the consumption of isoleucine (Ile) and leucine (Leu) in similar extent in both THP-1^{WT} and THP-1^{KD} and with respect to Valine (Val) we observed that the decrease in cell consumption is only statistically significant in THP-1^{WT} (Figure 4.1.9B). To complete the characterization of the BCAAs metabolism, we further looked at the gene expression of key enzymes in the BCAAs catabolism and we obtained that the expression of BCAT1 and Branched-Chain Keto Acid Dehydrogenase B

(BCKDHB) genes decreased under hypoxia in both cell lines. The inhibition on the expression levels of these enzymes was around 1.6-fold higher in THP-1^{KD} than in THP-1^{WT} (Figure 4.1.11) which is consistent with the observed higher decrease in Ile and Leu consumption induced by hypoxia in THP-1^{KD}.

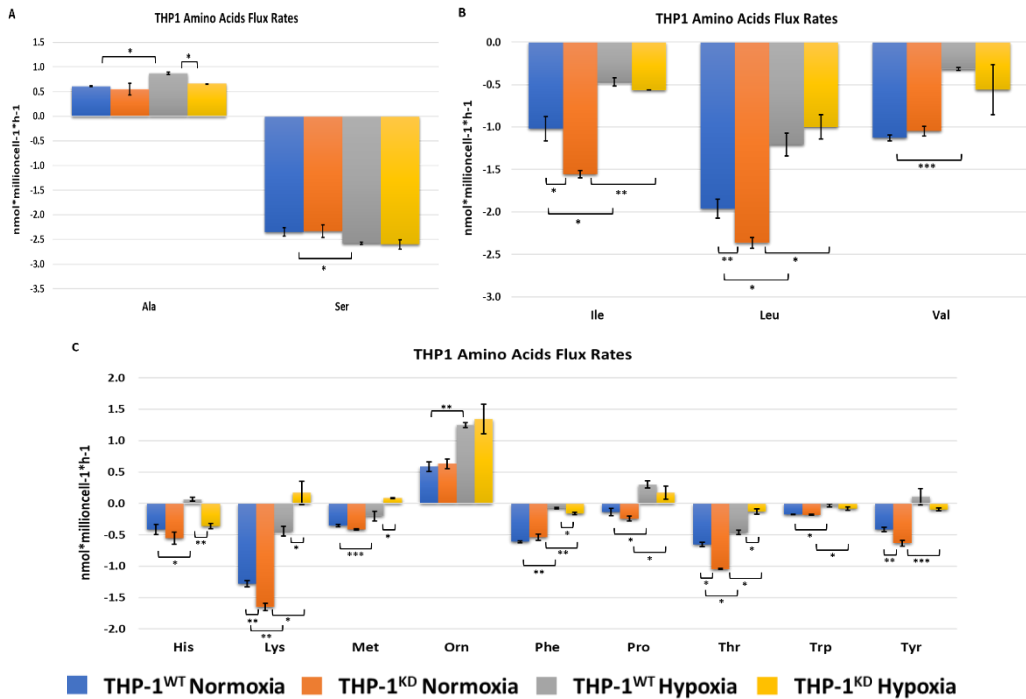


Figure 4.1.9- Flux rates of specific metabolites in extracellular media in normoxia and hypoxic conditions. (A) Alanine and Serine flux rates. (B) Branched-chain amino acids flux rates (Ile, Leu and Val). (C) Histidine, Lysine, Methionine, Ornithine, Phenylalanine, Proline, Threonine, Tryptophan and Tyrosine. Consumption and production rates obtained through Absolute 180 IQ Kit from Biocrates. Data represented is mean \pm SD for n=3 reads of n=3 samples of a single experiment. Statistically significant differences were determined by two-tailed independent sample Student's t-test, with p-values of $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***)

SLC7A8, an antiport transporter that intakes BCAAs as well as other neutral amino acids (tyrosine, phenylalanine, tryptophan, methionine and glutamine) through obligatory exchange mechanism, as well as

SLC7A7⁴⁶⁸, another antiport transporter that similarly exchanges neutral for cationic amino acids, were dramatically overexpressed in response to hypoxia only in THP-1^{WT} (Figure 4.1.11).

Methionine and Tyrosine consumption decreased under hypoxia, but the changes were statistically significant only in THP-1^{KD} cells. In the contrary, with respect to histidine, the decrease in consumption induced by hypoxia was significant only for THP-1^{WT}.

Lysine, threonine and phenylalanine consumption decreased significantly under hypoxia in both cell lines but the changes observed for lysine and threonine uptake were much larger in THP-1^{KD} (Figure 4.1.9C), whereas with respect to phenylalanine, the observed changes were larger in THP-1^{WT}. Worth to note that the consumption of α -amino adipic acid (α AAA), a biogenic amine generated in the intermediate steps of the catabolism of lysine^{279,284}, decreased under hypoxia in both cell lines (Figure 4.1.10). But the observed decrease was lower for THP-1^{KD}, consuming significantly more α AAA in hypoxia when compared to THP-1^{WT}. In normoxia conditions there were no significant differences in α AAA consumption between the cell lines.

Tryptophan uptake was slightly decreased in both cell lines under hypoxia (Figure 4.1.9C). Tryptophan can be metabolized to kynurenine, with indoleamine 2,3-dioxygenase 2 (IDO2) being the rate limiting step of this pathway, or to serotonin through TPH1 and TPH2^{198,312,314}. Both pathways produce biogenic amines and other active metabolic intermediates with signalling functions. Gene expression of IDO2 was strongly enhanced under hypoxia only in THP-1^{WT} which is consistent with a decreased consumption of kynurenine of these cells (Figure 4.1.10). On the contrary, kynurenine consumption was strongly enhanced under hypoxia in THP-

1^{KD} cells which is consistent with the inability of the KD cells to enhance IDO2 in response to hypoxia.

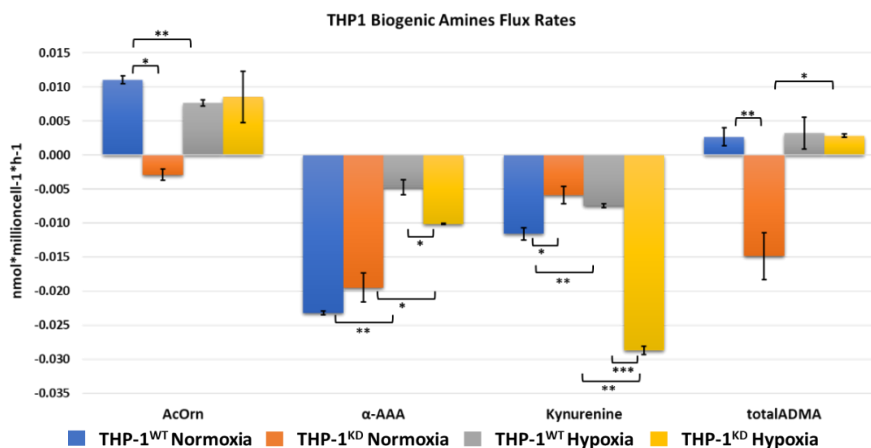


Figure 4.1.10- Flux rates of specific metabolites in extracellular media in normoxia and hypoxic conditions. Acetylornithine (AcOrn), alpha-aminoadipic acid (α AAA), kynurenine and total asymmetric dimethylarginine (total ADMA) flux rates. Consumption and production rates obtained through Absolute 180 IQ Kit from Biocrates. Data represented is mean \pm SD for n=3 reads of n=3 samples of a single experiment. Statistically significant differences were determined by two-tailed independent sample Student's t-test, with p-values of p< 0.05 (*), p< 0.01 (**), and p<0.001 (***)

Serine consumption was also slightly enhanced in hypoxia in both cell lines, but the increase was only statistically significant in THP-1^{WT} which is in accordance with the observed bigger increase in PHGDH and SHMT2 gene expression in THP-1^{WT} (Figure 4.1.11).

Secretion of alanine was also enhanced under hypoxia but only for THP-1^{WT} (Figure 4.1.9A), which is consistent with the enhanced glucose and glutamine consumption observed previously with respect to THP-1^{KD}.

Lastly, we measured intracellular amino acid content to see if the alterations to amino acids efflux/consumption pattern observed for THP-1^{KD} under hypoxic conditions, resulted in different net-total amino acids content intracellularly. The THP-1^{WT} cells had three times less aspartate and five times more serine in hypoxia whereas these metabolites levels did not

significantly change intracellularly under hypoxia in THP-1^{KD} cells (Appendix I Table 8.1-A). The observed THP-1^{WT} decrease of aspartate intracellularly under hypoxia is consistent with the reported evidence across different tumours of the negative correlation between hypoxia and aspartate intracellular content⁴⁶⁹.

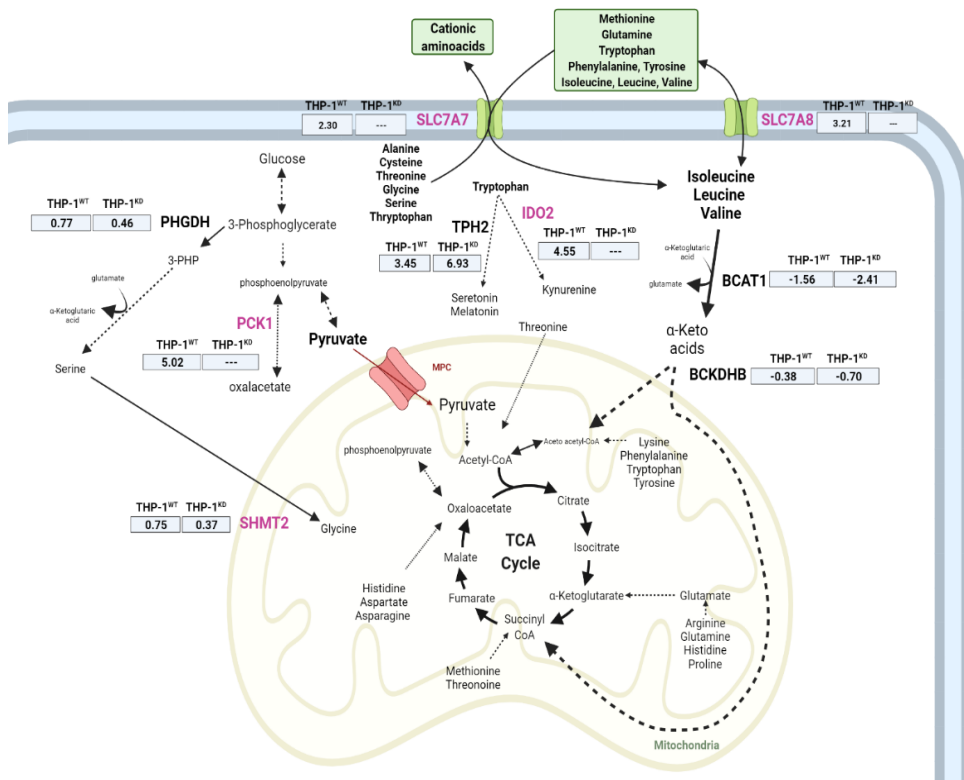


Figure 4.1.11- Gene expression changes of amino acids transport and catabolism between THP-1^{WT} and THP-1^{KD} in hypoxia. Significant changing genes due to TKTL1 highlighted in pink. Expression changes of normoxia versus hypoxia, indicated in log2-Fold-change of n=3 samples per condition. Abbreviations: BCAT1 (branched-chain amino acid transaminase 1), BCKDHB (branched-chain keto acid dehydrogenase B), IDO2 (indoleamine 2,3 dioxygenase 2), PCK1 (phosphoenolpyruvate carboxykinase 1), PHGDH (phosphoglycerate dehydrogenase), SHMT2 (serine hydroxymethyltransferase 2), SLC (solute carrier family), TPH2 (tryptophan hydroxylase 2). Created with BioRender.com.

The rise in intracellular serine content triggered by hypoxia in THP-1^{WT} together with the previously observed increase of serine consumption (Figure 4.1.9A) could be related with the use of this amino acid for pyrimidine synthesis and cell proliferation under hypoxia. In fact, it has been described that serine synthesis through PHGDH is crucial to ensure appropriate nucleotide levels⁴⁷⁰ and a similar effect through PCK1, a key player that drives pyrimidine biosynthesis under hypoxia⁴⁷¹. Worth to note that PCK1 gene expression was dramatically increased (log2FC=5.0) under hypoxia in THP-1^{WT} whereas hypoxia did not trigger any statistically significant change in THP-1^{KD} (Figure 4.1.11).

The fact that we did not observe these changes in intracellular amino acids content in hypoxia in THP-1^{KD} corroborates the importance of TKTL1 in triggering the switch to enhanced glycolysis and consequent adaptation of amino acids metabolism to support such response. Overall, this new role of TKTL1, beyond its enzymatic function on the non-oxidative branch of the PPP unveiled in this study, opens new opportunities for the design of combined therapies targeting TKTL1 and exploiting the impaired adaptation of these cells to hypoxic stress.

4.1.3 Discussion

Leukaemia is a type of cancer characterized by a rapid expansion of immature haematopoietic cells. This uncontrolled proliferation can arise from many distinct conjugations of mutations, whose compiled synergy drives tumorigenesis. The resulting phenotype presents a different metabolism from the surrounding normal cells, granting it advantages in proliferation and survival in the hypoxic conditions of the haematopoietic niche.

Transketolase-like 1 is a key enzyme of metabolism, linking glycolysis to the production of nucleotides, a necessary component for *de novo* synthesis of RNA and DNA, especially in rapidly proliferating cells. Although the

full scope of its functions is still unknown to this day, a clear correlation of its expression with cancer malignancy and poor prognosis has been established across many types of cancer. One such type is AML, where the putative role of TKTL1 overexpression remains to be properly pursued.

This study combined target metabolomics and transcriptomics profiling for a comparative analysis of a stable acute monocytic leukemic cell line with a TKTL1 knockdown and a wildtype counterpart, to discern its role on the metabolic adaptation under the state of hypoxia.

The main metabolic changes triggered by the hypoxic conditions, such as increased glucose and glutamine consumptions, are impaired by the TKTL1 knockdown. This difference occurs at the level of gene expression and protein regulation, resulting in less enzymatic activity, lower number of membrane transporters and a total decrease in utilization of glucose and glutamine. The decrease in glycolysis flux reflects in an impaired Warburg effect without TKTL1, with less lactate production and also an impaired inhibition of the PDH complex by the inability to properly upregulate the expression of PDKs isoenzymes and shift the metabolism from OXPHOS into anaerobic glycolysis.

Subsequently, we observed alterations of the use of amino acids in the metabolic pathways that feed the glutamine metabolism and supply substrates for pyrimidine synthesis, with the knockdown of TKTL1 reducing these behaviours in hypoxia. Specifically, the import of aspartate, a growth-limiting metabolite in hypoxia, and serine, which feeds the pyrimidine synthesis and One-Carbon metabolism. Altogether, this unveiled the role of TKTL1 in central metabolism, beyond the enzymatic function in the PPP.

Of note is that in response to TKTL1 knockdown, the cells in hypoxia initiated a compensatory mechanism in order to survive. Increased gene expression of SIRT5 stimulates the regulation of G6PD through post-translational modifications in order to maintain its activity in hypoxia.

Increased use of the isoenzyme HK3 and upregulation of PRMT8 are clear responses to ROS increase, since both have been reported to mediate mitochondrial biogenesis and stress tolerance, respectively.

Therefore, our results reveal that the role of TKTL1 in the adaptation to hypoxia is essential for the coordination of central metabolism to trigger the Warburg effect, of amino acid catabolism to maintain the glutamine metabolic pathways but also aiding on oxidative stress regulation.

Further studies should be developed to better understand the mechanisms involved in this new facet of TKTL1 functions on metabolism. Additional investigations of the changes identified here on other AML cell lines and primary patient-derived cells could reveal new approaches for therapies that combine targeting TKTL1 and one of the “weaknesses” that arise from its knock down.

4.2 Chapter II – Metabolic Characterization of TET2 loss of function in a CML cell model

4.2.1 Introduction

In the previous chapter we observed how TKTL1, an isoenzyme of TKT of the non-ox PPP plays a critical role on the switch to glycolysis and glutamine dependency of THP-1 cells to a severe hypoxia setting. Our results highlighted the need to investigate under hypoxic conditions the metabolic role of enzymes that are induced or silenced by the hypoxic response, to unveil new functions beyond the canonical enzymatic role as reaction catalysers.

In this chapter we investigated the role of TET2 in the metabolic adaptation of leukaemia cells to hypoxia. Leukemogenesis arises from many distinct mutations during the haematopoietic process. One of the most prevalent across multiple types of leukaemia is TET2, an epigenetic eraser that is involved in gene expression processes during haematopoietic differentiation (see chapter 1.1.5.2) and whose activity is proven to be directly influenced by metabolites of the TCA Cycle^{192,472}. Moreover, it has been widely demonstrated that hypoxia-induces TET2 loss of activity⁴⁷³. Here, taking into account that TET2 activity depends on α -ketoglutarate (α KG) as cofactor and that succinate and fumarate affect TET function by binding site competition, we questioned if the well documented loss of TET activity induced by hypoxia will impact cell metabolism, providing an advantage in metabolic adaptation to hypoxia.

To perform a proof of concept on the impact of the loss of TET2 activity on metabolic adaptation triggered by hypoxia we chose the dual cell lines model HAP1-Wild Type (Parental) and HAP1-TET2 knockout (TET2 KO). The HAP1 cell line is a near-haploid cell line, created from a KBM7 Male Chronic Myelogenous Leukaemia. It has lost most chromosome pairs, except for chromosome 8 and 15. Given this near-haploid nature, it is an

excellent CML cell line for genetic studies, with any mutation or genetic abnormality manifesting as phenotype⁴⁷⁴⁻⁴⁷⁶. It was chosen for that characteristic as the cell model to study the metabolic effects of having a simulated loss-of-function mutation in TET2 (see chapter 3.1). This is because most mutations of TET2 occur in only one allele and it is enough to stop TET2 activity overall.

