



UNIVERSITA' DEGLI STUDI DI TORINO

**DOCTORAL SCHOOL OF LIFE AND HEALTH SCIENCES
DEPARTMENT OF CLINICAL AND BIOLOGICAL SCIENCES**

PhD Program in Experimental Medicine and Therapy

Cycle: XXXII

**ASSESSMENT OF PATHOLOGICAL AND BIOLOGICAL ASPECTS OF THE LARGE
E3 UBIQUITIN LIGASE HERC1 IN MYELOID HAEMATOLOGICAL
MALIGNANCIES**

Submitted by
Muhammad Shahzad Ali

Supervisor: Prof Giuseppe Saglio

**Co-supervisor: Asst. Prof. Enrico Bracco
Asst. Prof. Barbara Pergolizzi**

PhD Coordinator: Prof. Pasquale Pagliaro

**Academic years of Enrolment
2016-2020
CODE OF SCIENTIFIC DISCIPLINE: MED/15**

ABSTRACT

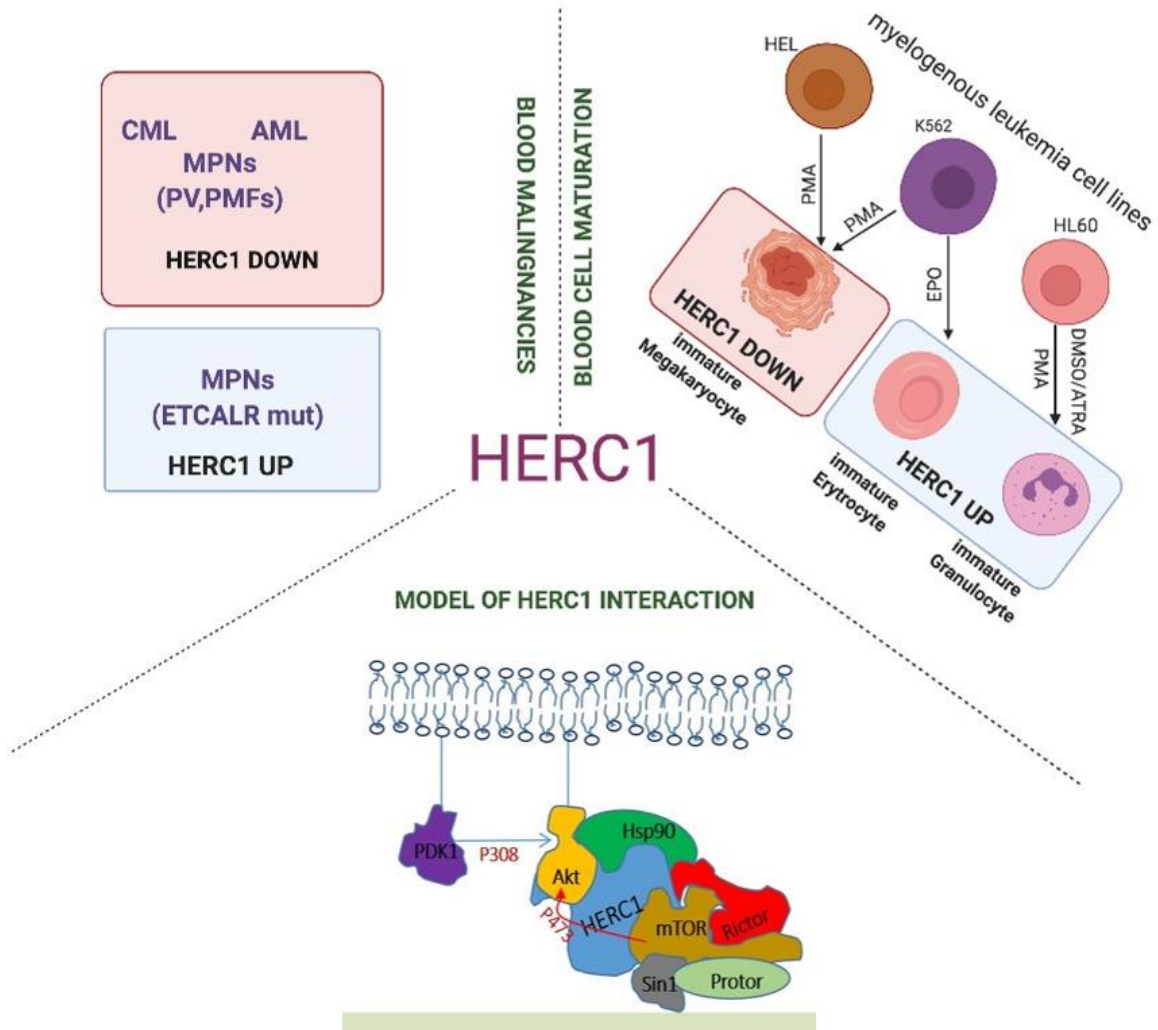
Normal blood cell production, a tightly regulated process known as hematopoiesis, is sustained by a trivial population of undifferentiated hematopoietic stem cells (HSCs) residing in our bone marrow. These cells inherent the self-renewal potential to give rise to itself without differentiation, but also able to differentiate, through balanced process, to generate all mature functional blood cell lineages, effectively fabricating around 100 million cells every second throughout the life of a healthy individual. Ubiquitination plays a major role in many aspects of hematopoiesis. Cellular protein degradation by the ubiquitin-proteasome system (UPS) regulates hematopoietic stem cell (HSC) self-renewal, differentiation, and survival. The balance between HSC self-renewal and differentiation is critical to maintain hematopoiesis throughout the life of the organism and any disruptions, such as random mutations to the critical genes in HSCs, of this balance often result in hematological malignancies.