4.2.2 Results

4.2.2.1 TET2 loss does not affect cell growth or glycolytic dependency

The first step in the characterization of the HAP1 cell lines was to attest how the knockout of TET2 was affecting cell growth, in both normoxia and hypoxia (Figure 4.2.1A). When comparing to the Parental cells, the knockout cells maintain a similar proliferation in both normoxia and hypoxia, with no significant differences arising. The similarity in cell numbers also translates into protein content, with the only change being due to hypoxia switch (Figure 4.2.1B).

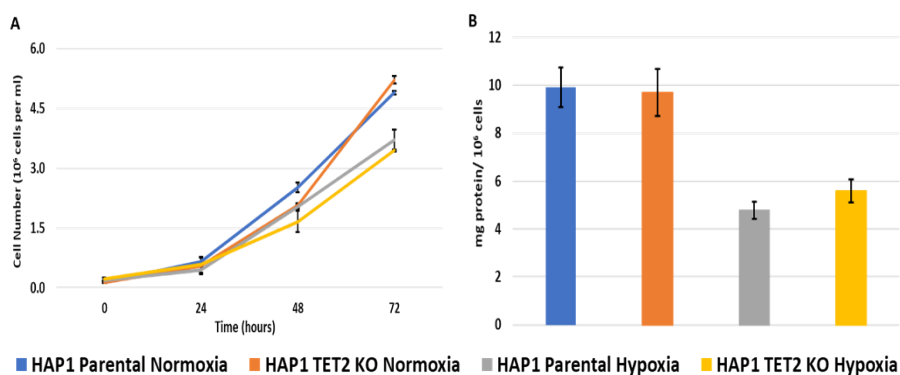


Figure 4.2.1- Effect of TET2 Knockout on Proliferation. Growth curves from 0 to 72h, in normoxia and hypoxia (A). Duplication times (hours) are: Parental Normoxia=25 ± 3.5; TET2 KO Normoxia=25 ± 3.4; Parental Hypoxia=22 ± 1.3 and TET2 KO Hypoxia=26 ± 0.5. Data shown as mean ± SD for n=2 reads of n=2 experiments. (B) Cell protein content of HAP1 Parental and TET2 Knockout under normoxia and hypoxia.

Next, we verify if in spite the similarities in duplication time, the loss of TET2 would translate into differences in cellular metabolic dependency on glucose/glutamine metabolism (Figure 4.2.2**Erro! A origem da referência não foi encontrada.**). First, we measured glucose consumption and lactate production in both HAP1 Parental and HAP1 TET2 KO cell lines incubated under either normoxic or hypoxic conditions. The consumption of glucose of TET2 KO was the same in normoxia than in hypoxia whereas Parental cells consume less glucose than TET2 KO cells but increase glucose consumption in response to hypoxia up to the same levels observed in Parental cells. (Figure 4.2.2A). The trend of glucose consumption is mirrored in the lactate production (Figure 4.2.2B).

Interestingly, the lactate produced per glucose consumed ratio (ratio lactate/glucose) obtained for each cell line under the different incubation conditions (normoxia or hypoxia) is around 2 which indicates that both cell lines rely strongly in direct cell glucose intake into lactate production even in normoxia conditions (Figure 4.2.2C).

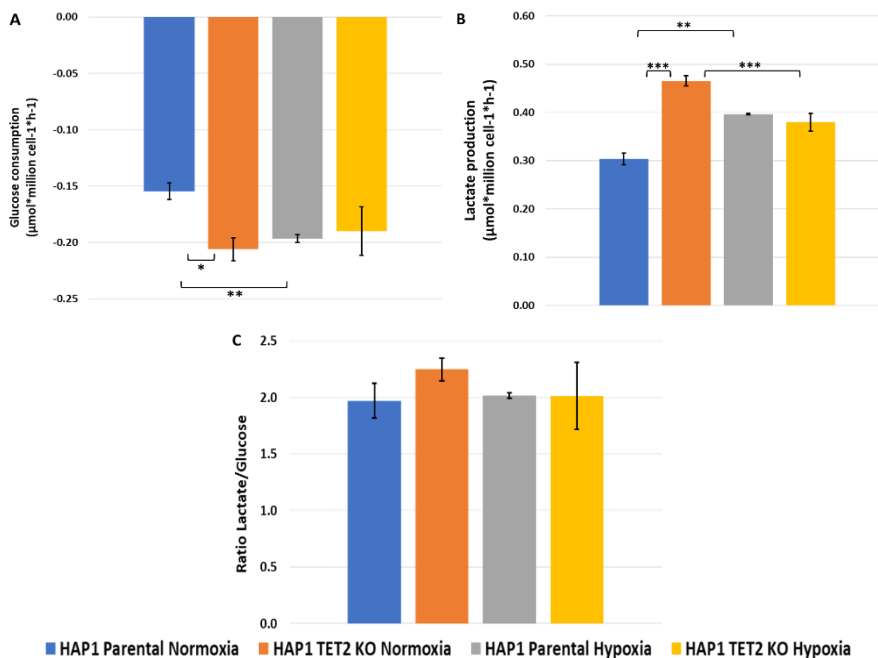


Figure 4.2.2- Loss of TET2 impact on Glucose and Lactate Metabolism. Consumption and production rates of glucose and lactate were determined after 48h incubation, in both normoxia and hypoxia conditions, as described in section 3.3. (A) Glucose consumption. (B) Lactate production. (C) Ratio of lactate produced per glucose consumed (Lac/Glc), determined by dividing the glucose consumption flux rate by lactate production flux rate. Data represented is mean \pm SD of n=3 reads of a representative experiment per condition. Statistically significant differences in all panels was determined by two-tailed independent sample Student's t-test: p<0.05 (*), p<0.01 (**), and p<0.001 (***).

Given it has been widely described that HK, GAPDH, PK and LDH are involved in the metabolic response to hypoxia of different cancer cells, we attested if the absence of TET2 would affect the total enzyme activities of these key glycolytic enzymes (Figure 4.2.3). We obtained an increase of activity levels of HK, GAPDH and PK, in response to the hypoxia challenge that was more pronounced in the Parental cell line (Figure 4.2.3A, B and C) which is in accordance with the increase in glucose intake observed in Parental cell line in response to hypoxia (see Figure 4.2.2A).

Altogether, the increase on glucose consumption and the more dramatic increase in HK, GAPDH and PK observed in Parental cells suggest that glucose-6-phosphate rerouting to pentose-phosphate pathway (PPP), operating as a cycle to generate NADPH, can be higher in Parental cells than in TET2 KO cells.

Worth to note that lactate dehydrogenase activity increase significantly under hypoxia in TET2 KO cells in spite there is not a net increase in the lactate production nor in the lactate/glucose ratio which is indicative of a different metabolic reprogramming due to the loss of TET2 (Figure 4.2.3D) suggesting the possibility that part of the lactate generated in the cytosol, either from glucose or from other sources, is recycled back into pyruvate.

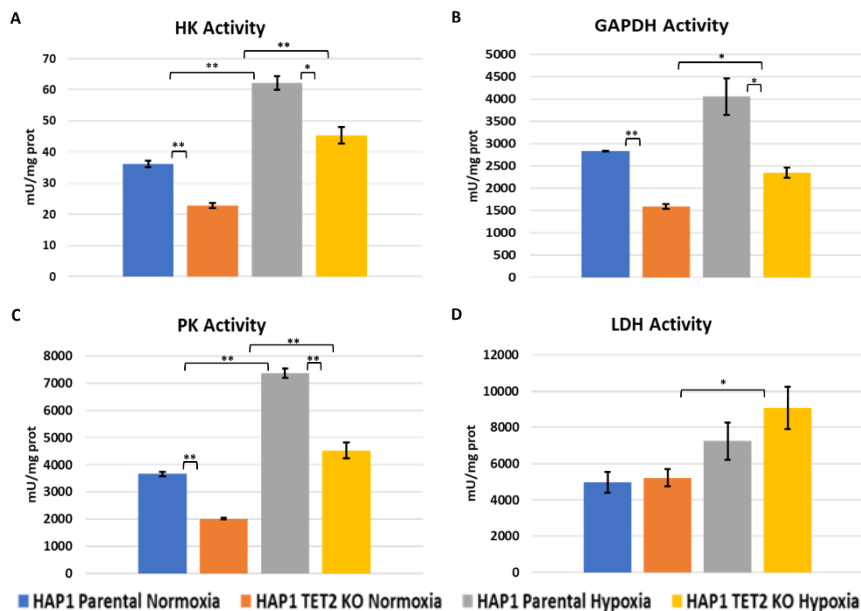


Figure 4.2.3- Impact of loss of TET2 on activity of glycolytic enzymes. Enzymatic Activity Assays performed through spectrophotometry in normoxia and hypoxia for enzymes: (A) Hexokinase (HK), (B) Glyceraldehyde-3-phosphate Dehydrogenase (GAPDH), (C) Pyruvate Kinase (PK) and (D) Lactate Dehydrogenase (LDH). Data represented is mean \pm SD for n=2 reads of n=2 independent experiments. Statistically significant differences in all panels was determined by two-tailed independent sample Student's t-test: p<0.05 (*), p<0.01 (**), p<0.001 (***)

To investigate more in depth if the differences in TET2 KO cells adaption to hypoxia can be due a different turnover of lactate and ribose pools, we performed a further characterization using ^{13}C -based metabolomics techniques.

To further explore glucose and lactate metabolism differences in Parental and TET2 KO cells, we incubated the cells with media containing U- ^{13}C -glucose and subsequently analysed the incorporation of ^{13}C into pyruvate and lactate using ^{13}C -based metabolomics techniques, under normoxia and hypoxia conditions. In brief, labelling contribution from glucose to pyruvate and lactate was analysed both intra- and extracellularly, using GCMS and NMR (methods in chapters 3.10.1 and 3.10.2, respectively) taking into account that metabolism of U- ^{13}C -glucose through glycolysis results in U- ^{13}C -pyruvate (pyruvate m3) and subsequent conversion of U- ^{13}C -pyruvate through LDH reaction results in U- ^{13}C -lactate (lactate m3). The obtained ^{13}C -label distributions for both metabolites showed a pattern of higher incorporation of ^{13}C when TET2 is lost, in both normoxia and hypoxia (see Figure 4.2.4). Since intracellular PK activity and extracellular lactate excretion are lower but LDH is higher in TET2 KO cells under hypoxia when comparing to Parental cells, the obtained labelling profile is indicative of a higher recycling of intracellular lactate-pyruvate in TET2 KO cells.

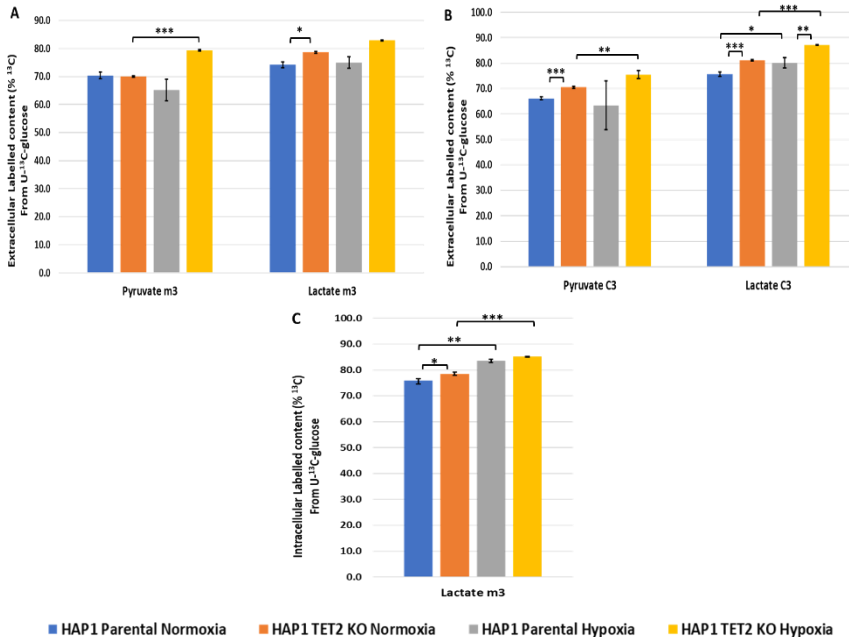


Figure 4.2.4- ¹³C-Labelling of Pyruvate and Lactate in HAP1 Parental and HAP1 TET2 KO cells incubated with U-¹³C-glucose. Measurement of ¹³C-labeled glucose contribution to ¹³C labelled pyruvate and lactate after 24h incubation with 22,5mM U-¹³C-glucose. (A) GCMS analysis of labelled extracellular Pyruvate and Lactate from media. m3 indicates that the metabolite is ¹³C uniformly labelled. Data represented is mean ± SD for n=2 reads of n=3 samples of a single experiment. (B) ¹³C-filtered ¹H-NMR spectroscopy analysis of labelled extracellular Pyruvate and Lactate. C3 refers to the carbon in position 3. Data represented is mean ± SD for n=1 reads of n=3 samples of a single experiment. (C) GCMS analysis of labelled intracellular Lactate. Data represented is mean ± SD for n=2 reads of n=3 samples of a single experiment. Statistically significant differences in all panels were determined by two-tailed independent sample Student's t-test: p< 0.05 (*), p< 0.01 (**), p<0.001 (***).

4.2.2.2 TET2 loss affects use of G6P and flux of Pentose Phosphate Pathway

Parallel to glycolysis and using its first product G6P is the Pentose Phosphate Pathway. G6PD is the first enzyme and the rate-limiting reaction of the oxidative branch of PPP and it has been recently described that G6PD activity⁴⁷⁷, beyond its canonical

role in ox-PPP. In brief, it has been recently reported that G6PD inhibition in hypoxic conditions resulted in α KG increase and SAM (a substrate for methylation reactions, see chapter 1.2.1) decrease playing a key role in regulating substrates for DNA methylation. To assess if TET2 knockout impacts ox-PPP either in normoxia or hypoxia, we next characterized the total enzyme activities of G6PD and 6PGD (a following enzyme in the ox-PPP). The results obtained showed that G6PD activity is dramatically decreased by the loss of TET2, in both normoxia and hypoxia whereas the following dehydrogenase of the ox-PPP only slightly increases when switching to hypoxia in TET2 KO cells (Figure 4.2.5B). Such impact of TET2 knockdown on G6PD activity could be related with the recently unveiled putative role of G6PD in regulating substrates for DNA methylation⁴⁷⁷ and will deserve further studies beyond the scope of the present work.

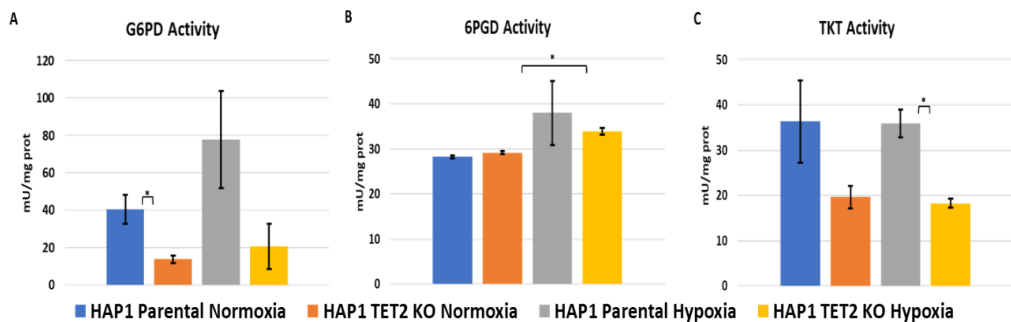


Figure 4.2.5- Decrease of key enzymatic activities of PPP due to loss of TET2. Enzymatic Activity Assays performed through spectrophotometry in normoxia and hypoxia for enzymes: (A) Glucose-6-phosphate Dehydrogenase (G6PD), (B) 6-phosphogluconate Dehydrogenase (6PGD) and (C) Transketolase (TKT). Data represented is mean \pm SD for n=2 reads of n=2 independent experiments. Statistically significant differences in all panels was determined by two-tailed independent sample Student's t-test: p<0.05 (*), p<0.01 (**), p<0.001 (***)

To also assess the impact of TET2 silencing on non-ox-PPP, we next analysed global TKT enzyme activity (Figure 4.2.5C) and found that

whereas hypoxia did not significantly alter TKT activity levels, knockout of TET2 caused a 35% reduction in total TKT activity. The observed result is in accordance with the fact that loss of TET2 results in higher degrees of DNA methylation⁴⁷⁸ and with the reported increase of TKT expression as a result of its promoter hypomethylation identified in hepatocellular carcinoma⁴⁷⁹. Worth to note that the fact that in Parental cells we observed a hypoxia-triggered increase in G6PD, which is in accordance with the observed increase on glucose consumption that prompt us to hypothesize a higher activity of PPP operating as a cycle. Thus the higher activity in hypoxia of G6PD, which is the key enzyme controlling PPP flux¹⁵⁷⁻¹⁵⁹, will result in an increased rerouting of glucose-6-phosphate through oxidative branch of PPP that will render NADPH and pentose-phosphate with the loss of one CO₂ from each glucose that enters the pathway. The excess of pentose-phosphate generated will return back to glycolytic intermediate metabolites, through non-oxidative PPP contributing to maintain the glucose/lactate optimal balance in hypoxia in Parental cells.

Next, through ¹³C-based metabolomics experiments in which we incubate the Parental and TET2 KO cells with U-¹³C-glucose (methods in chapter 3.10), we investigated how the G6P substrate was traveling from glycolysis to ribose pools or to other pathways. At the level of total ¹³C-label incorporation in ribose, we observed that TET KO cells display a lower percentage of ¹³C-labelled ribose both in normoxia and hypoxia conditions (Figure 4.2.6A), which is indicative of a lower turnover of the ribose pool and is in accordance with the lower G6PD and TKT activities reported in TET2 KO cells. Moreover, both cell lines display a decrease in ¹³C-labelled ribose intracellular content in response to hypoxia challenge being more pronounced for TET2 KO cells than for Parental cells (Figure 4.2.6C). Altogether these results demonstrated that either in normoxia or in hypoxia conditions, TET2 KO appears to impact PPP at both the levels of total

G6PD and TKT activities and the turnover of intracellular ribose metabolite pool.

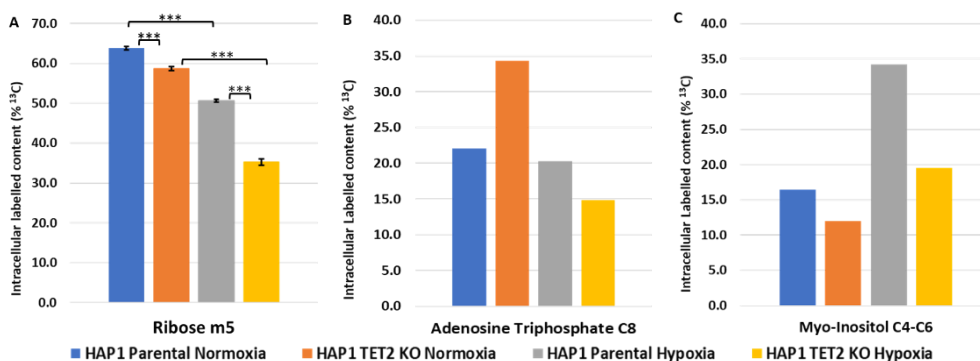


Figure 4.2.6- Loss of TET2 influences different use of PPP substrates. Measurement of glucose contribution to Myo-inositol, Adenosine Triphosphate (ATP) and Ribose after 24h incubation with 22,5mM U- ^{13}C -glucose. **(A)** GCMS analysis of labelled intracellular Ribose m5. Data represented is mean \pm SD for n=2 reads of n=3 samples of a single experiment. Statistically significant differences were determined by two-tailed independent sample Student's t-test: $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***). **(B)** ^1H - ^{13}C -HSQC NMR spectroscopy analysis of labelled intracellular ATP C8 and Myo-Inositol C4-C6 **(C)**. ^{13}C percentages were calculated as a ratio of the labelled and unlabelled sample and multiplied by the natural abundance of ^{13}C (1.1%). Data represented is n=1 read of n=1 sample of a single experiment. Statistical analysis could not be applied due to insufficient replicates.

Through NMR-based techniques, also using U- ^{13}C -glucose as a tracer, we also quantified the labelling of ATP (labelling in carbon C8 - one of the carbons of the ribose ring). Results showed that in normoxia TET2 KO cells display a higher percentage of ^{13}C -labelling in C8 of ATP molecule in (34.3%) than Parental cells (22%) (Figure 4.2.6B). By contrary, under hypoxic conditions we observed a strong decrease in ^{13}C -labelling in TET2 KO cells (14.9% of ATP C8) whereas Parental cells maintain a similar percentage of ^{13}C labelling that in normoxia (20.3% of ATP C8) (Figure 4.2.6B). The observed changes are also indicative of an altered ribose pool turnover in TET2 cells and evidenced that TET2 KO impact the metabolic

reprogramming triggered by hypoxia through altered PPP enzyme activities that result in an altered ribose-phosphate pool turnover.

Through NMR-based techniques, also using U-¹³C-glucose as a tracer, we identified also an increased ¹³C-labelling of Myo-Inositol, only in Parental cells in response to hypoxia (Figure 4.2.6C). Myo-Inositol is an important metabolite for the synthesis of cell signalling molecules (such as Phosphatidylinositol Phosphates, known as PI3P) and many nuclear functions, such as chromatin remodelling, gene transcription and DNA repair of double-strand breaks⁴⁸⁰⁻⁴⁸³. The fact that we see less labelling of this important precursor of Phosphatidylinositol Phosphates synthesis is indicative of an altered response to hypoxia of TET2 KO cells.

4.2.2.3 TET2 loss affects ROS homeostasis

Less G6PD activity directly affects the redox capacity of the cell, through less NADPH production and less reduction of GSSG. This in turn affects the detoxification of reactive oxygen species (ROS).

As such, we checked the ROS levels that showed lower levels on TET2 KO cells in normoxia (Figure 4.2.7). When switching to hypoxia, a rise of ROS levels is expected but the loss of TET2 leads to levels far above the Parental cell line.

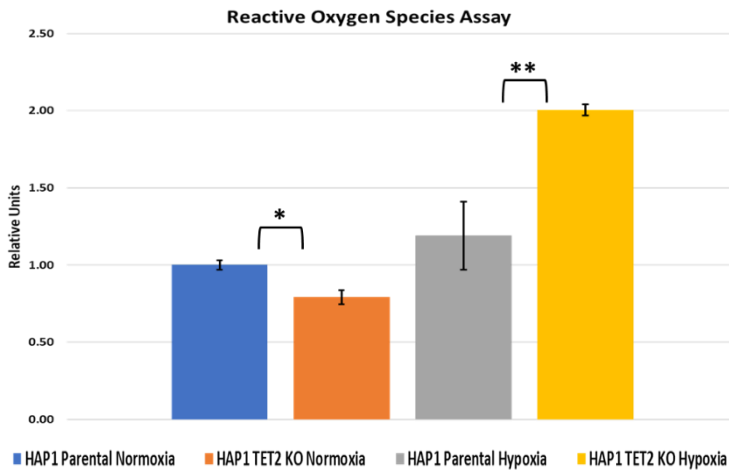


Figure 4.2.7- Loss of TET2 leads to increase of ROS levels. Flow cytometry gate count used was 5000 cells. Data represented is mean \pm SD for n=5 samples of n=2 experiments. Statistically significant differences were determined by two-tailed independent sample Student's t-test, with p-values of $p < 0.05$ (*) and $p < 0.01$ (**).

Investigations if this ROS production arises from mitochondrial activity showed that the hypoxia switch drives a similar decrease of oxygen consumption rates (OCR) (Figure 4.2.8A). This is a result of the scarceness of oxygen in this condition, regardless of the TET2 status of the cells.

As for the extracellular acidification rates (ECAR) from glycolytic activity and other non-glycolytic phenomenon, there was no significant change from the absence of TET2, only the influence of the hypoxic setting (Figure 4.2.8B).

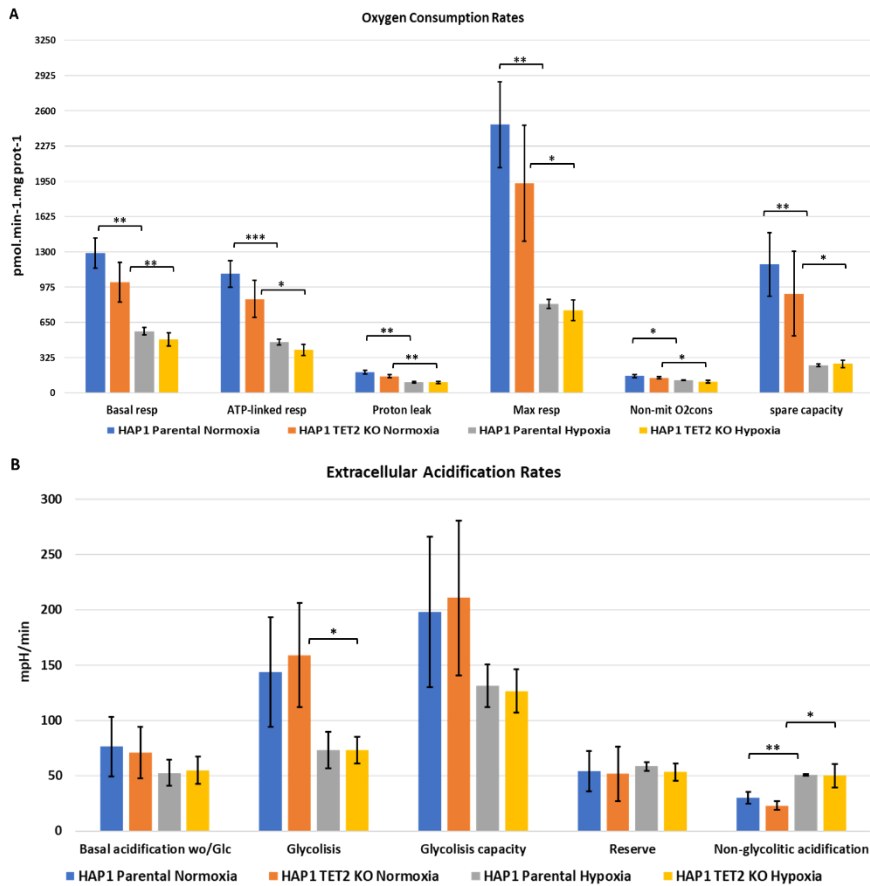


Figure 4.2.8- Loss of TET2 effects over Mitochondrial Activity. Seahorse assays performed using Seahorse XFe24, under Cell Mito Stress Assay and Glycolytic Rate Assay established protocol (Agilent ©). Oxygen Consumption Rate (OCR) assay (A), Extracellular Acidification Rate (ECAR) assay (B). Data represented is mean \pm SD for n=5 reads of n=3 experiments. Statistically significant differences were determined by two-tailed independent sample Student's t-test, with p-values of $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***)

As for the extracellular acidification rates (ECAR) from glycolytic activity and other non-glycolytic phenomenon, there was no significant change

from the absence of TET2, only the influence of the hypoxic setting (Figure 4.2.8B).

Next, the Crabtree Effect was quantified from the measurement of the OCR decrease after glucose addition (see chapter 3.7.3). At the timepoint of glycolysis injection (Δ OCR), we observe a significant difference between the cell lines, indicating that the lack of TET2 results in a partial loss of Crabtree effect in HAP1 cells (Figure 4.2.9). This result showed that HAP1 Parental cells have a higher preference for glycolysis and present lower mitochondrial respiration dependency than HAP1 TET2 KO cells in normoxia. The switch to hypoxia abolishes this difference, indicating that the hypoxia condition resulted in an adaptation to glycolysis that renders TET2 KO cells response to glucose addition similar to Parental cells.

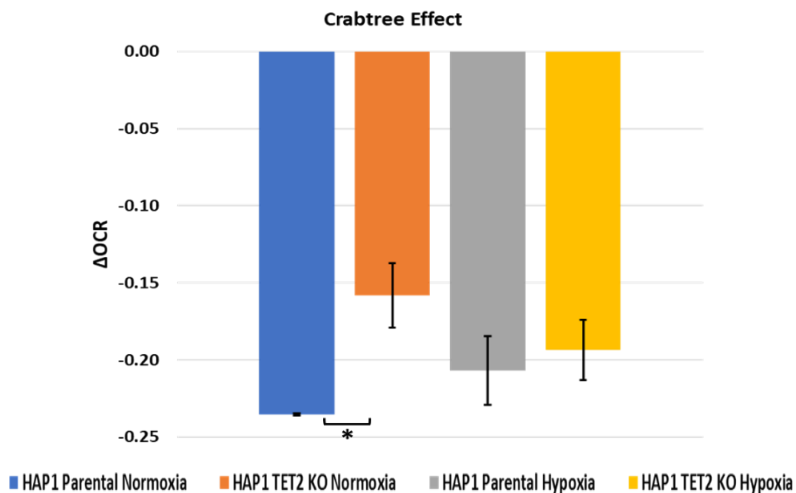


Figure 4.2.9- The Crabtree Effect on HAP1 cell lines. OCR rates measured for both cell lines under normoxia and hypoxia. Data represented is mean \pm SD of n=3 experiments. Significance was determined by two-tailed independent sample Student's test, with significance threshold at $p < 0.05$ (*).

The similarity of the mitochondrial parameters measured for both cell lines in hypoxia (Figure 4.2.8) let us to conclude that the increase of ROS levels observed in TET2 KO cells (Figure 4.2.7) could be due to the observed

decrease on G6PD total activity of these cells in hypoxia. The fact that TKT activity was also decreased as well as the percentages of ^{13}C enrichment in ribose reinforce this hypothesis as is indicative of a lower activity of ox-PPP and non-ox-PPP pathways acting as a cycle to generate NADPH. Altogether, the totality of the metabolic characterization results shows that the loss of TET2 is clearly driving the cells to manifest a different metabolism, with proliferation and energy production pathways affected, especially in hypoxia.

4.2.2.4 TET2 loss alters the entry point of pyruvate into TCA cycle

With the previous results indicating changes to mitochondrial activity and anaerobic glycolysis in hypoxia, we used tracer-based metabolomics to attest the transition of pyruvate after glycolysis into the TCA cycle. Said entry can be through the PDH complex, where pyruvate is broken down to acetyl-CoA. Another point of entry is through PC reaction, that converts pyruvate into OAA at the cost of ATP. Both paths converge in the formation of citrate, given that CS uses both acetyl-CoA and OAA to produce citrate. The routes of how labelled carbons from glucose can travel across one turn of the TCA cycle is represented in Figure 4.2.10.

Analysis of the isotopologues of citrate from the glucose source fed to the cells shows significant changes to citrate m2, m3, m4 and m5 (Figure 4.2.11 A). Citrate m2, which is the most produced labelled isotopologue and results from labelled pyruvate entering TCA cycle through PDH path, and Citrate m5 isotopologue, that results from condensation of labelled OAA and labelled acetyl-CoA, displayed the same percentage of occurrence in both Parental and TET2 KO cell lines under normoxia. Interestingly, under hypoxia challenge, citrate m2 decreases significantly only in TET2 KO cells, which is indicative of a decrease of PDH activity under hypoxia of TET2 KO cells. By contrary, citrate m5 increases in TET2 KO cells and

decreases in Parental cells under hypoxia. Taking into account that citrate m5 results from the condensation of acetyl-CoA m2 with OAA m3 (formed from carboxylation of pyruvate m3 catalysed by PC), the citrate m5 pattern obtained is indicative of a higher activity of pyruvate carboxylase in TET2 KO cells in response to hypoxia challenge. In the same line, citrate m3 results from the condensation of OAA m3 with an unlabelled acetyl-CoA, obtained from fatty acid metabolism.

Citrate m4, that can be formed in a second turn of TCA cycle either from condensation of OAA m2 with acetyl-CoA m2 or by condensation of OAA m4 with acetyl-CoA m2, increases under the hypoxia challenge in TET2 KO cells and decrease in Parental cells which is indicative of a higher TCA cycle turnover in TET2 KO cells under hypoxia fuelled by pyruvate carboxylase.

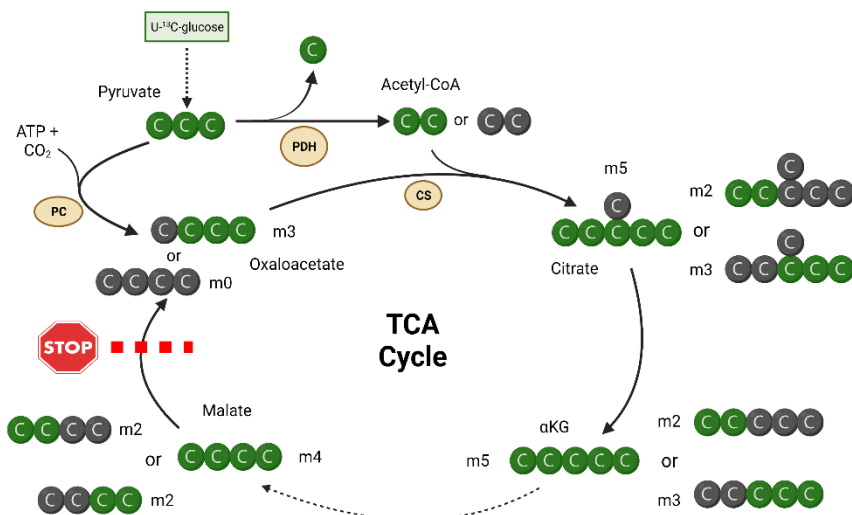


Figure 4.2.10- Scheme of labelling patterns expected in TCA cycle from U-¹³C-Glucose. Uniformly labelled glucose is converted into fully labelled pyruvate through glycolysis. Said pyruvate can then enter the TCA cycle via pyruvate dehydrogenase (PDH) or via pyruvate carboxylase (PC). In brief, the resulting oxaloacetate and acetyl-CoA is then used by citrate synthase (CS) to generate distinct isotopologues of citrate. Subsequent label patterns travel through α -ketoglutarate (α KG), succinate, fumarate and end up at malate (defined as the stop point of one loop of the cycle). Created with BioRender.com.

Corroborating the citrate labelling profile is the malate isotopologues pattern, given that malate can inherit both the labelling from citrate in the normal turn of the TCA cycle or from the cytosolic conversion from OAA. The significant changes observed were to malate m2, m3 and m4 (Figure 4.2.11B). Thus, consistent with the increase of labelling in citrate under the hypoxic challenge observed in TET2 KO cells, we see also an increase of labelling in malate in this cell line.

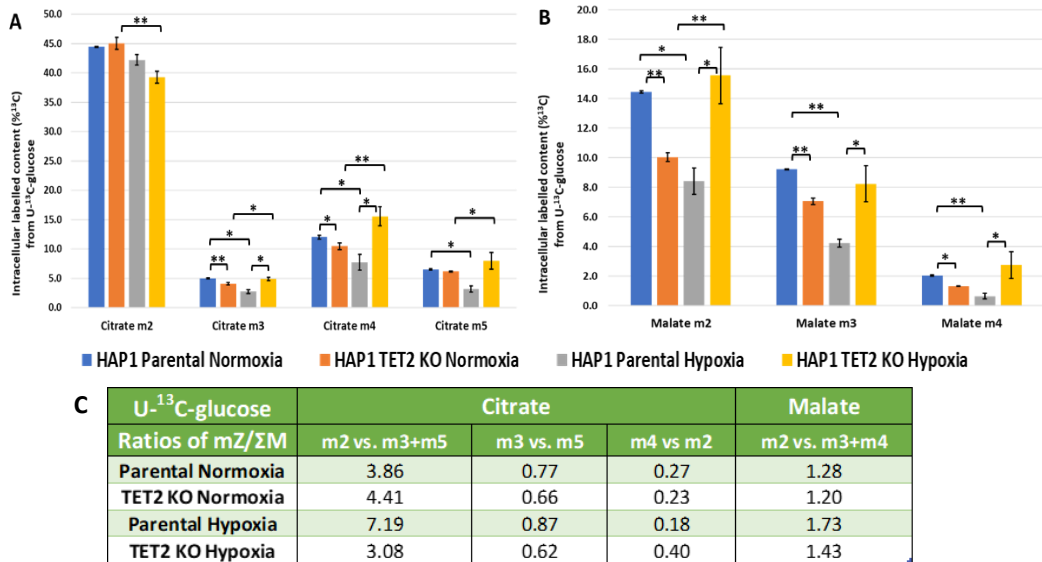


Figure 4.2.11- GCMS analysis of labelled intracellular Citrate and Malate from U-¹³C-glucose (only 1st turn). Measurement of glucose contributions (% labelled) to intracellular metabolites after 24h incubation with 22,5 mM U-¹³C-glucose. (A) Represented contribution of glucose to Citrate m2, m3, m4 and m5. (B) Represented contribution of glucose to Malate m2, m3 and m4. Representing labelled isotopologues above 2% labelling and with significant changes. Data represented is mean ± SD for n=3 reads of n=3 samples of a single experiment. Statistically significant differences were determined by two-tailed independent sample Student's t-test, with p-values of p < 0.05 (*), p < 0.01 (**), and p < 0.001 (***). (C) Ratios of labelled isotopologues from citrate and malate, from the same experiment represented in A and B. Calculations of PDH activity versus PC activity (m2 vs m3+m5 of citrate; m2 vs m3+m4 of malate); of TCA cycle velocity (m4 vs m2 of citrate) and of β-oxidation contribution of acetyl-CoA (m3 vs m5 of citrate).