In this thesis, to gain deeper insights into the mechanisms underlying transformation to malignant cell during HSCs self-renewal and differentiation/hematopoiesis, we for the first time investigated the potential association of the giant HECT E3 ubiquitin ligase HERC1 with differentiation, and its pathological role in myeloid related disorders such as, Chronic Myeloid Leukemia (CML), Myeloproliferative neoplasms (MPNs) and Acute Myeloid Leukemia (AML). Our findings revealed that the *HERC1* gene expression was severely down-regulated both in acute and in chronic myelogenous leukemia, whereas in Philadelphia chromosome-negative neoplasms its transcript level was peculiarly controlled. We also observed a differential *HERC1* gene expression when leukemic cell lines were induced to differentiate which revealed the pivotal role of HERC1 E3 ligase in the regulation of blood cells differentiation.

We also examined gene expressional interplay and physical association of HERC1 and BCR-ABL1. Our results revealed an inverse correlation between the gene expression of *HERC1* and *BCR-ABL1* in the evolution of CML. Interestingly the HERC1 protein physically interacts with BCR-ABL1 onco-protein and likely forming a very large supramolecular complex. Furthermore, we uncovered that HERC1 is a putative BCR-ABL1 tyrosine kinase substrate. Additionally, we also identified HSP90 and the components of mTORC2 complex (Rictor, mTOR), pAkt(S473) as novel binding partners of large HERC1.

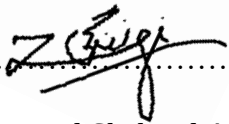
In conclusion, we have identified for the first time an association of large E3 ligase HERC1 with haematological malignancies in which *HERC1* transcript level was mostly down-regulated, thus likely acting as an onco-suppressor. Moreover, we noticed that there are few remarkable exceptions suggesting that its role is tightly context-dependent. HERC1 protein also represents a novel BCR-ABL1 interacting partner and substrate. Taken together with our findings that HERC1 as a novel regulator of blood cell differentiation and likely a potential mediator of Akt activation, might possibly open up a critical issue for further study to learn more about HERC1 biology and its role in human diseases.

GRAPHICAL ABSTRACT



AUTHOR'S DECLARATION

I undersigned declare that, the content of this thesis is the result of my own original work during the last three years. No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification in this or any other university or another institute of learning.



.....

Muhammad Shahzad Ali

To my lovely Mother, and the memory of my Father (late).

TABLE OF CONTENTS

ABSTRACT	i
GRAPHICAL ABSTRACT	iii
AUTHOR'S DECLARATION	iv
LIST OF FIGURES	ix
LIST OF TABLES	xi
ACKNOWLEDGMENTS	xii
LIST OF PUBLICATIONS	xiii
ABBREVIATIONS	xiv
Chapter1: INTRODUCTION	01
1. Hematopoiesis	01
1.1 A Brief Overview on Hematopoiesis	01
1.2 Myeloproliferative Neoplasms (MPNs)	03
1.3 Philadelphia Chromosome-Positive Myeloproliferative Neoplasms.....	05
1.3.1 Chronic Myeloid Leukemia Etiology.....	05
1.3.2 BCR-ABL1 Fusion Onco-proteins Function and Structure	07
1.3.3 Clinical aspects and CML Treatment	09
1.3.4 CML Stem Cells	11
1.4 Classic Philadelphia Chromosome-Negative MPNs.....	12
1.4.1 Polycythemia Vera (PV)	12
1.4.2 Essential Thrombocythemia (ET)	13
1.4.3 Primary Myelofibrosis (PMF)	15
1.5 Acute Myeloid Leukemia	17
1.5.1 Clinical Aspects of AML	17
1.5.2 AML Treatment	22
1.6 Ubiquitination	24
1.6.1 HERC protein family.....	27

1.6.2	Domain Organization of HERC1	27
1.6.3	<i>HERC1</i> Gene Variants (SNP and Mutations)	29
1.6.4	HERC1 Interactome and its Function	30
AIMS OF THE STUDY		32
Chapter2: MATERIAL AND METHODS		33
2.1 Patient Cohorts and Cell Lines		33
2.1.1	Clinical Samples	33
2.1.2	Cell Lines	33
2.1.3	Freezing and Thawing Cells	34
2.1.4	Cell Counting and Trypan Blue Exclusion	34
2.1.5	General Reagents	34
2.2 Separation of Cellular Fractions		36
2.2.1	Samples Preparation from Patients' PB and BM	36
2.2.2	CD34 ⁺ Cells Separation	36
2.2.3	CD3 ⁺ and CD19 ⁺ Cells Separation	37
2.2.4	<i>In Vitro</i> Treatment	37
2.2.5	Cytospin Preparation	37
2.2.6	Immunofluorescence	37
2.3 Analysis of Gene Expression		38
2.3.1	Isolation of RNA	38
2.3.2	Reverse Transcription and Gene Expression Analysis by qPCR	38
2.3.3	Isolation of Protein from Cells	40
2.3.4	Bradford Assay	41
2.3.5	SDS Gel Electrophoresis	41
2.3.6	Antibody Binding and Visualization	41
2.3.7	Estimation of Protein Loading	42
2.3.8	Antibodies for Western Blotting and Flow Cytometry	42
2.3.9	Immunoprecipitation	43
2.3.10	<i>In-vitro</i> Kinase Assay	43
2.3.11	Plasmids Amplification	44

2.3.12 Plasmid Construction and Transfection	44
2.4 Differentiation Analysis	45
2.4.1 Induction of Differentiation and Flow Cytometry	45
2.4.2 Benzidine Staining	45
2.4.3 Apoptosis Assay.....	46
2.5 Statistical Analysis	46
Chapter 3: RESULTS	47
3.1 <i>HERC1</i> Gene Expression is Aberrantly Regulated in Myeloid Related Disorders and Leukemias	47
3.2 Newly Diagnosed AML Display <i>HERC1</i> Downregulation Independently from the Genetic Alterations	48
3.3 MPNs Display a Peculiar Pattern of <i>HERC1</i> mRNA Abundance	50
3.4 <i>HERC1</i> Expression is Sensitive to the BCR-ABL1 Tyrosine Kinase Activity.....	51
3.5 <i>HERC1</i> is Severely Downmodulated in Myeloid Cell Population of CML Patient	53
3.6 <i>HERC1</i> gene Expression is Linked to Imatinib Treatment in CML Leukemia Cells..	55
3.7 <i>HERC1</i> is a Novel Binding Partner and a Substrate of BCR-ABL1.....	58
3.8 <i>HERC1</i> Gene Expression is Associated to Differentiation Arrest in Myeloid Leukemia Cells	60
3.9 <i>HERC1</i> Gene Expression Linked to the Differentiation of Ph ⁺ (K562) Myeloid Leukemia Cells with Erythropoietin (EPO) and PMA	63
3.10 <i>HERC1</i> as a Novel and Emerging Binding Partner of mTORC2 Complex	66
Chapter 4: DISCUSSION	68
Chapter 5: CONCLUSION AND FUTURE PERSPECTIVES	74
Chapter 6: REFERENCES	75
ANNEX	94