Next, we verified the ratios of labelled citrate and malate with the U-¹³C-glucose tracer to highlight how much the loss of TET2 was influencing multiple parameters (Figure 4.2.11C). Using the percentage of labelled isotopologues (mZ) per the total labelling occurring in each sample (ΣM), we compared the amount of citrate m2 (from PDH path) versus citrate m3 and m5 (that require OAA made through PC). In normoxia, the contribution of PC and PDH to the TCA cycle is similar in both cell lines (Figure 4.2.11C). But in the hypoxic condition, the Parental cells increase the PDH activity while the TET2 KO sees this ratio decrease, indicating less PDH and more PC activity. Similar ratio calculations using the malate labelling corroborates this tendency of higher PC activity with the loss of TET2 (Figure 4.2.11C).

Another point that confirms the shift in the production of citrate is the contribution of acetyl-CoA from β -oxidation (i.e. unlabelled carbons). This ratio (m3 vs m5 of citrate) shows that in hypoxia the Parental has more entry of acetyl-CoA from β -oxidation while the TET2 KO cells do not change between conditions. This indicates less contribution of fatty acid metabolism to TCA cycle activity in TET2 KO cells in hypoxia.

Lastly, we can also determine how “fast” the TCA cycle reactions are occurring in regard to the absence of TET2. Again, there are equal behaviours between the cell lines in normoxia but in hypoxia, the Parental cells slow down their TCA activity while the cells without TET2 actually increase it. All these results show that the loss of TET2 is altering the mitochondrial activity, increasing the TCA cycle reactions in hypoxic conditions.

4.2.2.5 TET2 loss impairs the hypoxia-triggered switch to reductive carboxylation of glutamine

Glutamine is a non-essential amino acid in normal cells but found to be essential for cancer cells, especially in hypoxic conditions. Given this fact, we inquired how the loss of TET2 would affect the glutamine metabolism. Measurements of glutamine consumption and glutamate production through spectrophotometry-based methods (see chapter 3.3) indicated that the hypoxic condition induces a major alteration to these processes (Figure 4.2.12).

Glutamate secretion is slightly higher in cells without TET2 in normoxia and when switching to hypoxia both cell lines increase glutamate production (Figure 4.2.12A).

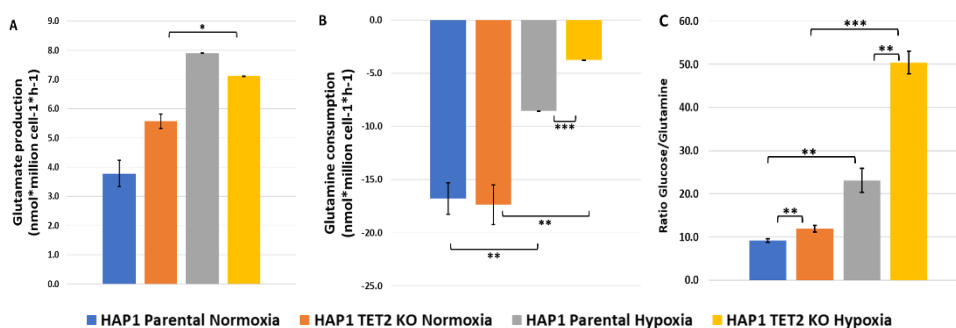


Figure 4.2.12- Loss of TET2 impact on glutamine metabolism. Consumptions and production rates measurements through spectrophotometry from cell culture media after 48h incubation, in both normoxia and hypoxia conditions. (A) Glutamate production and (B) Glutamine consumption and production. (C) Ratio of glucose consumed versus glutamine consumed. Data represented by mean \pm SD of n=3 reads of a representative experiment per normoxia and hypoxia. Statistically significant differences in all panels was determined by two-tailed independent sample Student's t-test: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***)

Net glutamine consumption/production is similar between the cell lines in normoxia, (Figure 4.2.12B). with both cell lines consuming much less glutamine than glucose (Figure 4.2.12B-C). In the hypoxia setting, the metabolic adaptation leads to a decreased consumption of glutamine. This

decrease is more accentuated in the TET2 KO cells, this indicates that the loss of TET2 could result in a different glutamine metabolism in response to hypoxia in the TET2 KO cells.

This is further evidenced by the ratios of glucose consumption versus glutamine consumption (Figure 4.2.12C) that display a higher increase under hypoxia in TET2 KO cells.

To further investigate cell glutamine dependencies in due to TET2 knockout, we analysed the labelling profile of the metabolites involved in glutaminolysis, using tracer-based metabolomics.

For this purpose, we incubated Parental and TET2 KO cells in parallel biochemical equivalent experiments, with either 22.5 mM U-¹³C-glucose and unlabelled 4 mM glutamine or with 4 mM U-¹³C-glutamine and unlabelled 22.5 mM glucose, followed by isotopologue distribution GCMS-based analysis (see chapter 3.10.1).

The route of incorporation of ¹³C label into α KG, from U-¹³C-glucose source has already been depicted in Figure 4.2.10 and the conversion of the ¹³C-labelled α KG into glutamate and glutamine is catalysed respectively by glutamate dehydrogenase (reversible reaction) and glutamine synthetase. The routes of how isotopologues of glutamate and TCA cycle intermediates originated from the U-¹³C-glutamine source of labelled carbons are depicted in Figure 4.2.13A. In brief the ¹³C-label from glutamine can be directly incorporated into glutamate through glutaminase (or through transamination reactions) and into α KG through glutamate dehydrogenase reaction. Next, α KG can enter TCA cycle and be converted into citrate after one turn of the cycle or, alternatively, can follow the reductive carboxylation pathway and be converted into citrate through a reverse IDH reaction. Reductive carboxylation can occur either in cytosol or in the mitochondria and has been described to occur mainly in hypoxic conditions or in cells with defective mitochondria¹⁸⁷.

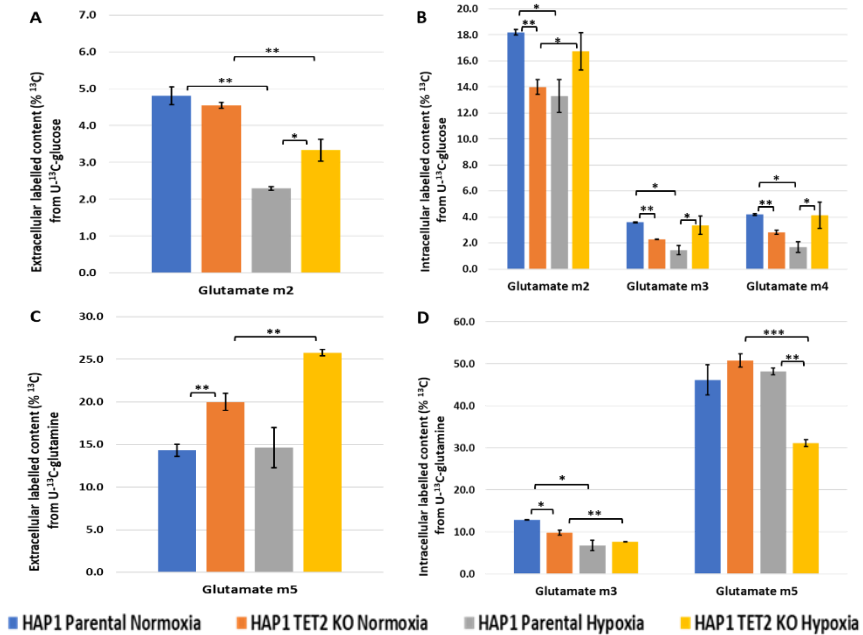


Figure 4.2.13- GCMS analysis of Glutamate isotopologues. Measurement of glucose contribution (% ¹³C labelled) to extracellular glutamate after 24h incubation with 22.5 mM U-¹³C-glucose (A) and 4 mM U-¹³C-glutamine (C). Similar measurements to intracellular glutamate from glucose (B) and glutamine (D) tracer source. Representing labelled isotopologues above 2% labelling and with significant changes. Data represented in all panels is mean ± SD for n=3 reads of n=3 samples of a single experiment. Statistically significant differences were determined by two-tailed independent sample Student's t-test, with p-values of p<0.05 (*), p<0.01 (**), and p<0.001 (***).

Glutamate m2, from U-¹³C-glucose source, excreted to the extracellular media is around 5% in normoxia in both Parental and TET2 KO cells and decreases in both cell lines under hypoxia, being the decrease less pronounced in the KO cells (Figure 4.2.13). The intracellular glutamate m2 is higher in Parental cells in normoxia with a decrease after the switch to hypoxia but an increase in the TET2 KO cells. The same tendencies are observed for intracellular glutamate m3 and m4 (Figure 4.2.13A-B). The higher percentage of glutamate m3 and m4 observed in TET2 KO cell line in hypoxia conditions is in accordance with the ¹³C-label pattern observed in citrate (see Figure 4.2.11) and corroborate that TET2 KO cells have

higher entry of pyruvate into TCA cycle through pyruvate carboxylase than Parental cells under hypoxia conditions.

Next, we investigated the ^{13}C -label incorporation of glutamate from U- ^{13}C -glutamine source. Analysing the glutamate m5, which is the direct product of this tracer source, we observe that under hypoxia TET KO cells the secreted glutamate is more enriched as m5 whereas Parental cells secreted glutamate maintain the same percentage of m5 enrichment in hypoxia than in normoxia. This result is indicative of an increase of glutamine synthetase activity and subsequent export of glutamate triggered by hypoxia only in TET2 KO cells (Figure 4.2.13C).

Intracellular glutamate m5 levels are only lower in TET2 KO cells under hypoxia (Figure 4.2.13D), which is indicative of a higher export of glutamate that can be exchanged for other amino acids through antiport transporters or/and of a higher use of glutamate for glutathione synthesis.

As for glutamate m3 from glutamine source, its formation happens after more than one turn of the TCA cycle and we see much less occurring in hypoxia than normoxia (Figure 4.2.13D).

Put together, these results indicate that without TET2, the cells in hypoxia perform the first deamination of glutamine to obtain nitrogen, with the resulting glutamate being partly directed to the TCA cycle and partly being secreted to the extracellular media.

Following this, we investigated ^{13}C -label incorporation into citrate and malate from U- ^{13}C -glutamine. We obtained that citrate m5 and malate m3 increase under hypoxia challenge in Parental cells whereas in TET2 KO cells we observed a decrease of these two isotopologues in response to hypoxia (Figure 4.2.14A-B). These results show that the knockout of TET2 impairs the switch to reductive carboxylation triggered by hypoxia in Parental cells. As depicted in Figure 4.2.15, citrate m5 and malate m3 (generated from oxaloacetate m3) are produced from U- ^{13}C -glutamine

through reductive carboxylation pathway and are excellent indicators of the activity of this pathway.

Citrate m4 and Malate m4 are the products of the TCA cycle from the entry point of labelled carbons from α KG, with the hypoxia levels lower than in normoxia for both cell lines (Figure 4.2.14A-B).

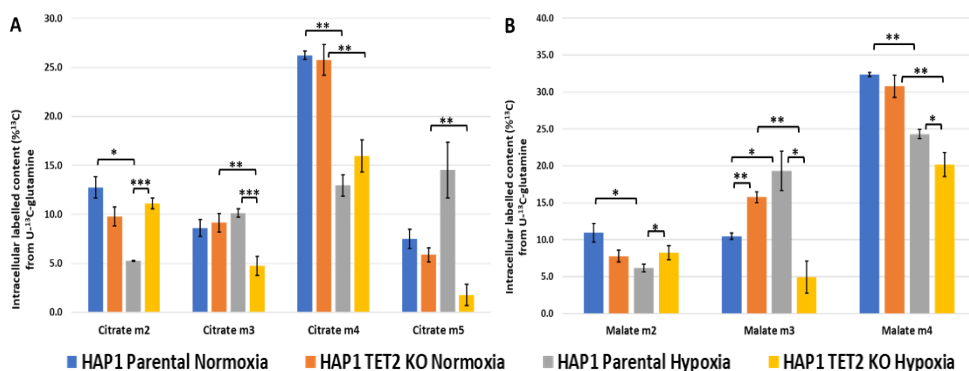


Figure 4.2.14- GCMS analysis of labelled intracellular Citrate and Malate. Measurement of glutamine contributions (% labelled) to intracellular citrate and malate after 24h incubation with 4 mM of U-¹³C-glutamine. **(A)** Represented contribution of glutamine to Citrate m2, m3, m4 and m5. **(B)** Represented contribution of glutamine to Malate m2, m3 and m4. Representing labelled isotopologues above 2% labelling and with significant changes. Data represented is mean \pm SD for n=3 reads of n=3 samples of a single experiment. Statistically significant differences were determined by two-tailed independent sample Student's t-test, with p-values of p<0.05 (*), p<0.01 (**) and p<0.001 (***).

Lastly, we observed the ratios of the isotopologues of citrate and malate to corroborate our observations (Figure 4.2.15B). Comparing citrate m4 versus citrate m5 indicates the preference for oxidative carboxylation or reductive, respectively. This ratio is similar in normoxia but drastically changes in hypoxia, with the Parental cells favouring the reductive carboxylation while the cells without TET2 favour the oxidative reaction. From the malate ratios, we can infer the preference from reductive carboxylation or normal TCA cycle, by comparing malate m3 versus m4. This again tells us that while in normoxia there is not much difference, in hypoxia the Parental cells favour the reductive carboxylation path.

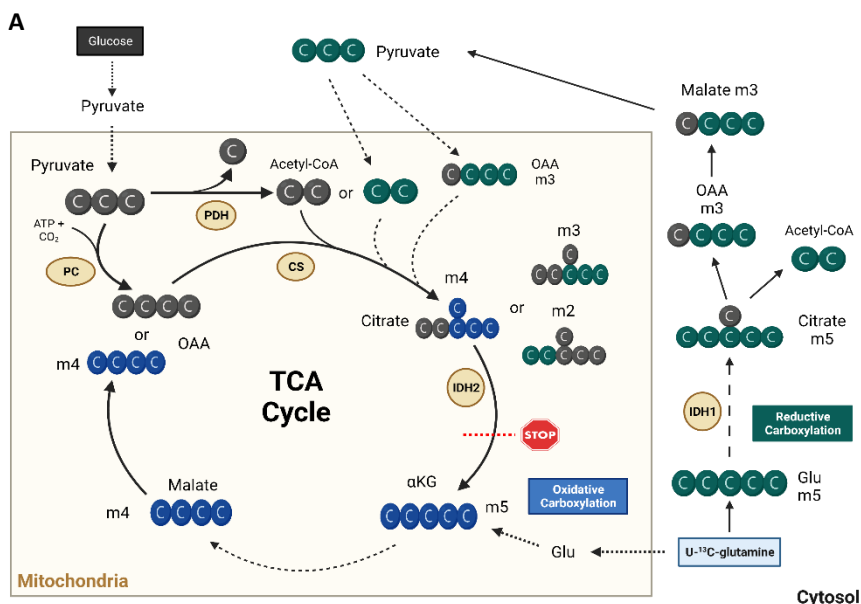


Figure 4.2.15- Scheme of labelling patterns expected in TCA cycle from U-¹³C-Glutamine. (A) Uniformly labelled glutamine is converted into fully labelled glutamate (Glu) through glutaminolysis, which then enters the TCA cycle in the form of α -ketoglutarate (α KG), the denominated oxidative carboxylation path (highlighted in blue). Subsequent label patterns travel through succinate, fumarate, malate and end up at oxaloacetate (OAA). This can be used by citrate synthase (CS) to form citrate (defined as the stop point of one loop of the cycle). Alternatively, glutamine can suffer reductive carboxylation (highlighted in green), generating differently labelled Glu, α KG, citrate, OAA, acetyl-CoA and malate. Said malate can be transformed into pyruvate and feed the TCA cycle, in the form of acetyl-CoA (from PDH) or in the form of OAA (from PC). Created with BioRender.com. (B) Ratios of labelled isotopologues from citrate and malate, from the same experiment represented in Figure 4.2.14. Calculations of Reductive carboxylation versus Oxidative carboxylation (m4 vs m5 of citrate; m4 vs m3 of malate).

4.2.2.6 TET2 loss directs One-Carbon Metabolism for Glutathione production

The metabolic characterization results arising from the knockout of TET2 all point to a higher mitochondrial activity and ROS increase. For the cells to maintain their proliferation with these parameters under hypoxia, we can hypothesize that the detoxification systems such as glutathione are also changing.

As mentioned in chapter 1.3.6, glutathione production is dependent of various other metabolic pathways. It requires glutamate from glutaminolysis, glycine and homocysteine from the One-Carbon metabolism and NADPH to recover GSSG back to GSH, supplied by the PPP and TCA cycle.