LIST OF FIGURES

Figure 1.1. Simplified hematopoietic tree and hierarchy of differentiation in the haematopoietic system	02
Figure 1.2. Schematic diagram of MPNs classification	04
Figure 1.3. The <i>BCR-ABL1</i> gene fusion leading to CML malignant cells	06
Figure 1.4. Schematic map of the BCR, ABL, and <i>BCR-ABL</i> fusion variants	08
Figure 1.5. Polycythemia Vera vs Healthy subject bone marrow biopsy.....	12
Figure 1.6. Essential Thrombocythemia vs Healthy subject bone marrow biopsy.....	14
Figure 1.7. Primary Myelofibrosis and Healthy subject bone marrow biopsy.....	15
Figure 1.8. Schematic illustration of the development of AML	18
Figure 1.9. The Protein Ubiquitination Pathway	25
Figure 1.10. Structural features of human large HERC1	28
Figure 1.11. Structural domains of HERC1 and HERC1 Interactome	31
Figure 3.1. The <i>HERC1</i> gene expression differently regulated in BM and PB and it is dysregulated in haematological malignancies	48
Figure 3.2. The <i>HERC1</i> transcript is severely downmodulated in acute myeloid leukemia independently from genetic alteration	49
Figure 3.3. <i>HERC1</i> mRNA is differentially and peculiarly expressed in MPNs patients	51
Figure 3.4. <i>HERC1</i> gene expression is differently regulated in CML patients during the evolution of the disease	53
Figure 3.5. HERC1 is mainly downregulated in myeloid lineage	55
Figure 3.6. Effect of Imatinib treatment on <i>HERC1</i> gene expression in CML primary and K562 (Ph+) cells	56

Figure 3.7. HERC1 mRNA and protein amounts are regulated by the BCR-ABL1 tyrosine kinase activity	57
Figure 3.8. HERC1 is a novel interactor and a potential substrate of BCR-ABL1.....	59
Figure 3.9. <i>HERC1</i> gene expression modulated upon HL60 cell differentiation, a tight association between the HL60 leukemic cell differentiation and HERC1 gene expression	62
Figure 3.10. Effect of <i>HERC1</i> gene expression on differentiation of Ph ⁺ (K562) myeloid leukemia cell line with PMA and EPO	65
Figure 3.11. HERC1 mediate Akt activation by establishing a complex with Akt and the components of mTORC2 complex along with Hsp90	67
Figure A1. <i>HERC2</i> gene expression at different phases of CML patients	94
Figure A2. Quantification of HERC1 signal of immunofluorescence by measuring the integrated intensities of each sample	95
Figure A3. <i>HERC1</i> transcript level in normal human blood cells population according to the Human Differentiation Map Portal databases	96
Figure A4: HERC1 is tyrosine phosphorylated in chronic myeloid leukemia K562 cells	96

LIST OF TABLES

Table 1.1: 2016 WHO Diagnostic Criteria for (PV).....	13
Table 1.2: 2016 WHO Diagnostic Criteria for ET	14
Table 1.3: 2016 Who Diagnostic Criteria for PMF	16
Table 1.4: French-American-British and 2016 revised WHO classification of acute myeloid leukemia	21
Table 2.1: Composition of Solutions and Buffers	34
Table 2.2: Reagents and PCR programs used for cDNA synthesis	39
Table 2.3: Reagents and PCR programs used for gene expression analysis by qPCR	40
Table 2.4: Antibodies	42
Table A2.1: Chronic Myeloid Leukemia (CML) patients's characteristics	97
Table A2.2: Characteristics of Acute Myeloid Leukemia (AML) at diagnosis	100
Table A2.3: Details of MyeloProliferative Neoplasms (MPNs) patients	102

ACKNOWLEDGEMENTS

The last four years in my life have been an extraordinary experience and for this, I have many people to thank. Firstly, I would like to express my gratitude to my wonderful supervisor, Prof. Giuseppe Saglio. I admire him very much for his dedication to science as well as to family, and I have always felt very fortunate and lucky to be his student. Being an international PhD student he always treats me as a son to make me as stronger as a father does for his real son. Thanks for considering me as a family member and I love to thanks to your lovely wife Lucia for her motherly care and hospitality. Thank you for all your wisdom and guidance over the years, and for giving me the independence and freedom to make some of my own mistakes. I am also very aware and appreciative of the opportunities you have provided me. The real journey started when you introduced me to very fantastic and friendly natured co-tutors, Dr. Enrico Bracco and Dr. Barbara Pergolizzi, both of them contributed greatly to the scientist I am today, they were great sources of knowledge and inspiration and I really think of myself as the luckiest one to have them who always support me to establish HERC1 research project. In particular, I'll never forget their generous support not only scientifically but for also taking care of all things administrative. They have always been there for discussions on research projects, providing useful feedback on the thesis. In particular, I thank to Dr. Cristina Panuzzo for taking me on as a colleague to work elbow to elbow and for her supportive and defensive behavior that always encourage me to gather mainly positive energy. Thank you for always knowing where to find the silver lining. I gratefully acknowledge the contribution of Prof. D. Cilloni, Emilia Giuliano, and all others for providing invaluable help in obtaining clinical information of patients for this study. I am grateful to all the patients who agreed to participate in the research presented in this thesis. I wish to thank Prof. Dr. Patrick Forde for hosting me in his lab as an international researcher for one year at Cork Cancer Research Center, University College Cork Ireland. I appreciate the opportunity he gave me to discuss and exchange ideas with his group members and opening the doors of all the available facilities to establish a project. I would like to thank all of my other friends, one of our genius thesis student S. Magnati, and my relatives, who have shown support one way or another. Last but not least, I would like to thank all my family members especially my parents and my sister, Zunaira, for being my best companion and for always showing a smile when I needed one. They always believe in my abilities and encourage me to pursue new challenges.

LIST OF PUBLICATIONS

1. **Ali, M. Shahzad**; Panuzzo, Cristina; Calabrese, Chiara; Maglione, Alessandro; Piazza, Rocco; Cilloni, Daniela; Saglio, Giuseppe; Pergolizzi, Barbara; Bracco, Enrico. **(2021)**. "The Giant HECT E3 Ubiquitin Ligase HERC1 Is Aberrantly Expressed in Myeloid Related Disorders and It Is a Novel BCR-ABL1 Binding Partner" *Cancers* 13, no. 2: 341. <https://doi.org/10.3390/cancers13020341>.
2. Calabrese C, Panuzzo C, Stanga S, Andreani G, Ravera S, Maglione A, Pironi L, Petiti J, **Ali, M. Shahzad**, Scaravaglio P, Napoli F, Fava C, De Gobbi M, Frassoni F, Saglio G, Bracco E, Pergolizzi B, Cilloni D. **(2020)**. Deferasirox-Dependent Iron Chelation Enhances Mitochondrial Dysfunction and Restores p53 Signaling by Stabilization of p53 Family Members in Leukemic Cells. *International Journal of Molecular Sciences*.; 21(20):7674. <https://doi.org/10.3390/ijms21207674>.
3. Panuzzo, Cristina; Signorino, Elisabetta; Calabrese, Chiara; **Ali, M. Shahzad**.; Petiti, Jessica; Bracco, Enrico; Cilloni, Daniela. **(2020)**. "Landscape of Tumor Suppressor Mutations in Acute Myeloid Leukemia" *J. Clin. Med.* 9, no. 3: 802. <https://doi.org/10.3390/jcm9030802>
4. Pergolizzi, B., Panuzzo, C., **Ali, M. Shahzad**., Iacono, M. L., Levron, C. L., Ponzzone, L., & Bracco, E. **(2019)**. Mammals and Dictyostelium rictor mutations swapping reveals two essential Gly residues for mTORC2 activity and integrity. *Journal of Cell Science*. 132(22) <https://doi: 10.1242/jcs.236505>
5. **Ali, M. Shahzad**, Kheshwant S. Gill, Giuseppe Saglio, Daniela Cilloni, Declan M. Soden, and Patrick F. Forde. **(2018)** Expressional changes in stemness markers post electrochemotherapy in pancreatic cancer cells. *Bioelectrochemistry*, 122, 84-92. <https://doi.org/10.1016/j.bioelechem.2018.03.009>

ABBREVIATIONS

HSC: Hematopoietic stem cells

MPP: Multipotent Progenitors

MEP: Megakaryocyte-Erythroid Progenitor

CMP: Common Myeloid Progenitor

GMP: Granulocyte-Macrophage Progenitors

LMPP: Lymphoid-Primed Multipotent Progenitors

CLP: Common Lymphoid Progenitors

NK: Natural Killer cells

LSC: Leukemic Stem Cell

MPN: Myeloproliferative Neoplasm

PV: Polycythemia Vera

ET: Essential Thrombocytosis

PMF: Primary Myelofibrosis

CML: Chronic Myeloid Leukemia

CNL: Chronic Eutrophilic Leukaemia

CEL: Chronic Eosinophilic Leukaemia

AML: Acute Myeloid Leukemia

APL: Acute Promyelocytic Leukemia

MDS: Myelodysplastic Syndrome

WHO: World Health Organization

JAK2: Janus Kinase 2

CALR: Calreticulin

MPL: Myeloproliferative Leukemia

TPO: Thrombopoietin

EPO: Erythropoietin

G-CSF: Granulocyte Colony Stimulating Factor

PI3K: Phosphatidylinositol-3-Kinase

MAPK: Mitogen-Activated Proteins Kinase
ERK: Extracellular signal- Regulated Kinase
BCR: Breakpoint Cluster Region
SH: SRC homology
CP: Chronic Phase
AP: Accelerated Phase
BC: Blast Crisis
TKI: Tyrosine Kinase Inhibitor
MRD: Measurable Residual Disease
IL1RAP: Interleukin 1 Receptor Accessory Protein 1
IL1R1: Interleukin 1 Receptor
IL2RA: Interleukin 2 Receptor Subunit Alpha
DPP4: Dipeptidyl Peptidase 4
SDF1: Stromal Cell-Derived Factor 1
CXCL12: C-X-C motif chemokine 12
MSTNpp: Myostatin pro-peptide
VTE: Venous Thromboembolism
CBF: Core Binding Factor
ATO: Arsenic Trioxide
ATRA: All-Trans Retinoic Acid
GVHD: Graft-Versus-Host Disease
CART: Chimeric Antigen Receptor T-cells
PTMs: Posttranslational Modifications
RING: Really Interesting New Gene
HECT: Homologous to the E6AP Carboxyl Terminus
RBR: Ring Between Ring
DUBs: Deubiquitinases
CBL: Casitas B-cell Lymphoma

FLT3: fms-related tyrosine kinase 3

RCC1: regulator of chromosome condensation

ARF1: ADP-Ribosylation Factor 1

RCC: Regulator of Chromatin Condensation

RLD: RCC-like domain,

WD40: W-D dipeptide repeat

BH3: Bcl-2 homology 3

HECT: Homologous to E6AP C-Terminus

SPRY: SPIA and ryanodine receptor

IQGAP1: IQ-motif-containing GTPase activating protein 1

1. INTRODUCTION

1. Hematopoiesis

1.1 A Brief Overview on Hematopoiesis

Hematopoiesis, derived from Greek word where “hema” means “blood” and “poies” means “to produce, the process of generating the cellular components of the blood. Blood is a connective tissue with a liquid form that allows it to reach and penetrate virtually every part of the human body [1]. Hematopoietic stem cells (HSCs) give rise to all type of blood cells which are generally divided into three groups; erythrocytes (red blood cells), responsible for delivering oxygen and removing carbon dioxide; thrombocytes, also known as platelets, responsible for coagulation, which help to stop bleeding during an injury, and leukocytes (white blood cells), including immune cells such as lymphoid and myeloid cells. HSCs are functionally defined by their ability to differentiate into all functional blood cells, and self-renewal ability to give rise itself. Cells that have multipotency but lack self-renewal ability are termed multipotent progenitors (MPP) [2].