From GCMS and NMR-based tracer-based metabolomics approaches and incubating the cells as above described with 22.5 mM U-¹³C-glucose, we can investigate the flow between glycine and serine. The results presented all the possible isotopologues of serine, both intra and extracellularly (Figure 4.2.16A-B). Serine m3 is a direct product from glycolysis, receiving the labelled carbons from 3-phosphoglycerate (3PG) whereas serine m2 and serine m1 can result from the interconversion of serine and glycine through the reversible serine hydroxymethyltransferase (SHMT) reaction with the participation of labelled or unlabelled substrates. The most significant differences observed, at the level of intracellular isotopologues distribution between the two cell lines in response to hypoxia, are that the Parental cells only increase m3 whereas in TET2 KO cells we observe a significant increase of all serine isotopologues (m1, m2 and m3) under the hypoxic challenge. Worth to note that the percentage of labelled enrichment of the serine isotopologues in extracellular serine also increased in TET2 KO cells in response to hypoxia whereas it is not altered in Parental cells (Figure 4.2.16B). Taking into account that the serine

consumption of the cells is not statistically significant, neither between the two cell lines nor in the different oxygen levels (Figure 4.2.16C) and that glycine m2 (Figure 4.2.16A) is also increased in TET2 KO cells in hypoxia, we concluded that there is a higher interconversion between serine and glycine through SHMT reaction due to an increase in One-Carbon Metabolism in TET2 KO cells under hypoxia. At the level of total glycine consumption, we observed a dramatic increase of glycine consumption in hypoxia in both cell lines that could be due to the fact that hypoxia increased the need of glycine for glutathione synthesis (Figure 4.2.16C).

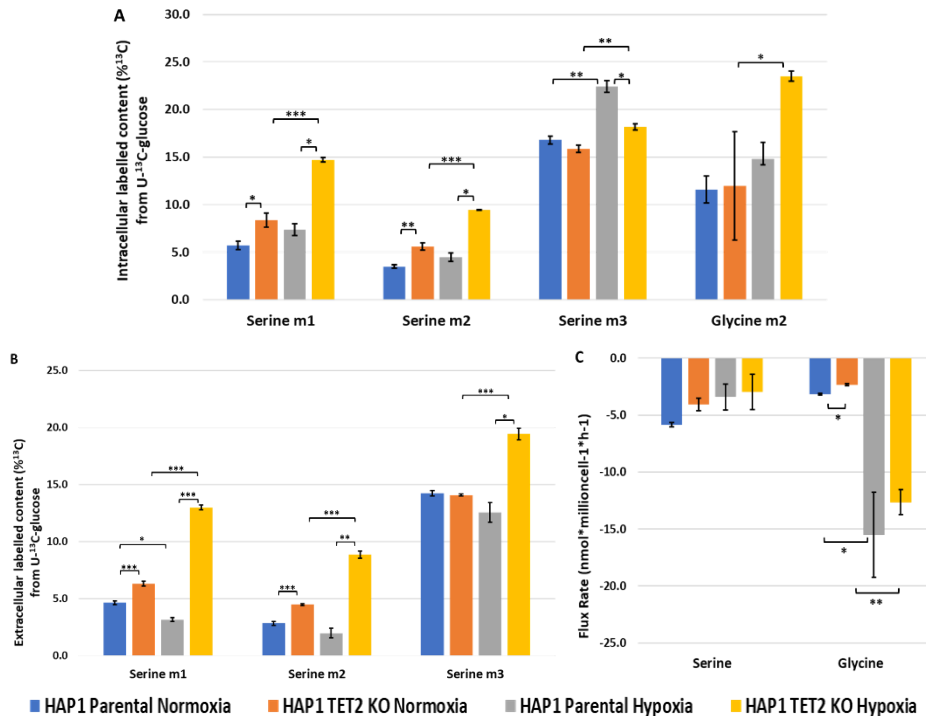


Figure 4.2.16- GCMS analysis of labelled Serine and Glycine. Measurement of glucose contribution (% labelled) to intracellular (A) and extracellular (B) metabolites after 24h incubation with 22.5 mM U-¹³C-glucose. Representing labelled isotopologues above 2% labelling and significant changes. Data represented is mean \pm SD for n=3 reads of n=3 samples of a single experiment. (C) Consumption rates of Serine and Glycine obtained through Absolute 180 IQ Kit from Biocrates. Data represented is mean \pm SD for n=3 reads of n=3 samples of a single experiment. Statistically significant differences were determined by two-tailed independent sample Student's t-test, with p-values of p<0.05 (*), p<0.01 (**), and p<0.001 (***).

The results obtained using ^{13}C -based NMR metabolomics showed that the percentage of ^{13}C enrichment in carbon C2 of glycine and carbon C3 of serine are also increased under hypoxic challenge in TET2 KO cells corroborating the results obtained by ^{13}C -based GC-MS (Figure 4.2.17). Worth to note that SHMT catalysed conversion of serine into glycine generates 5,10me-THF that can be used as substrate by the 5,10me-THF dehydrogenase activity of MTHFD resulting in the reduction of NADP to NADPH. As TET2 KO cells are not able to increase G6PD under the hypoxia challenge, we hypothesized that the increase observed in glycine m2 can serve the purpose of generating NADPH.

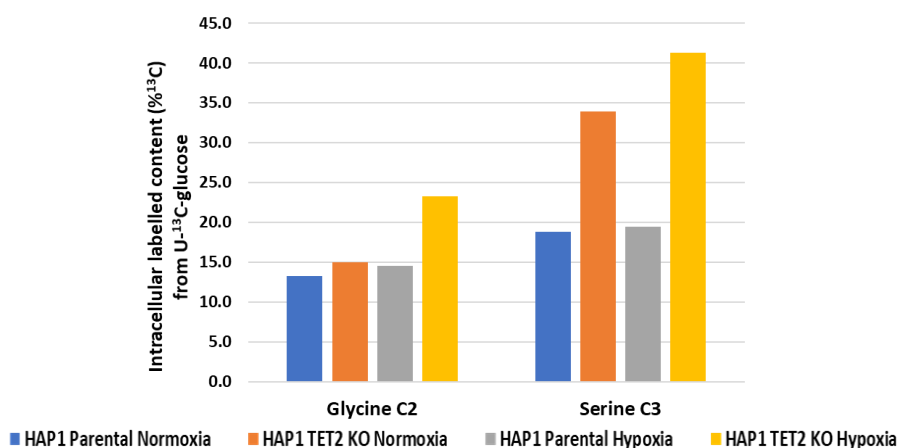


Figure 4.2.17- Labeled Glycine and Serine Isotopomers using ^{13}C -tracer based Metabolomics. Measurement of glucose contribution to intracellular metabolites after 24h incubation with 22,5mM U- ^{13}C -glucose, processed and analysed by ^1H - ^{13}C -HSQC NMR spectroscopy (full procedure in Methods 4.11). ^{13}C percentages were calculated as a ratio of the labelled and unlabelled sample and multiplied by the natural abundance of ^{13}C (1.1%). Data represented is n=1 read of n=1 sample of a single experiment. Statistical analysis could not be applied due to insufficient replicates.

To further investigate if the differences in glutamate and glycine ^{13}C enrichment observed due to TET2 knockout are reflected in the mirror carbons in the glutathione molecule we analysed the differences in ^{13}C enrichment in selected carbons of glutathione. As mentioned before,

glutathione is the joining of three other amino acids, glutamate, cysteine and glycine. Through NMR we are able to see the incorporation of labelled carbons to each specific constituent of GSH (Figure 4.2.18). The carbons C2 and C3 correspond to the 2nd and 3rd carbon of a glutamate molecule. From a glucose source, the C2 labelling correspond to the profile of glutamate m2 attested by GCMS (Figure 4.2.13B). Equal similarities can also be seen on the comparison from the glutamine tracers (Figure 4.2.13D). These results indicate that the use of glutamate for glutathione production is higher in Parental cells, especially in the normoxia setting.

Glutathione C9 correspond to one of the carbons from the glycine utilized in its synthesis. From a glutamine source, the incorporation of this labelled carbon is barely above the natural occurrence of ¹³C (Figure 4.2.18). As for the carbons from a glucose source, we see high levels in TET2 KO cells, both in normoxia and hypoxia (Figure 4.2.18A). Furthermore, it matches the profile seen of serine C3 and glycine C2 (Figure 4.2.17), showing how this carbon travelled all the way to integrate in glutathione.

Altogether, the obtained results show that the loss of TET2 is affecting glycine and serine metabolism adaptation to the hypoxia challenge which is also reflected in the incorporation of carbons of glucose and glutamine into glutathione. The fact that TET2 KO cells lost the capacity to increase total G6PD activity in response to hypoxia, diminishing its capacity to generate NADPH, is here unveiled as the putative event that trigger the enhanced One-Carbon Metabolism in these cells. Thus, consecutive SHMT and MTHFD reactions will generate glycine and NADPH providing an alternative important source of reductive equivalents to compensate the deficient G6PD total activity of TET KO cells in hypoxia. The enhanced ROS levels observed in TET KO cells in hypoxia could be also related with this primary event of impaired G6PD enhancement under the hypoxic condition of TET2 KO cells.

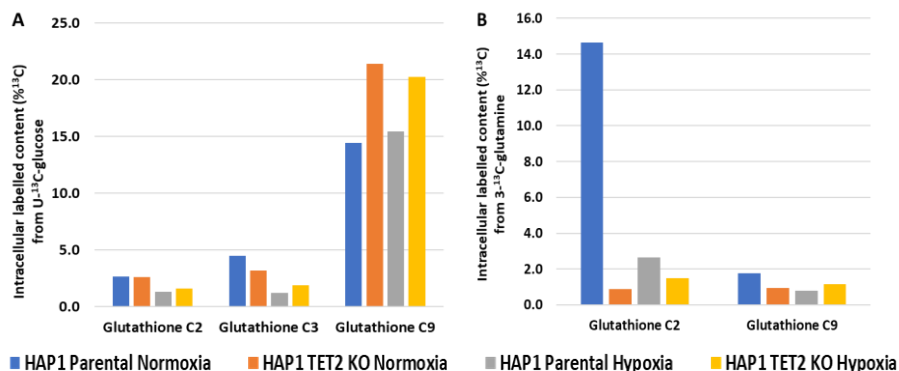


Figure 4.2.18- Label incorporation of intracellular Glutathione using ¹³C-tracer based Metabolomics. Measurements obtained using cellular extracts after 24h incubation with U-¹³C-glucose (A) and 3-¹³C-glutamine (B) tracer, processed and analysed by ¹H-¹³C-HSQC NMR spectroscopy (full procedure in Methods 4.11). ¹³C percentages were calculated as a ratio of the labelled and unlabelled sample and multiplied by the natural abundance of ¹³C (1.1%). Data represented is n=1 read of n=1 sample of a single experiment. Statistical analysis could not be applied due to insufficient replicates.

4.2.2.7 TET2 loss influences metabolism of amino acids

With the changes observed to glycolysis, glutamine metabolism and TCA cycle, we pondered how the metabolism of amino acids that interconnects with these pathways were changing as well in response to the loss of TET2. Alanine is a non-essential amino acid that connects the glycolysis pathway with glutamine metabolism because it can be produced from pyruvate carbon-skeleton through alanine aminotransaminase reaction between glutamate and pyruvate that renders αKG in addition to alanine.

Through tracer-based metabolomics, we investigated the incorporation of ¹³C-label from U-¹³C-glucose. The incorporation of ¹³C in C3 position of alanine, attested by NMR (Figure 4.2.19A-B), both intra- and extracellularly, shows the same pattern as pyruvate (see Figure 4.2.4), with higher levels in TET2 KO in hypoxia. This indicates that like the recycling of pyruvate-lactate, a similar process is occurring between alanine and pyruvate. The isotopologues analysed by GCMS corroborate the same

tendency, with alanine m3 being the more present isotopologue for TET2 KO cells, extracellularly (Figure 4.2.19C).

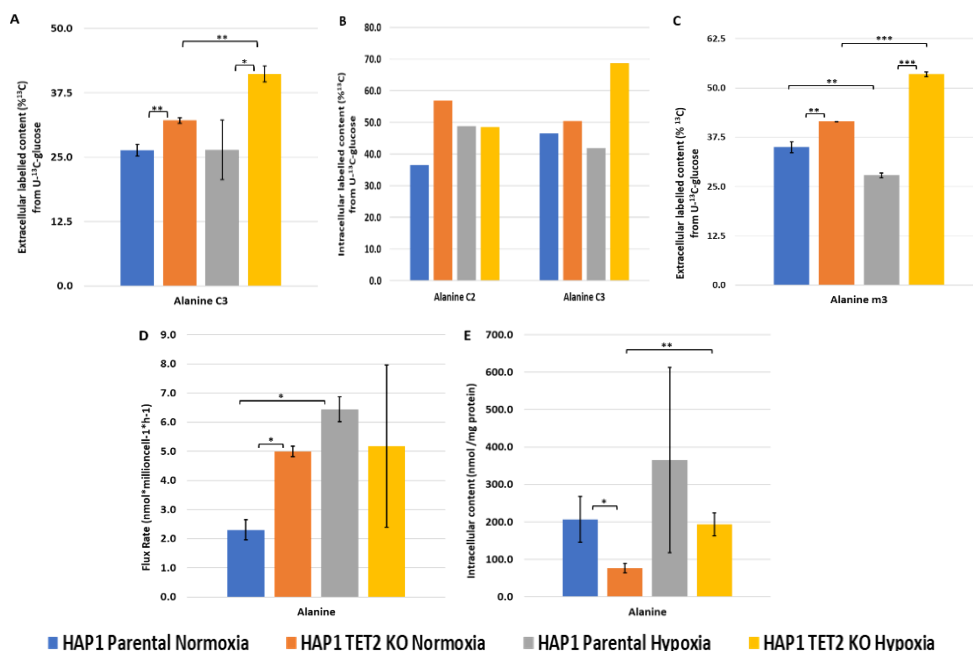


Figure 4.2.19- Impact of TET2 knockout on Alanine metabolism through targeted and ¹³C-based Metabolomics. (A) ¹³C-filtered ¹H-NMR spectroscopy analysis of labelled extracellular alanine. Data represented is mean ± SD for n=1 reads of n=3 samples of a single experiment. (B) ¹H-¹³C-HSQC NMR spectroscopy analysis of labelled intracellular Alanine. ¹³C percentages were calculated as a ratio of the labelled and unlabelled sample and multiplied by the natural abundance of ¹³C (1.1%). Data represented is n=1 read of n=1 sample of a single experiment. Statistical analysis could not be applied due to insufficient replicates. (C) GCMS analysis of labelled extracellular Alanine. Data represented is mean ± SD for n=3 reads of n=3 samples of a single experiment. (D) Consumption and production rates obtained through Absolute 180 IQ Kit from Biocrates. Data represented is mean ± SD for n=3 reads of n=3 samples of a single experiment. (E) Intracellular levels of Alanine (Ala) through Absolute 180 IQ Kit from Biocrates. Data normalized by protein content. Data represented is mean ± SD for n=3 reads of n=3 samples of a single experiment. Statistically significant differences in related panels were determined by two-tailed independent sample Student's t-test: p < 0.05 (*) and p < 0.01 (**).

The flux rates of alanine show a clear secretion from cells to the extracellular media, with TET2 KO secreting more in normoxia while in hypoxia the differences are not statistically significant as there is a high

error associated to the alanine measurements in hypoxia (Figure 4.2.19D). This higher alanine production in normoxia correlates with the higher glucose consumption observed also in TET2 KO cells in normoxia.

Aspartate is one of the amino acids that had significant changes in our tracer experiments. Given that ^{13}C -labelled aspartate is generated in transamination reactions from oxaloacetate, that in turn is interconverted with malate through malate dehydrogenase reaction, it is expected that aspartate ^{13}C -isotopologues distribution mirror that observed in malate (see the paths of ^{13}C incorporation into oxaloacetate, malate from U- ^{13}C -glucose and U- ^{13}C -glutamine depicted in Figure 4.2.10 and Figure 4.2.15).

From the experiments using U- ^{13}C -glucose we see that m2 and m3 aspartate labelling pattern in Parental and TET2 KO cell lines, in normoxia and hypoxia conditions (Figure 4.2.20A), perfectly mirror those obtained for malate (see Figure 4.2.11B).

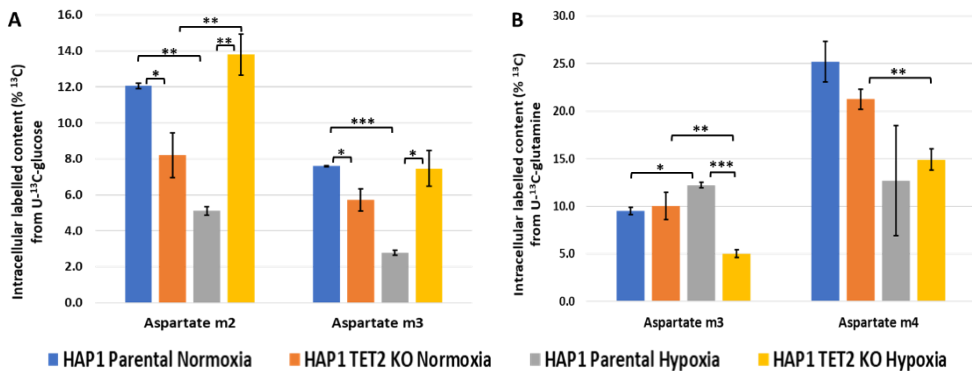


Figure 4.2.20- GCMS analysis of labelled intracellular Aspartate. Measurement of glucose and glutamine contributions (% labelled) to intracellular aspartate after 24h incubation with 22,5 mM U- ^{13}C -glucose and 4 mM U- ^{13}C -glutamine, respectively. (A) Represented contribution of glucose to Aspartate m2 and m3. (B) Represented contribution of glutamine to Aspartate m3 and m4. Representing labelled isotopologues above 2% labelling and with significant changes. Data represented is mean \pm SD for n=3 reads of n=3 samples of a single experiment. Statistically significant differences were determined by two-tailed independent sample Student's t-test, with p-values of p< 0.05 (*), p< 0.01 (**), and p<0.001 (***).

When using the U-¹³C-glutamine tracer, we obtained changes in aspartate m3 and m4 (Figure 4.2.20B). Again, these are mirrored labels from its precursor malate (see Figure 4.2.14B) as aspartate m4 comes from the oxidative metabolism of glutamine through the TCA cycle whereas aspartate m3 results from the conversion of glutamine into citrate (reductive metabolism of glutamine) and its conversion into oxaloacetate and acetyl-CoA by the action of ATP-citrate lyase (see Figure 4.2.15). These results corroborate that Parental cells enhance the reductive carboxylation in hypoxia and that TET2 knockout impairs the switch to reductive carboxylation of HAP1 cells in response to hypoxia.