The cells downstream of the HSCs and MPPs are divided into two main subgroups, the myeloid and the lymphoid lineage [1]. The mature myeloid cells arise through different maturation pathways and originate from different progenitors (**Figure 1.1**). Megakaryocyte-erythroid progenitor (MEP), progenies of a common myeloid progenitor (CMP), differentiate to erythrocytes and megakaryocytes which ultimately terminate to thrombocyte. Megakaryocytes can also directly arise from MPPs. CMPs also give rise to granulocyte-macrophage progenitors (GMP) which in turn, give rise to granulocytes, monocytes, macrophages and dendritic cells [1,3]. The mature lymphoid cells arise from lymphoid-primed multipotent progenitors (LMPP) that, passing a cellular state denominated common lymphoid progenitors (CLP), give rise to T and B-lymphocytes, and natural killer cells (NK-cells) [4]. Dendritic cells can also arise from LMPPs [2]. The lymphoid system, excluding NK-cells, make up the adaptive immune system, whereas the leukocytes of the myeloid branch constitute the innate immune system. Even though the exact relationship between lineage commitment and self-renewal potential is not fully understood the self-renewal and differentiation of HSCs is critical to maintain hematopoiesis throughout the life of the organism [5].

The mature blood cells show the highest complexity, having a variable half-life but are predominantly short-lived, HSCs continuously provide more differentiated progenitors while properly maintaining the HSC pool size throughout life by precisely balancing self-renewal and differentiation [5,6]. HSCs in an adult human generate approximately 10^{11} - 10^{12} every day or around 100 million cells every second throughout the lifetime of a human being [7]. Due to the high turnover rate, hematopoiesis is required to be tightly regulated to allow for a daily production of trillions of cells to rapidly replenish old cells or to meet demands during stress such as blood loss or infection [8]. With the passage of time random mutations in HSCs are known to be accumulated which strike to the critical genes, that cause HSCs to transform into malignant cells, known as leukemic stem cell (LSC) [9,10].

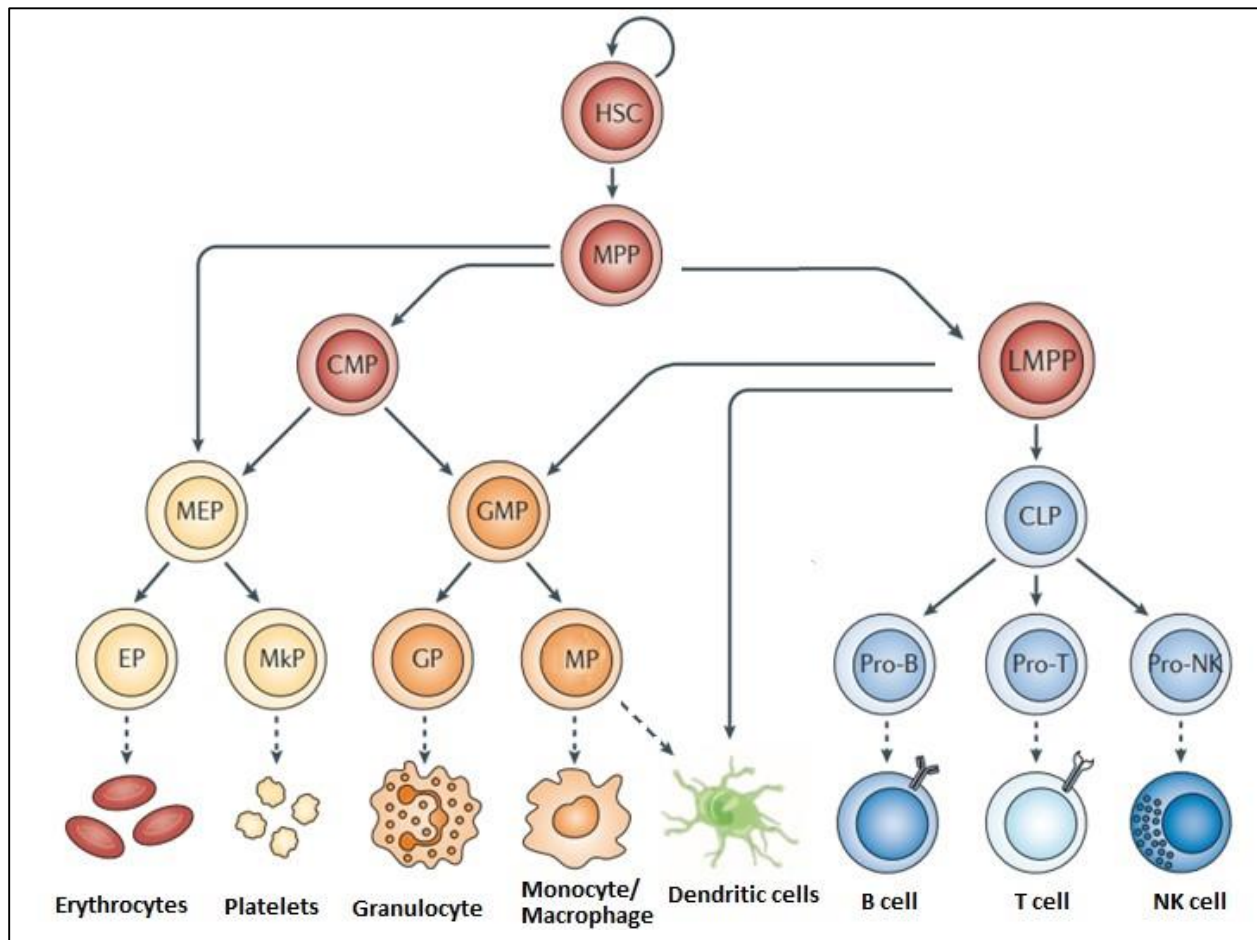


Figure 1.1. Simplified hematopoietic tree and hierarchy of differentiation in the haematopoietic system. The hematopoietic tree shows the hierarchical arrangement in a stepwise manner from a self-renewing, multipotent hematopoietic stem cell (HSC) at the apex with subsequently more restricted progenitors downstream which choose a specific branch of the

haematopoietic system to rise to a specific lineage (myeloid and lymphoid). CLP, common lymphoid progenitor; CMP, common myeloid progenitor; EP, erythrocyte progenitor; GMP, granulocyte-macrophage progenitor; GP, granulocyte progenitor; LMPP, lymphoid primed multipotent progenitor; MP, macrophage progenitor; MEP, megakaryocyte-erythrocyte progenitor; MkP, megakaryocyte progenitor; MPP, multipotent progenitor; NK, natural killer [11].

Compared to solid-state tumors, hematological malignancies are a group of non-epithelial cancers, with a different dynamic of malignant transformation, originate from hematopoietic stem cells of blood-forming tissue, the bone marrow, as a result of mutations or chromosomal translocations [12]. Due to the inherent high proliferative state of blood cells often, only two mutations or a single chromosomal translocation is enough. Interestingly in blood malignancies, mutations in the “classic” cancer genes are less common (such as *TP53*, *RB1*, *ATM*, *BRCA1/2*), while mutations in epigenetic regulators, chromatin modifiers and the spliceosome machinery are prominent in the genetic landscape [9,13]. Finally, in some cases, the leukemic driver is not known. However, it is very complex, still ambiguous and subject to improvements as new facts about hematological malignancies are being discovered.

1.2 Myeloproliferative Neoplasms (MPNs)

The myeloproliferative neoplasms (MPN) are clonal hematopoietic stem cell disorders characterized by uncontrolled proliferation and expansion of myeloid lineage cells, described by William Damashek for the first time in 1951 as myeloproliferative disorders (MPDs) [14]. MPNs are categorized into two classical groups on the basis of current diagnostic criteria: Philadelphia chromosome-positive (Ph+), including chronic myeloid leukemia (CML), and Philadelphia chromosome-negative (Ph-), including polycythemia vera (PV) characterised by a raised red cell mass, essential thrombocythemia (ET) characterised by an isolated increase in platelet count, and primary myelofibrosis (PMF), in which the haematopoietic compartment is gradually replaced with collagen fibers, leading to bone marrow failure and extramedullary haematopoiesis. Additionally, MPN patients display risk of AML transformation [15,16].

Over the years, based on important discoveries in molecular and cellular pathology, MPNs have led to latest WHO classification which includes seven subcategories of MPNs: CML Ph⁺, Chronic neutrophilic leukaemia (CNL), PV, PMF, ET, Chronic eosinophilic leukaemia (CEL) and

MPNs-unclassifiable [17,18]. Mutations in different genes such as *JAK2*, *MPL*, *TET2*, *ASXL1*, *NF1*, *SF3B1*, *RUNX1*, *SUZ12*, *IDH1*, *IDH2*, *DNMT3a*, *CBL*, *IKZF1*, *LNK* and *CALR* have been described in Ph⁻ MPN patients, that normally are not characterized by a crucial driver [19–22]. While majority of MPNs cases have mutations in *JAK2*, *CALR* or *MPL* that are always mutually exclusive [23]. In 95% of patients with PV and 50-60% of those with ET and PMF, have a gain-of-function somatic point mutation due to a G>T substitution in the Janus kinase 2 (*JAK2*) gene on chromosome 9p, resulting in constitutive activation of *JAK2* (*p.V617F*) [24,25]. Mutations in exon 12 of *JAK2* also reported in a small proportion of patients [26]. *JAK2* is a cytoplasmic tyrosine kinase, required for signal transduction from a number of cytokine receptors, including those for thrombopoietin (TPO), erythropoietin (EPO) and granulocyte colony-stimulating factor (G-CSF), and which therefore plays a vital role in haematopoiesis [27]. Impairment of *JAK2* regulation results in the constitutive activation of *JAK2*- *STAT5*, phosphatidylinositol-3-kinase (*PI3K*) and mitogen- activated 10 proteins kinase/extracellular signal- regulated kinase (*MAPK/ERK*) downstream signaling pathways in a cytokine- independent or hypersensitive manner [28]. MPNs are also amenable to targeted therapy, and several inhibitors of mutated *JAK2* have been developed including ruxolitinib, lestaurtinib and pacritinib [29,30].