Another metabolite which interacts with intermediates of the TCA cycle is proline. ¹³C-Labelled carbons can incorporate proline when synthesized from glutamate, produced from U-¹³C-glucose or from U-¹³C glutamine. From the glucose contribution, proline m2 levels are higher in normoxia than hypoxia, with the Parental cell line displaying higher ¹³C-enrichment than the TET2 KO cells in both conditions (Figure 4.2.21A). Worth to note that the synthesis of proline molecule from glutamate requires a net consumption of 2 molecules of NADPH. The fact that TET2 KO cells have less capacity to generate NADPH through G6PD due to the decrease of total activity both in normoxia and hypoxia could be the possible mechanism underlying the observed differences.

On the side of U-¹³C-glutamine contribution to proline, we observe significant labelling changes in isotopologues m2 and m4 of the fragment C2C5 of proline (Figure 4.2.21B). The m4 labelling occurring in the fragment of proline C2C5 is directly from glutaminolysis producing glutamate m5. We can confirm this since the labelling profile is shared with glutamate m5.

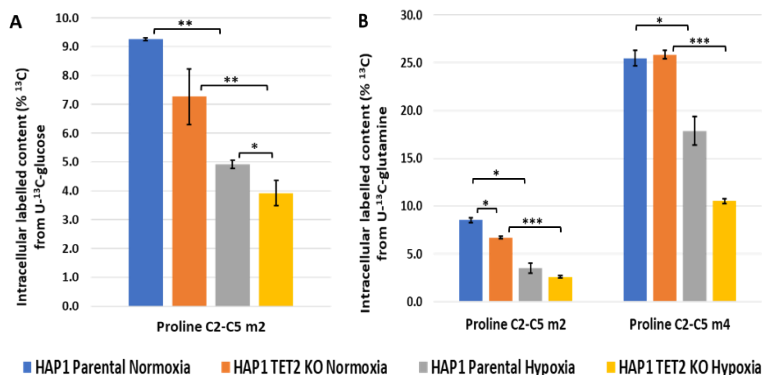


Figure 4.2.21- GCMS analysis of labelled intracellular Proline. Measurement of glucose and glutamine contributions (% labelled) to intracellular proline after 24h incubation with 22,5 mM U-¹³C-glucose and 4 mM U-¹³C-glutamine, respectively. Proline fragment C2C5 is represented. (A) Represented contribution of glucose to Proline m2. (B) Represented contribution of glutamine to Proline m2 and m4. Representing labelled isotopologues above 2% labelling and with significant changes. Data represented is mean ± SD for n=3 reads of n=3 samples of a single experiment. Statistically significant differences were determined by two-tailed independent sample Student's t-test, with p-values of p< 0.05 (*), p< 0.01 (**), and p<0.001 (***).

Proline m2 labelling percentages in the C2C5 fragment of proline are much lower than m4 (Figure 4.2.21B), given that such isotopologue results from glutamate coming from second turn of TCA cycle, thus generating forms of glutamate with 2 or 3 ¹³C. This proline m2 would correlate with glutamate m2 or glutamate m3 molecules (Figure 4.2.13D). The lowering levels of proline m2 attested in hypoxia are then another evidence that the first steps of the TCA cycle are being reduced in both cell lines (Figure 4.2.21B), with less label traveling from αKG back to glutamate.

At the level of the branched chain amino acids (BCAAs) consumptions in normoxia (isoleucine, leucine and valine), we do not see any difference between Parental and TET2 KO cells (Figure 4.2.22B). By contrary, we observed that hypoxia triggers a dramatic increase in isoleucine and leucine consumptions in Parental cells whereas in TET2 KO cells only leucine slightly increase consumption in hypoxia. At the level of intracellular BCAAs content we see higher intracellular levels in Parental cells in

normoxia (Figure 4.2.22A). Interestingly, hypoxia triggers a dramatic increase in isoleucine and leucine content in Parental cells and only small changes in TET2 KO cells. The same tendency in lesser extension is observed for Valine.

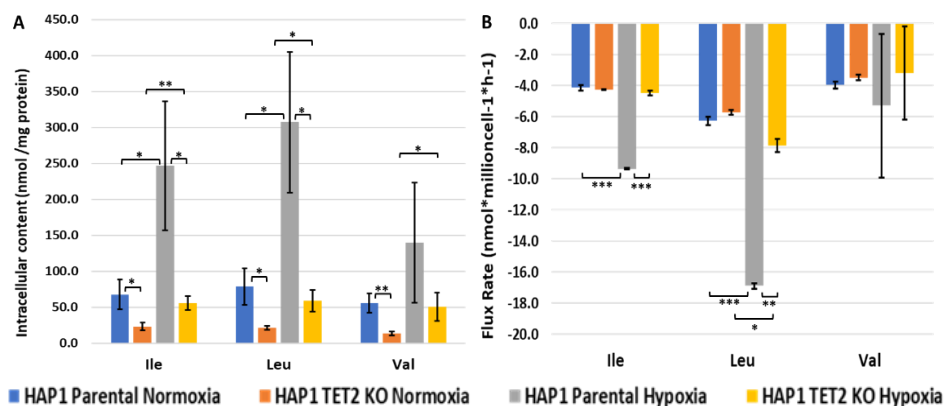


Figure 4.2.22- TET2 knockout effect on Branched-chain amino acids metabolism. (A) Intracellular levels of Isoleucine (Ile), Leucine (Leu) and Valine (Val) through Absolute 180 IQ Kit from Biocrates. Data normalized by protein content. Data represented is mean \pm SD for n=3 reads of n=3 samples of a single experiment. (B) Consumption and production rates of Isoleucine (Ile), Leucine (Leu) and Valine (Val), obtained through Absolute 180 IQ Kit from Biocrates. Data represented is mean \pm SD for n=3 reads of n=3 samples of a single experiment. Statistically significant differences in related panels were determined by two-tailed independent sample Student's t-test: $p < 0.05$ (*) and $p < 0.01$ (**).

Taking into account that the knockout of TET2 not affect cell proliferation rate neither in normoxia non in hypoxia, the differences observed in BCAAs intracellular content and consumption cannot be related with cell growth needs. The obtained results could be explained at the light of the recent evidence reported in the literature on the role of BCAAs catabolism in activating mTOR and regulating α KG homeostasis. In brief, it has been reported that downregulation of TET2 results in activation of mTOR signalling pathways^{484,485} and that the first step in BCAAs catabolism (catalysed by BCAT1 enzyme), is critical for maintaining low levels of α KG that decrease TET2 activity and result in DNA-hypermethylation⁹⁵.

Thus, according to these reported evidences the lack of increase of BCAAs consumption in hypoxia and the low intracellular levels can be due to the fact that leucine and BCAAs are not required by TET2 KO cells neither for increasing mTOR nor to decrease α KG available for TET2-catalysed DNA demethylation.

Finally, we analysed the differences at the level of methionine, which is indirectly related with glutathione synthesis, and polyamines, which are metabolites involved in arginine metabolism and the synthesis of SAM (a key substrate for methylation reactions). We observed that methionine, ornithine and spermine intracellular contents are higher in Parental cells in normoxia and increase under the hypoxic challenge in these cells whereas this effect is not observed in TET2 KO cells (Figure 4.2.23A and C). At the level of cell metabolite consumption/production, the results are not conclusive for neither Methionine and spermine, due to a high experimental error, but a higher net secretion of ornithine to extracellular media is observed for TET2 KO cells which is indicative of a lower utilization of this metabolite for the synthesis of other polyamines or SAM (Figure 4.2.23B). Taking into account that it has been described that exogenous spermine augmented DNA methyltransferase activity and that abnormal gene methylation is triggered by polyamine deficiency⁴⁸⁶, it is plausible to conclude that the obtained results, on altered polyamine intracellular levels and consumption/production between the two cell lines, are a consequence of the loss of TET2 activity in KO cells.

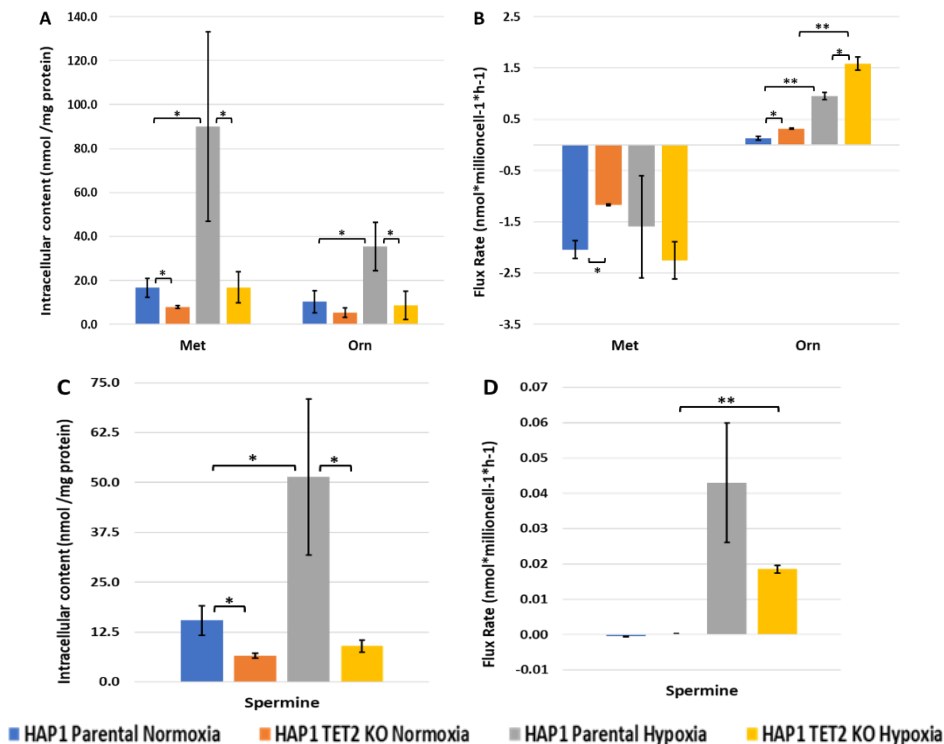


Figure 4.2.23- TET2 knockout effect on Methionine, Ornithine and Spermine metabolism. (A) Intracellular levels of methionine (Met) and ornithine (Orn). Data normalized by protein content. (B) Consumption and production rates of methionine (Met) and ornithine (Orn). (C) Intracellular levels of Spermine. Data normalized by protein content. (D) Consumption and production rates of Spermine. All results obtained through Absolute 180 IQ Kit from Biocrates. Data represented in all panels is mean \pm SD for n=3 reads of n=3 samples of a single experiment. Statistically significant differences in all panels were determined by two-tailed independent sample Student's t-test: $p < 0.05$ (*) and $p < 0.01$ (**).

4.2.3 Discussion

The many characterization experiments performed to verify the metabolic phenotype of these CML cell lines must be compiled, for a proper analysis of exploitative weaknesses for future therapeutics.

In general, the differences attested in normoxia were minor, with the loss of TET2 barely affecting the cell survival and proliferation. As such, we

focused in creating a metabolic map of the alterations seen in hypoxia (Figure 4.2.24). For the main reason that the loss of TET2 is one of the first hit mutations to occur to haematopoietic cells, who dwell in hypoxic conditions during most of their existence.

First major difference is that most of the enzymes in glycolysis and PPP are less active without TET2. This is an impediment to energy production and generation of the necessary nucleotides for proliferation. Given the standard hypoxic response generally described in the majority of cancers is to increase the gene expression of these specific enzymes, their lack of activity in TET2 KO would mean that the demethylation process is necessary in the hypoxic response.

At the junction point of pyruvate, the loss of TET2 results in a distinct use of this metabolite. Lactate production increases in Parental, having no hindrance in making it and expelling it to the microenvironment. The same difference occurs in alanine production. As for the remaining pyruvate that is transported into the mitochondria, there is the second major difference between Parental and TET2 KO. The Parental cells break down pyruvate in the PDH complex, producing acetyl-CoA and CO₂, which are then used in other reactions. But without TET2, the cells are using pyruvate through Pyruvate carboxylase to make oxaloacetate, at the expense of ATP.

This leads to the third major difference, the use of the TCA cycle. Besides the distinct entry point of the cycle to form citrate, there are different equilibriums with anaplerotic reactions. One of the most important processes for anaplerosis is glutaminolysis. The conversion of glutamine into glutamate, and subsequently α KG, is higher in Parental cells. But instead of feeding that α KG into the TCA cycle, it performs reductive carboxylation to form citrate, in other words, the reverse reactions of the cycle. On the other hand, Parental cells have increased uptake of BCAAs, which catabolism produce acetyl-CoA and succinyl-CoA, which can feed the cycle at different points.

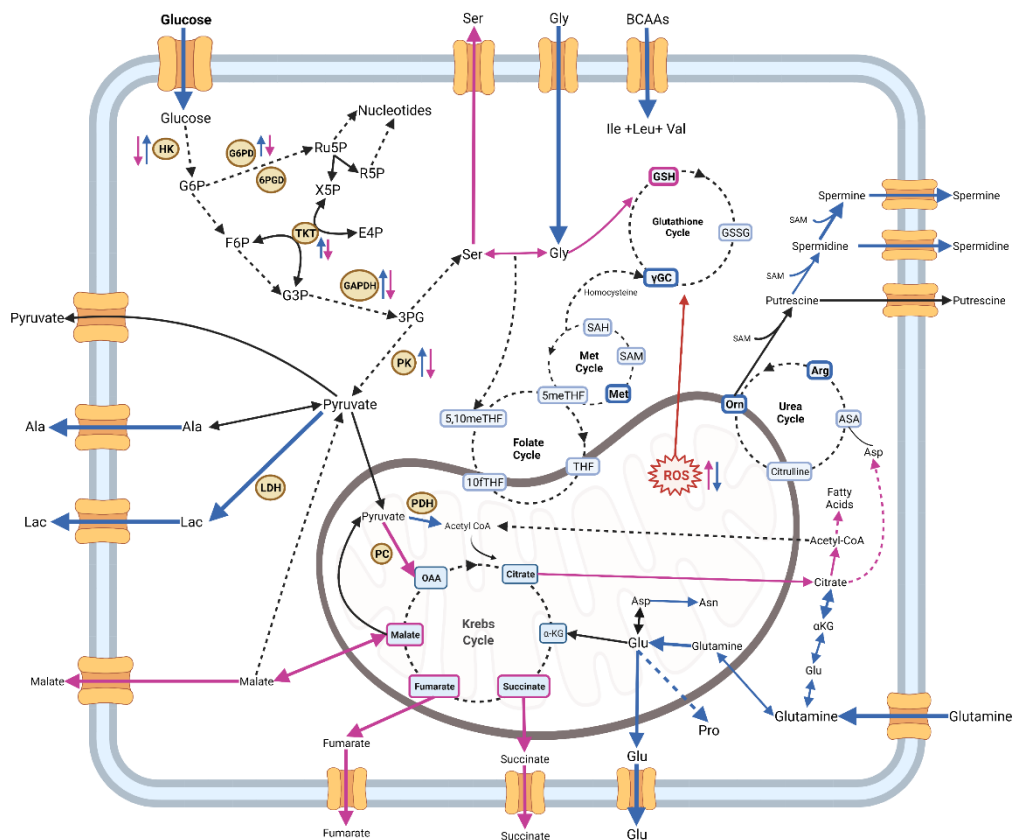


Figure 4.2.24- Metabolic map of changes induced in HAP1 cell lines in hypoxia. Built with the metabolic characterization experiments of these body of work. Blue arrows correspond to paths favoured by HAP1 Parental and pink arrows correspond to TET2 KO cell line. Created with BioRender.com. Abbreviations: 3-phosphoglycerate (3PG), 5-methyl-tetrahydrofolate (5meTHF), 5,10-methylene-tetrahydrofolate (5,10meTHF), 6-phosphogluconate dehydrogenase (6PGD), 10-formyl-tetrahydrofolate (10fTHF), Alanine (Ala), α -Ketoglutarate (α KG), Arginine (Arg), Argininosuccinic acid(ASA), Asparagine (Asn), Aspartate (Asp), Branched-chain amino acids (BCAAs), Erythrose-4-phosphate (E4P), Fructose-6-phosphate (F6P), γ -Glutamyl cysteine (γ GC), Glucose-6-phosphate (G6P), Glucose-6-phosphate dehydrogenase (G6PD), glyceraldehyde-3-phosphate (G3P), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Glutamate (Glu), Glutathione (GSH), Glutathione disulphide (GSSG), Glycine (Gly), Hexokinase (HK), Isoleucine (Ile), Lactate (Lac), Lactate dehydrogenase (LDH), Leucine (Leu), Methionine (Met), Ornithine (Orn), Oxaloacetate (OAA), Proline (Pro), Pyruvate carboxylase (PC), Pyruvate dehydrogenase (PDH), Pyruvate kinase (PK), Reactive oxygen species (ROS), Ribose-5-Phosphate (R5P), Ribulose-5-phosphate (Ru5P), S-adenosyl-homocysteine (SAH), S-adenosyl-methionine (SAM), Serine (Ser), Transketolase (TKT), Tetrahydrofolate (THF), Valine (Val), Xylulose-5-phosphate (X5P).

The loss of TET2 appears to drive the cell to continue the TCA cycle, with increased expulsion of citrate, succinate, fumarate and malate. This goes in accordance that TET2 and IDH1/2 mutations are mutually exclusive, resulting in a similar phenotype. IDH mutations produce 2HG, an oncometabolite that blocks dioxygenases such as TET2, with consumption of NADPH instead of production^{47,93,487,488}. Given that the mutated enzyme does not produce succinate, anaplerotic reactions are necessary to feed the cycle but limited because BCAAs catabolism is impaired since BCATs are also dioxygenases affected by 2HG. The solution appears to be a reverse cycling, from oxaloacetate into succinate, explaining the preference for pyruvate carboxylase. A side consequence is that more fumarate and succinate also exert a blocking effect in dioxygenases, such as epigenetic writers/erasers, BCATs and PHDs, among many others. This leads to more impediment to metabolic routes, genomic instabilities and a boosting to HIFs-mediated gene expression.