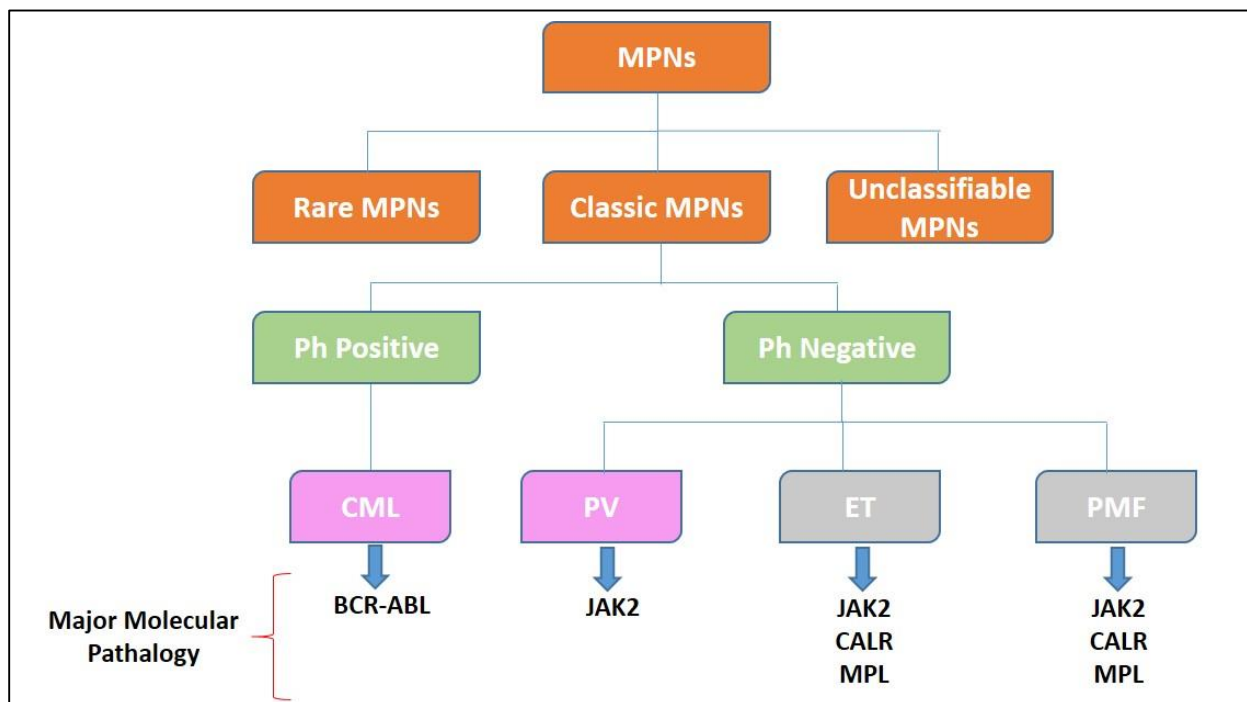


Figure 1.2. Schematic diagram of MPNs classification. The 2016 WHO latest classification of MPNs in three major categories: rare MPNs, classic MPNs and unclassifiable MPNs. Classic

MPNs are further classified as Philadelphia chromosome (Ph) positive (CML) or Ph negative, includes Polycythemia Vera (PV) with major molecular pathological feature JAK2 mutations or Essential thrombocythemia (ET) and primary myelofibrosis (PMF) characterised by JAK2, CALR or MPL mutations. Gray shades identify the MPNs affected by CALR mutations.

1.3 Philadelphia Chromosome-Positive Myeloproliferative Neoplasms

1.3.1 Chronic Myeloid Leukemia Etiology

Chronic myeloid leukemia (CML) accounts for 15-20% of all cases of leukemia in adults. It is a stem cell (SC) neoplasm sustained by a rare population of quiescent cells characterized by the *BCR-ABL1* oncogene. The *BCR-ABL1* gene results from balanced, reciprocal t(9;22)(q34;q11) translocation (**Figure 1.3**) which occurs in a hematopoietic stem cell [31]. The *BCR* gene is located on chromosome 22 and spans over 130 kilobases (kb) and encodes two proteins, which are 160kDa and 130kDa in size [32]. The *c-ABL* (human homologue of the Abelson Murine Leukaemia virus) is a tyrosine kinase encoding oncogene, located on chromosome 9, has 11 exons, encodes a 145 kDa protein, which is a member of non-receptor kinases [33]. The both *BCR* and *cAbl* genes normally encode ubiquitously expressed kinases, the serine-threonine and tyrosine respectively. This translocation results in a fusion gene between the breakpoint cluster region gene (*BCR*) on chromosome 22 and the *c-Abl* oncogene 1 (*ABL1*), a tyrosine kinase encoding oncogene, on chromosome 9 that lead to the synthesis of an abnormal chimeric *BCR-ABL1* mRNA transcript and protein [32]. The fusion onco-protein BCR-ABL1, derived from the translocation, is a constitutively activated tyrosine kinase, which elicits a variety of downstream survival and proliferative pathways in order to protect leukemia cells from apoptosis and to promote uncontrolled proliferation [31]. The disease defining event was first described, but not fully understood, in 1960 by Hungerford and Nowell [34]. Thirteen years later the observed aberrant chromosome, named as Philadelphia chromosome based on the city of discovery, was the first example of a specific DNA abnormality that could be linked directly to cancer [35]. Subsequently, the molecular basis of chimeric *BCR-ABL1* fusion gene was described in 1983 [36].