These changes to mitochondrial activity result in the 4th major alteration, an increase in ROS levels and necessary detoxification through glutathione. Without TET2, the cells require more GSH, which drives the production of glycine through the One-Carbon metabolism. This also influences the levels of serine and most probably the folate cycle.

The Parental cells do not possess the same requirements of ROS detoxification but still have production of γ -glutamyl cysteine. This arises from a more active methionine cycle, which results in more homocysteine available to make precursors of GSH. Instead, the Parental cells appear to have more detoxification through the urea cycle, to remove the excess of ammonia from the increased glutaminolysis.

With more activity of the methionine and urea cycles, the Parental cells have more precursors to generate polyamines, that help maintaining DNA stability and cellular homeostasis.

The different methionine levels could be a consequence of the loss of TET2, for it lowers the requirements of SAM. While SAM is used by DNMTs, a constant state of DNA hypermethylation arises from the absence of TET2, meaning that less *de novo* methylation is requiring methyl groups from SAM.

All these differences summarized are possible venues for treatment, for both wildtype and TET2 mutated leukaemias, that should be investigated. The increased glutaminolysis can be counteracted with glutaminase inhibitors, such as BPTES and CB-839, both under testing for clinical application across many types of cancer with glutamine addiction⁴⁸⁹. The use of PDH and pyruvate carboxylase inhibitors could help to confirm which is the preferred entry point of pyruvate into the TCA cycle with the loss of TET2⁴⁹⁰⁻⁴⁹².

BCAAs metabolism has been linked with leukemogenesis, with overexpression of BCAT1 resulting in a similar phenotype to mutated IDH^{95,251,252}. Inhibition of BCAT1 was reported to impair cancer-initiating cells but leaves normal HSPCs unaffected⁹⁵, being a possible treatment to early on stage before the TET2-mutated status induced more genetic alterations.

With a mutated TET2, targeting the apoptotic signalling is another option, given the fragility of the mitochondria under oxidative stress. One such example is Venetoclax, an inhibitor of Bcl-2 function, allowing apoptosis to occur and a standard treatment in CLL and AML^{45,52}.

Many studies have shown that TET2 can have its activity recovered by administrating ascorbate (vitamin C). As mentioned in chapter 1.1.5.2, vitamin C is a cofactor of TET enzymes and many other dioxygenases and α KG-dependent enzymes^{80,87}. Its administration can activate the other TETs (TET1 and TET3), increase the 5hmC levels and induce the DNA response repair⁸⁷. It is transported by its specific Sodium Vitamin C co-transporters (SVCTs) in reduced form or by GLUT3 in its oxidised form²⁷⁶. It was

proven to impede tumour proliferation and induce myeloid differentiation^{86,87,97}. Its activity remains effective in the presence of many other prevalent leukemic mutations, such as p53, IDH, WT1 and EZH2⁴⁹³⁻⁴⁹⁵. In fact, vitamin C treatment alters the methylation status of promoters of many transcription factors which are leukaemia-initiators such as CEBP β and RUNX1⁴⁹⁶.

Other drugs with proven results in recovering TET2 function are the hypomethylating agents 5-azacytidine and its deoxy derivative Decitabine^{497,498}. These molecules can incorporate the DNA and when DNMTs try to apply the methylation labelling, it binds irreversibly and forces DNA repair. The use of these drugs has great synergy with ascorbate administration⁴⁹⁸ and inhibitors of DNA repair mechanisms⁴⁹⁹, activating apoptosis.

Confirmation of the effect of all these possible treatments would require an enormous pipeline of experiments that was not possible given the worldwide restrictions imposed on the past two years for health security reasons. But continuation of these investigations is of the upmost necessity, as a means to develop a standardized response for leukaemia patients that present TET2 mutations and at high risk of acquiring more mutations that will result in resistance to standard treatments.

5. GENERAL DISCUSSION

5 General Discussion

Leukaemia is one of the cancers that despite the great advancements in carcinogenesis, diagnostic and treatment^{19,22,23,31}, the understanding of how the synergy of the many distinct mutations that give rise to this haematological disease is still lacking. Another layer of knowledge that only now is starting to be peeled is how leukemic cells alter their metabolism with each mutation during tumorigenesis in order to augment their proliferation under the challenge of hypoxia. In fact, metabolic adaptation is one of the main Hallmarks of Cancer⁴, due to the important role in allowing cancer proliferation across many distinct types.

Since leukaemia treatment decisions nowadays accounts for the mutational profile of the patient, the elucidation of how specific mutations drive the metabolism of leukemic cells in hypoxia is of most importance. Specially to account for possible resistance to therapy arising from leukemic stem cells that avoid the cytotoxic effects by remaining deep in the hypoxic conditions of the haematological niche of the bone marrow^{13,373,390,500}.

For this reason, the aim of this thesis was to perform metabolic characterization of leukemic cell models presenting specific mutations that are reported to be involved in the metabolic reprogramming of hypoxia adaptation and also involved in tumorigenesis and poor prognosis.

Transketolase-like 1 (TKTL1) is a key enzyme of metabolism, linking glycolysis to the production of nucleotides, a necessary component for *de novo* synthesis of RNA and DNA, especially in rapidly proliferating cells^{54,442}. Correlation of its expression with cancer malignancy and poor prognosis has been established across many types of cancer but how it impacts these functions remain unclear to this day. One such type is AML, where the putative role of TKTL1 overexpression remains to be properly pursued.

Equal in status is the ten-to-eleven Methylcytosine dioxygenase 2 (TET2), with clear established causation during the leukemogenesis process of various types of haematological malignancies and loss of function mutations induced by hypoxia^{86,477}. This epigenetic eraser is tightly linked to the metabolic behaviour of cancer cells because it requires α -ketoglutarate as its co-factor and its binding site can suffer competition by succinate, fumarate and the oncometabolite 2-hydroxyglutarate, produced by mutated IDH, another very common mutation in leukaemia^{47,94,488,501}. But to this day is still unclear how the loss of TET2 activity can alter the cellular metabolism, in order to contribute for cancer proliferation in the hypoxic niche.

Our analysis of the cell models THP-1 wildtype and THP-1 with a knockdown of TKTL1 using a combination of targeted-metabolomics and transcriptomic profiling under the state of chronic hypoxia have elucidated that this isoenzyme plays a role on the adaptation triggered by hypoxia.

The standard hypoxic changes to metabolism, such as increased glycolysis and glutamine consumption are impaired without TKTL1. This impact was seen at various levels, from gene expression, protein regulation, decreased intake due to less membrane transporters expressed. These changes reflected on the total flux of glycolysis, leading to less lactate production and less Warburg Effect, ultimately resulting in less proliferation of the cells with knockdown of TKTL1.

On the other hand, the metabolic characterization of the HAP1 cell models Parental and TET2 knockout exhibit similar proliferation rates under hypoxic conditions, as well as equal glucose intake and lactate production, showing that the TET2 mutation does not impair cancer proliferation.

A common point between both these functionally distinct proteins under analysis was the profile of enzymatic activity. Both for TKTL1 knockdown cells and TET2 KO, the enzymatic response of the glycolysis and PPP key proteins are affected in both normoxia and hypoxia, never reaching the

same capacity as the wildtype counterparts. This indicates that a major of protein regulation of these processes is dependent of another factor that is clearly affected by the loss of both TKTL1 and/or TET2, which results in an impaired response to hypoxia reprogramming that would increase the expression of these enzymes to increase the glycolytic and PPP flux. Curiously, the enzyme that is not affected at all by the removal of TKTL1 or TET2 is LDH. In fact, the knockdown of TKTL1 and the knockout of TET2 in hypoxia sees both an increase of LDH activity over their control pairings. This indicates that LDH regulation is independent of the other glycolytic and PPP enzymes. Another consideration is that LDH is an enzyme that drives acidic conditions of the microenvironment, which affects the immune response to cancer cells. These changes in pH of haematological niche leads to favourable conditions for immune response evasion^{502,503}. Noteworthy is the fact that high LDH levels are considered a tumoral burden parameter, across different types of cancer⁵⁰²⁻⁵⁰⁵. We can then infer that both the mutations under study increase the LDH activity levels, aiding the haematological malignancies that lie in the hypoxic niche not only energetically but also to manipulate the microenvironment in order to proliferate and survive.

The changes of G6PD activity for both the proteins under study arise from different roots and end in the same result. G6PD is one of the main enzymes involved in maintaining the pool of NADPH/NADP⁺ and as such is tightly regulated, both at gene and protein level. We saw that the TKTL1 knockdown lead to a compensatory mechanism in hypoxia to maintain G6PD activity by expressing SIRT5⁴⁵². This deacylase has been reported to regulate many proteins by different post-translational modifications in a NAD⁺-dependent manner⁵⁰⁶, such as IDH2, another producer of NADPH, and CPS1, the rate-limiting enzyme to initiate the urea cycle. This expression of SIRT5 induced by the knockdown of TKTL1 indicates that the cell requires compensatory mechanisms that manage oxidative stress

response, opening new opportunities for therapy development focused to impair this process.

G6PD was found recently to be linked in supplying methyl groups for DNA methylation⁴⁷⁷. The knockout of TET2 results in dysregulation of the methylation status of DNA, with both hyper- and hypomethylated areas. The less G6PD activity can be a consequence of this abnormal methylation status, since we observed a shift in lesser PPP flux to distinct substrates in both normoxia and hypoxia in TET2 KO cells, but further investigations are required.

Another central point of metabolism that is changing in our analysis of these models was how pyruvate travels to the mitochondria. The TKTL1 knockdown impaired the inhibition of the PDH complex by less expression of PDKs isoenzymes, which are a common feature of the hypoxic response. This adds to the impaired Warburg Effect without TKTL1, by not properly shifting from OXPHOS into anaerobic glycolysis.

Our ¹³C-tracer based metabolomics analysis of TET2 KO cells was able to clarify that the entry point of pyruvate into the mitochondria shifts due to this mutation. Without TET2, the cells use Pyruvate Carboxylase as the means to convert pyruvate into oxaloacetate to enter the TCA cycle while the Parental cells favour the use of PDH.

Regarding the glutamine metabolism, our cell models present totally distinct behaviours. THP-1 cells with TKTL1 have a dependency of glutamine in hypoxia, increasing its consumption through expression of transporters and also augmenting its synthesis through GS. It also shifts many other amino acids metabolism in order to obtain more glutamine as well as substrates for proliferation, like pyrimidine synthesis from serine.

Of note is the increase in gene expression of IDO2, responsible for the production of kynurenine, a known biogenic amine used by leukemic cells to suppress the immune response because it is toxic to T-cells and Natural Killers (NK) cells^{198,315}.

All these advantages are affected by the knockdown of TKTL1, unveiling its role on the central metabolism beyond its standard enzymatic function. In fact, it is necessary for the metabolic reprogramming to hypoxia to occur with the oxidative stress under control. The compensatory mechanisms that arise on TKTL1 knockdown cells, such as SIRT5, HK3 isoenzyme⁴⁶² and PRMT8⁴⁶⁷ are reported to help with ROS homeostasis, mitochondrial biogenesis and stress tolerance, further confirming this function for TKTL1.

On our HAP1 cell models, the glucose metabolism is highly favoured over glutamine, with neither of the cell lines increasing their glutamine consumption in hypoxia. The glutamine metabolism that occurs in hypoxia is coursed through distinct paths according to the status of TET2. The ¹³C-tracer based metabolomics analysis revealed that cells that retain TET2 have a predilection for reductive carboxylation of glutamine while the loss of TET2 activity drives the cells to perform the oxidative path through the TCA cycle. Of note is the fact that glutamate production on TET2 KO in the hypoxic condition analysed through ¹³C-labelling was decreasing from glucose and glutamine sources, meaning that more transamination reactions are occurring to produce unlabelled glutamate.

These changes in combination with the distinct entry path of pyruvate indicate that cells that suffer mutations to TET2 have a distinct TCA cycle activity. Since TET2 is an α KG-dependent enzyme, reductive carboxylation is a metabolic route to obtain this metabolite for its activity without producing succinate and fumarate, the competitors to α KG binding site on TET2. Loss of TET2 no longer imposes this consideration, leading the cells to have more TCA cycle activity.

Like on our THP-1 cell models, the changes in glutaminolysis and TCA cycle induced by TET2 knockout are dysregulating the oxidative stress of the cells in hypoxia, as seen by the probing of ROS levels. The metabolism of other amino acids is changing in response to this, trying to feed

intermediates to the TCA cycle, with additional production of glutamate off transamination reaction from BCAAs catabolism²²². Another change to amino acids metabolism is the use of One-Carbon metabolism, to compensate the lack of NADPH from G6PD decrease but also to produce glycine. Both the increase in glycine and glutamate production was reflected on glutathione synthesis in our analysis of ¹³C-tracer metabolomics. We can conclude from this that the loss of TET2 forces the cells in hypoxia to redirect their amino acid metabolism for glutathione synthesis, as a mechanism to try to regulate the higher levels of ROS. This in turn translates into less serine and PPP substrates available for nucleotide synthesis.

Interestingly, the common points of change between a PPP enzyme overexpression and the loss of an epigenetic marker are all related to the generation of redox power and substrates for proliferation, with G6PD appearing at the centre of those changes. TET2 mutations have been reported to lead to genomic instability, by altering the DNA methylation status of many oncogenes and TSGs⁸¹. TKTL1 has also been linked with epigenetic alterations, with reports of promoter hypomethylation during carcinogenesis of neck squamous cell carcinoma and melanoma^{58,59}. Both enzymes of our study were also reported to have synergy with other typical mutations in carcinogenesis^{67,77,83,91}.

The effect seen on hypoxia indicates that both TKTL1 and TET2 play a role on the reprogramming of the metabolism for adapting to chronic hypoxia. While TKTL1 is a clear advantage in proliferation, granting more oxidative stress control for the cells in hypoxia, the loss of TET2 appears to be a mutation that favours the development of haematological malignancies. Despite the shift in metabolism seen in our characterization that more detoxification of ROS is required, it has been reported that continued oxidative stress favours carcinogenesis, explaining the genomic instability

reported by TET2 mutations and also by its inhibition by the oncometabolite 2-HG.

Recent investigations of the use of epigenetic drugs and their effect on these enzymes have started to surface. For TKTL1, to this day the only report using the epigenetic drug Azacitidine (5-Aza) was that this hypomethylating agent induced its expression⁵⁹, exactly the effect we do not want in a leukaemia patient. TET2 has much more promising potential, by use of the deoxy-derivative of 5-Aza, Decitabine^{35,109,507}, as well as administration of vitamin C (ascorbic acid)^{86,87} showing improvements across various types of leukaemia^{87,493–497,508,509}. As a proof of potential of vitamin C, a case of an AML patient that failed to respond to induction chemotherapy had a clinical remission of over 2-years by ascorbate treatment, eventually relapsing due to new acquirement of mutations⁴⁹⁴. Many of the studies of vitamin C treatment showed no additional cytotoxic effects when combined to other therapies, with the combination of decitabine and vitamin C presenting good synergy^{498,508,510}. Another combination that presents high potential was highlighted from a synthetic lethals study of mutated TET2 with inhibitors of Poly-ADP-ribose Polymerase 1 (PARP1) and Topoisomerase 1 (TOP1) targeted drugs, resulting in DNA double-strand breaks and inability to repair it, resulting in cell death⁴⁹⁹.

One point that shows that epigenetic targeting for treatment must be a personalized medicine approach is the use of sirtuin activators. Guan et al reported TET2 reactivation by sirtuin activators, that lead to deacylase of TET2 and subsequent activation⁵¹⁰. But given our discovery of the use of sirtuins, more specifically SIRT5, as a coping mechanism for cells with TKTL1 knockdown, the use of these activators must be carefully tailored for the leukemic genotype of each patient.

For all those reasons, metabolic characterization must be included on a leukemic patient process of diagnostic, to properly stratify its response to

the broad array of possible therapy combinations that modern medicine has produced in the last years.

Further investigations of the new role of TKTL1 over metabolism in hypoxic conditions must be pursued, with proof of concept on other AML cell lines and primary patient-derived cells, that could reveal new approaches for therapies that combine targeting TKTL1 and one of the “weaknesses” that arise from its knock down.

Similarly, further studies of TET2 must continue with transcriptomic and epigenetic analysis to verify the metabolomic changes arising from the distinct DNA methylation status resulting from loss of TET2. Subsequent comparative analysis in other types of leukaemia cell lines would be required to confirm our hypothesis of the advantages that arise from such mutation in a hypoxic environment.

The results of this thesis add to the pool of knowledge for the inclusion of metabolic characterization as a diagnostic tool for personalised medicine, to aid the betterment of quality of life of patients undergoing the difficult process of surviving leukaemia.

6. CONCLUSIONS

6 Conclusions

1. TKTL1 is essential for the metabolic adaptation of leukemic cells to hypoxia as well as the mutation of TET2, driving the Warburg Effect and cell proliferation.
2. TKTL1 knockdown showed impairment of the gene expression of membrane transporters and key enzymes of the hypoxia response, altering the flux of glycolysis as well as the use of pyruvate to OXPHOS by impaired inhibition of PDH. The loss of TET2 had a similar effect, shifting the use of pyruvate from PDH and into pyruvate carboxylation.
3. Both enzymes of this study are influential on the Pentose Phosphate Pathway flux in hypoxia, with their loss resulting in an impaired production of NADPH and nucleotides.
4. TKTL1 also influences the glutamine metabolism, by increasing the intake and catabolism of amino acids for synthesis of glutamine and nucleotides.
5. Mutation of TET2 impairs the leukemic cells from triggering the hypoxic switch to reductive carboxylation of glutamine, leading to ROS dysregulation through higher TCA cycle activity.
6. Metabolic characterization of loss of TET2 and impaired TKTL1 highlight coping mechanisms against oxidative stress, through protein regulation and One-carbon metabolism to obtain redox power, in the form of NADPH and GSH, opening new avenues for therapy development.

7. BIBLIOGRAPHY

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8. APPENDIX I

8 Appendix I

8.1 Targeted metabolomics experiments for THP-1 cell models.

The Biocrates Absolute IDQ™ p180 kit (Biocrates Life Sciences AG, Austria) was performed as indicated in chapter 3.9 (full experimental procedure). Of the metabolites obtained, we analysed the intracellular concentrations and consumption/production rates of 21 amino acids (Table 8.1-A and Table 8.1-C, respectively) and 19 biogenic amines (Table 8.1-B and Table 8.1-D, respectively).

Metabolite	Concentration (nmol/mg protein)							
	THP-1 ^{WT} Normoxia		THP-1 ^{KD} Normoxia		THP-1 ^{WT} Hypoxia		THP-1 ^{KD} Hypoxia	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Ala	402.3	143.5	473.6	144.6	346.5	5.6	279.0	79.0
Arg	273.4	77.0	272.0	150.9	292.7	144.4	111.3	86.2
Asn	771.5	228.9	563.3	54.5	474.1	39.6	702.8	395.8
Asp	118.2	37.5	136.5	46.4	37.0	10.1	55.3	44.1
Cit	0.2	0.2	3.2	4.7	2.5	2.9	0.5	0.9
Gln	1636.5	205.7	1760.4	510.5	1377.2	295.8	1100.2	204.7
Glu	855.5	1481.8	1090.0	943.9	0.0	0.0	1609.7	571.9
Gly	915.6	375.1	1075.9	575.8	583.6	285.9	779.0	222.7
His	72.8	13.7	46.9	17.3	59.0	8.2	36.6	16.4
Ile	185.4	38.6	200.0	39.0	210.7	76.2	153.9	44.0
Leu	236.2	55.2	248.5	52.7	249.8	63.0	168.8	66.5
Lys	40.3	17.6	53.4	8.7	63.6	23.2	39.0	25.7
Met	71.6	20.6	66.4	25.4	63.1	21.3	41.6	22.5
Orn	22.3	10.4	21.3	9.0	35.9	6.4	29.1	16.4
Phe	42.5	7.4	39.6	11.2	59.4	22.2	34.3	9.9
Pro	705.6	139.5	744.0	116.1	249.7	37.7	191.4	38.5
Ser	70.2	23.9	129.2	90.8	353.9	116.4	186.9	34.3
Thr	173.8	92.9	142.2	28.2	200.7	89.8	111.9	40.3
Trp	11.0	3.7	12.8	1.8	16.1	6.5	9.7	1.0
Tyr	76.1	26.8	91.2	14.1	89.8	32.8	82.1	19.3
Val	54.4	19.1	40.2	5.7	60.3	7.3	51.9	20.8

Table 8.1-A- Intracellular concentrations of amino acids in THP-1^{WT} and THP-1^{KD} cell models. The intracellular content of these metabolites was obtained using the Biocrates Absolute IDQ™ p180 kit (Biocrates Life Sciences AG, Austria) after 48h incubation with RPMI 1640 10mM Glc and 4mM Gln, 5% S/P and 1% FBS, in both normoxia and hypoxia conditions.

Concentration (nmol/mg protein)								
Metabolite	THP-1 ^{WT} Normoxia		THP-1 ^{KD} Normoxia		THP-1 ^{WT} Hypoxia		THP-1 ^{KD} Hypoxia	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Ac-Orn	2.4	2.3	1.2	1.9	3.3	1.8	2.6	3.0
ADMA	2.2	1.5	1.6	0.8	1.3	0.4	0.7	0.7
alpha-AAA	7.1	2.4	6.3	2.8	5.4	5.4	4.6	0.5
c4-OH-Pro	0.0	0.0	0.3	0.5	0.0	0.0	0.0	0.0
Carnosine	0.8	0.6	0.5	0.2	0.4	0.2	0.2	0.1
Creatinine	16.7	6.9	15.2	2.3	10.4	2.3	11.0	1.9
DOPA	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Dopamine	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Histamine	0.3	0.3	0.2	0.4	0.0	0.0	0.3	0.4
Kynurenine	0.4	0.2	0.4	0.1	0.4	0.2	0.5	0.2
Met-SO	1.9	0.6	2.0	1.0	2.8	1.5	2.8	1.4
Nitro-Tyr	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
PEA	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Putrescine	3.0	1.3	3.6	1.8	3.0	0.9	3.2	0.5
SDMA	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Serotonin	0.1	0.0	0.2	0.1	0.0	0.0	0.0	0.0
Spermidine	45.4	16.2	36.5	5.3	23.8	9.0	26.5	7.4
Spermine	20.9	11.7	14.6	2.4	13.8	7.3	9.7	4.5
t4-OH-Pro	221.3	22.6	202.1	40.3	130.6	4.6	130.6	58.5
Taurine	469.1	82.5	464.1	362.0	196.0	188.3	219.2	200.8
total DMA	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.2

Table 8.1-B- Intracellular concentrations of biogenic amines in THP-1^{WT} and THP-1^{KD} cell models. The intracellular content of these metabolites was obtained using the Biocrates Absolute IDQTM p180 kit (Biocrates Life Sciences AG, Austria) after 48h incubation with RPMI 1640 10mM Glc and 4mM Gln, 5% S/P and 1% FBS, in both normoxia and hypoxia conditions.

Flux Rates (nmol*millioncell-1*h-1)								
Metabolite	THP-1 ^{WT} Normoxia		THP-1 ^{KD} Normoxia		THP-1 ^{WT} Hypoxia		THP-1 ^{KD} Hypoxia	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Ala	0.6	0.0	0.6	0.1	0.8	0.1	0.7	0.0
Arg	-0.4	1.5	-3.2	0.2	-1.0	1.0	-2.7	0.1
Asn	-0.5	0.0	-2.0	0.3	-0.1	0.0	0.7	0.1
Asp	-0.4	0.3	-0.4	0.0	-0.5	0.1	-0.4	0.2
Cit	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Gln	-7.6	0.2	-7.0	0.4	-4.6	1.0	-4.9	0.3
Glu	2.2	0.1	1.6	0.0	1.3	0.1	1.0	0.0
Gly	-0.4	0.1	-0.2	0.6	2.7	3.5	1.1	0.2
His	-0.4	0.1	-0.6	0.1	0.1	0.0	-0.4	0.0
Ile	-1.0	0.1	-1.6	0.0	-0.5	0.1	-0.6	0.0
Leu	-2.0	0.1	-2.4	0.1	-1.2	0.1	-1.0	0.1
Lys	-1.3	0.1	-1.7	0.1	-0.4	0.1	0.2	0.2
Met	-0.4	0.0	-0.4	0.0	-0.2	0.1	0.1	0.0
Orn	0.6	0.1	0.6	0.1	1.3	0.0	1.4	0.2
Phe	-0.6	0.0	-0.5	0.1	-0.1	0.0	-0.2	0.0
Pro	-0.1	0.1	-0.2	0.0	0.3	0.1	0.2	0.1
Ser	-2.4	0.1	-2.3	0.1	-2.6	0.0	-2.6	0.1
Thr	-0.7	0.0	-1.1	0.0	-0.5	0.0	-0.1	0.0
Trp	-0.2	0.0	-0.2	0.0	0.0	0.0	-0.1	0.0
Tyr	-0.4	0.0	-0.6	0.0	0.1	0.1	-0.1	0.0
Val	-1.1	0.0	-1.1	0.1	-0.3	0.0	-0.6	0.3

Table 8.1-C- Consumption and production rates of amino acids in THP-1^{WT} and THP-1^{KD} cell models. The consumption/production rates of these metabolites was obtained using the Biocrates Absolute IDQTM p180 kit (Biocrates Life Sciences AG, Austria) to measure culture medium at timepoints 0h and 48h incubation with RPMI 1640 10mM Glc and 4mM Gln, 5% S/P and 1% FBS, in both normoxia and hypoxia conditions. Calculations were done according to chapter 3.3.

Flux Rates (nmol*millioncell-1*h-1)								
Metabolite	THP-1 ^{WT} Normoxia		THP-1 ^{KD} Normoxia		THP-1 ^{WT} Hypoxia		THP-1 ^{KD} Hypoxia	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Ac-Orn	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
ADMA	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
alpha-AAA	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
c4-OH-Pro	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Carnosine	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Creatinine	0.0	0.0	-0.1	0.0	0.0	0.0	0.0	0.0
DOPA	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Dopamine	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Histamine	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Kynurenine	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Met-SO	0.0	0.0	0.0	0.0	-0.2	0.0	-0.2	0.0
Nitro-Tyr	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
PEA	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Putrescine	3.0	1.3	3.6	1.8	3.0	0.9	3.2	0.5
SDMA	NA	NA	NA	NA	NA	NA	NA	NA
Serotonin	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Spermidine	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Spermine	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
t4-OH-Pro	-0.1	0.0	-0.4	0.1	0.5	0.1	0.2	0.1
Taurine	0.0	0.0	-0.1	0.0	0.0	0.0	0.0	0.0
total DMA	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Table 8.1-D- Consumption and production rates of biogenic amines in THP-1^{WT} and THP-1^{KD} cell models. The consumption/production rates of these metabolites was obtained using the Biocrates Absolute IDQTM p180 kit (Biocrates Life Sciences AG, Austria) to measure culture medium at timepoints 0h and 48h incubation with RPMI 1640 10mM Glc and 4mM Gln, 5% S/P and 1% FBS, in both normoxia and hypoxia conditions. Calculations were done according to chapter 3.3.

8.2 Targeted metabolomics experiments for HAP-1 cell models.

The Biocrates Absolute IDQ™ p180 kit (Biocrates Life Sciences AG, Austria) was performed as indicated in chapter 3.9 (full experimental procedure). Of the metabolites obtained, we analysed the intracellular concentrations and consumption/production rates of 21 amino acids (Table 8.2-A and Table 8.2-C, respectively) and 19 biogenic amines (Table 8.2-B Table 8.2-D, respectively).

Metabolite	Concentration (nmol/mg protein)							
	HAP1 Parental Normoxia		HAP1 TET2 KO Normoxia		HAP1 Parental Hypoxia		HAP1 TET2 KO Hypoxia	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Ala	206.7	61.1	76.5	11.9	364.8	247.2	193.3	30.4
Arg	41.5	5.4	12.7	4.0	99.6	50.8	84.8	48.8
Asn	82.4	21.2	37.6	11.0	318.2	185.7	102.2	40.2
Asp	79.7	138.0	129.8	34.2	212.5	154.5	160.8	19.0
Cit	0.8	0.2	0.1	0.2	0.0	0.0	0.0	0.0
Gln	680.3	350.7	311.6	61.1	2182.0	254.7	520.7	57.7
Glu	0.0	0.0	102.3	177.2	1503.7	1621.7	398.9	691.0
Gly	328.7	284.7	105.4	44.0	497.1	134.0	217.9	197.1
His	36.0	6.9	6.7	3.1	77.2	26.1	22.3	11.4
Ile	68.0	21.1	23.4	5.3	246.9	89.4	55.9	9.7
Leu	78.9	25.2	21.5	2.5	307.4	98.0	59.1	15.3
Lys	147.1	53.0	47.1	13.4	424.8	114.2	91.9	22.0
Met	16.6	4.3	8.0	0.7	90.0	43.1	16.9	7.1
Orn	10.4	5.0	5.4	2.1	35.4	11.0	8.7	6.4
Phe	34.5	12.7	8.2	1.2	107.8	42.8	26.7	8.0
Pro	231.1	48.3	75.7	16.0	321.6	160.8	87.6	8.3
Ser	106.9	28.7	51.1	22.4	412.8	241.0	80.6	67.0
Thr	82.7	143.2	95.0	25.0	408.0	103.0	174.5	96.5
Trp	8.1	1.1	1.0	0.1	20.1	13.8	3.6	0.3
Tyr	37.9	10.4	9.7	1.7	147.3	62.8	28.8	5.2
Val	56.3	13.3	13.8	2.5	140.1	83.7	50.5	19.6

Table 8.2-A- Intracellular concentrations of amino acids in HAP-1 Parental and HAP-1 TET2 KO cell models. The intracellular content of these metabolites was obtained using the Biocrates Absolute IDQ™ p180 kit (Biocrates Life Sciences AG, Austria) after 48h incubation with IMDM 25mM Glc and 4mM Gln, 5% S/P and 1% FBS, in both normoxia and hypoxia conditions.

Concentration (nmol/mg protein)								
Metabolite	HAP1 Parental Normoxia		HAP1 TET2 KO Normoxia		HAP1 Parental Hypoxia		HAP1 TET2 KO Hypoxia	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Ac-Orn	0.8	0.6	0.0	0.0	0.0	0.0	0.0	0.0
ADMA	1.2	0.2	0.6	0.3	4.2	2.5	0.6	0.2
alpha-AAA	6.8	2.7	11.5	1.9	8.5	5.3	6.8	3.8
c4-OH-Pro	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Carnosine	0.2	0.1	0.0	0.0	0.5	0.5	0.1	0.0
Creatinine	10.0	2.4	1.7	0.6	27.8	14.8	10.4	1.0
DOPA	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Dopamine	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Histamine	0.1	0.1	0.0	0.0	0.0	0.0	0.1	0.1
Kynurenine	0.1	0.1	0.1	0.0	0.6	0.2	0.1	0.1
Met-SO	0.5	0.3	0.1	0.1	2.3	0.6	0.5	0.0
Nitro-Tyr	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
PEA	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Putrescine	4.7	1.4	9.9	1.5	11.1	3.5	2.5	0.4
SDMA	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Serotonin	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Spermidine	29.9	10.0	16.4	2.2	72.4	24.2	16.8	2.8
Spermine	15.4	3.8	6.6	0.6	51.4	19.6	9.0	1.5
t4-OH-Pro	4.4	1.4	1.2	0.1	6.8	1.9	2.3	0.8
Taurine	0.0	0.0	87.0	36.1	898.5	591.1	230.6	207.5
total DMA	0.8	0.2	0.1	0.1	0.3	0.5	0.2	0.1

Table 8.2-B- Intracellular concentrations of biogenic amines in HAP-1 Parental and HAP-1 TET2 KO cell models. The intracellular content of these metabolites was obtained using the Biocrates Absolute IDQ™ p180 kit (Biocrates Life Sciences AG, Austria) after 48h incubation with IMDM 25mM Glc and 4mM Gln, 5% S/P and 1% FBS, in both normoxia and hypoxia conditions.

Flux Rates (nmol*millioncell-1*h-1)								
Metabolite	HAP1 Parental Normoxia		HAP1 TET2 KO Normoxia		HAP1 Parental Hypoxia		HAP1 TET2 KO Hypoxia	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Ala	2.3	0.3	5.0	0.2	6.4	0.4	5.2	2.8
Arg	-4.0	0.0	-4.9	0.4	-5.5	1.5	-4.4	1.8
Asn	-1.1	0.1	-6.2	0.3	-1.8	1.6	-1.9	0.7
Asp	-0.6	0.2	-0.6	0.1	-2.8	1.8	-2.9	0.8
Cit	0.0	0.0	0.1	0.1	0.0	0.2	-0.1	0.0
Gln	-19.6	0.2	-17.5	0.9	-7.6	19.6	-21.0	2.1
Glu	1.8	0.8	5.1	0.2	4.1	11.1	3.0	3.6
Gly	-3.2	0.1	-2.3	0.1	-15.5	3.7	-12.7	1.1
His	-0.8	0.2	-1.5	0.2	-1.1	0.0	1.1	0.2
Ile	-4.1	0.2	-4.3	0.0	-9.4	0.0	-4.5	0.2
Leu	-6.3	0.3	-5.8	0.2	-16.9	0.2	-7.9	0.4
Lys	-3.0	1.0	-3.9	0.3	-11.1	3.2	-2.9	0.1
Met	-2.0	0.2	-1.2	0.0	-1.6	1.0	-2.3	0.4
Orn	0.1	0.0	0.3	0.0	1.0	0.1	1.6	0.1
Phe	-1.4	0.1	-1.1	0.2	-2.2	1.1	-0.4	0.1
Pro	1.6	0.1	1.8	2.0	3.6	2.8	1.8	0.0
Ser	-5.9	0.2	-4.1	0.5	-3.4	1.1	-3.0	1.5
Thr	-1.8	0.1	1.4	1.2	7.2	1.5	1.9	0.1
Trp	-0.4	0.0	-0.5	0.2	-0.9	0.3	-0.2	0.0
Tyr	-0.8	0.3	-2.0	0.1	-1.7	0.2	-1.3	0.2
Val	-4.0	0.2	-3.5	0.2	-5.3	4.6	-3.2	3.0

Table 8.2-C- Consumption and production rates of amino acids in HAP-1 Parental and HAP-1 TET2 KO cell models. The consumption/production rates of these metabolites was obtained using the Biocrates Absolute IDQ™ p180 kit (Biocrates Life Sciences AG, Austria) to measure culture medium at timepoints 0h and 48h incubation IMDM 25mM Glc and 4mM Gln, 5% S/P and 1% FBS, in both normoxia and hypoxia conditions. Calculations were done according to chapter 3.3.

Flux Rates (nmol*millioncell-1*h-1)								
Metabolite	HAP1 Parental Normoxia		HAP1 TET2 KO Normoxia		HAP1 Parental Hypoxia		HAP1 TET2 KO Hypoxia	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Ac-Orn	0.0	0.0	0.1	0.0	0.1	0.0	0.1	0.0
ADMA	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
alpha-AAA	0.0	0.0	0.1	0.0	0.2	0.1	0.2	0.0
c4-OH-Pro	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Carnosine	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Creatinine	0.0	0.0	0.0	0.0	0.3	0.0	0.1	0.0
DOPA	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Dopamine	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Histamine	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Kynurenine	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Met-SO	0.0	0.0	0.0	0.0	0.0	0.0	-0.1	0.0
Nitro-Tyr	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
PEA	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Putrescine	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SDMA	NA	NA	NA	NA	NA	NA	NA	NA
Serotonin	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Spermidine	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Spermine	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
t4-OH-Pro	0.0	0.0	0.1	0.1	0.1	0.2	0.1	0.0
Taurine	-0.1	0.0	-0.1	0.0	0.4	0.4	0.1	0.0
total DMA	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Table 8.2-D- Consumption and production rates of biogenic amines in HAP-1 Parental and HAP-1 TET2 KO cell models. The consumption/production rates of these metabolites was obtained using the Biocrates Absolute IDQ™ p180 kit (Biocrates Life Sciences AG, Austria) to measure culture medium at timepoints 0h and 48h incubation IMDM 25mM Glc and 4mM Gln, 5% S/P and 1% FBS, in both normoxia and hypoxia conditions. Calculations were done according to chapter 3.3.

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