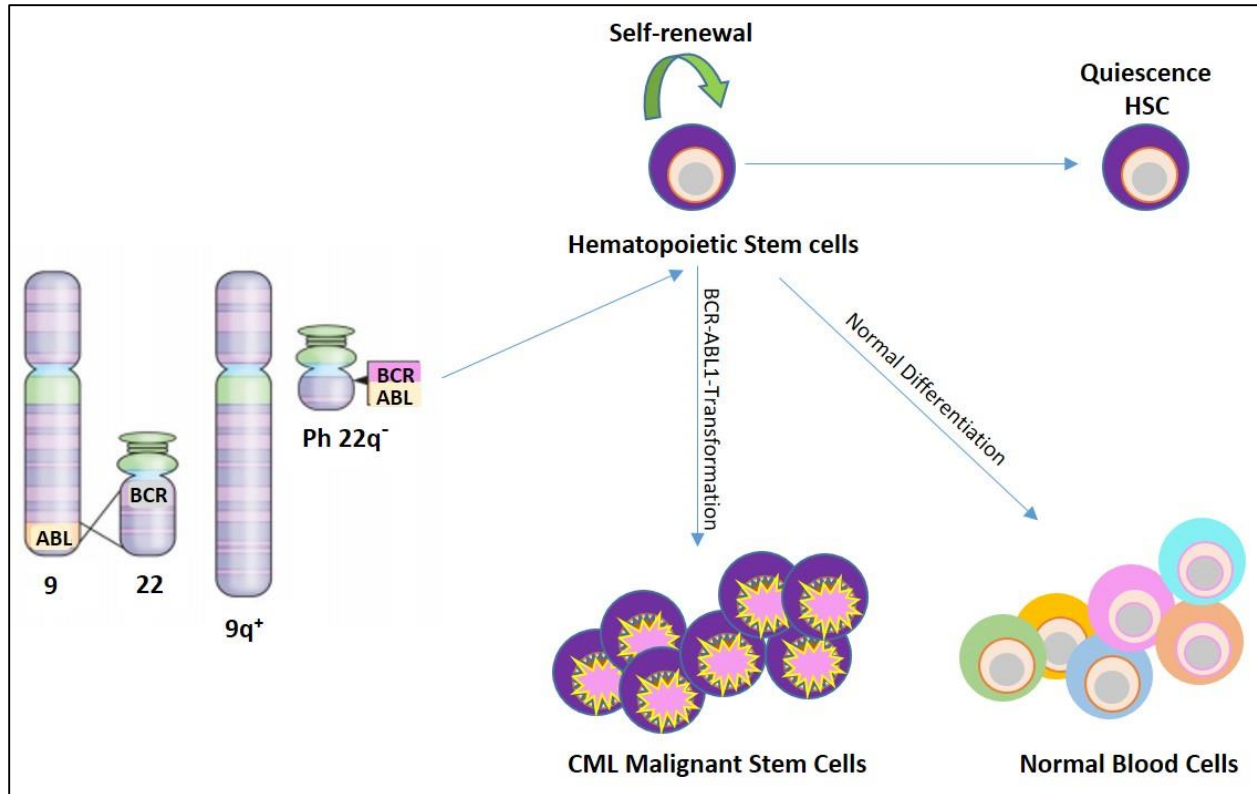


Figure 1.3. The *BCR-ABL1* gene fusion leading to CML malignant cells. *BCR-ABL1* rearrangement, is believed to acquire in hematopoietic stem cells. The resulting constitutively active tyrosine kinase leads to uncontrolled cell proliferation and the development of malignant CML leukemic cells. While the HSC without t(9;22)(q34;11) translocation differentiates normally to different cell lineage.

The incidence in Europe is 1.10 per 100,000 in 2000 to 2002 (increase to ~4 per 100 000 in aged patients 75-99) and about 1-2 in 100,000 cases per year globally. The median age for patients at diagnosis is 45 to 55 years, however in the UK, the Cancer Research UK states that 50% of diagnosed patients are 65 and over. The hallmark feature of CML chronic phase is leukocytosis, thrombocytosis, and splenomegaly (50-60% of the cases), hepatomegaly in 10-20% [37].

CML has been divided into three phases in which 90-95% are diagnosed as chronic phase (CP), that may evolve in an accelerated phase (AP) (5%), and 3% as blast phase which is much more aggressive, associated with differentiation block characterized by an accumulation of undifferentiated blasts in blood and bone marrow [38,39].

According to WHO classification, CP patients typically have less than 10% blasts in their peripheral blood or BM, therefore the blood cells are mainly differentiated and minimally invasive.

CML accelerated phase is defined as: **a)** the blast count in peripheral blood or bone marrow of 10-19%, **b)** more than 20% basophils in peripheral blood or other criteria based on therapy response, splenomegaly persistence, thrombocyte levels, or additional genetic abnormalities. More than 20% of the blasts in PB or bone marrow or alternatively extramedullary blast accumulation define the CML in blast phase. CML blast crisis is often myeloid resembling AML (70%) and less frequently lymphoid (B-lymphoid) where the disease resembles ALL (30%) [38,40]. Due to newly acquired cytogenetic changes blast cells exhibit enhanced proliferation and have acquired the ability to evade apoptosis. At this end stage, blast cells have become severely aggressive that they have infiltrated extramedullary sites. Patients in BC have a very poor prognosis with a median survival rate of 3-6 months [41]. The molecular events that occur to trigger disease progression from CP CML to advanced AP or BC are poorly known. However, the accumulation of various different chromosomal abnormalities have been implicated in the transition from CP to an advanced disease (AP or BC), such as double Ph chromosome, trisomy 8, and isochromosome 17 [41,42]. During progression to accelerated phase and blast crisis the cells acquire additional genetic aberrations including larger chromosomal alterations and point mutations [39]. Most common mutations at molecular level in cells during blast crisis are found in *ABL1*, *IKZF1*, *RUNX1*, *ASXL1* and *TP53* with an overrepresentation of *ABL1* and *IKZF1* mutations in lymphoid blast crisis [43]. The genes *JAK1*, *FLT3*, *NF1*, *ERG* and *MLL3*, known to involved in hematopoiesis or leukemogenesis have also been implicated in disease progression [44].

1.3.2 BCR-ABL1 Fusion Onco-proteins Function and Structure

There are three different variants of *BCR-ABL1*, P210, P190 and P230 [45], arising from distinct breakpoints in the *BCR* gene on chromosome 22. Depending on the translocation breakpoint in *BCR*, different BCR-ABL1 protein isoforms are expressed, which all contain exons 2–11 of the *ABL1* gene, but differ in the length of their BCR part (**Figure. 1.4**). Indeed, the most common *BCR-ABL1* variant in CML is P210, made up of *BCR* exons 12-16 (the major breakpoint cluster) fused to the 5'-end of the *ABL* gene [32,45]. The cABL1 protein contains SRC homology (SH)3,2, and 1 along with NLS and actin-binding domains. The SH1 domain, possesses the tyrosine kinase activity of p210 BCR-ABL1, while SH2, SH3 and actin binding domains are involved in protein-protein interactions required for the protein signaling and actin cytoskeleton dynamics, respectively [46–48]. Upstream fusion of the BCR domain is thought to disrupt SH-

domain-mediated regulation of the ABL kinase. Being constitutively active, BCR-ABL has much higher tyrosine kinase activity compared to the native c-ABL protein which results in malignant transformation [49]. BCR has multiple functional domains, the most critical are: the dimerization domain, which has a coiled-coil (CC) motif, that responsible for the constitutive ABL tyrosine kinase activity; a serine-threonine kinase domain, which activates signaling pathways regulated by ABL tyrosine kinase activity, as well as BCR-ABL1; a Rho guanine-nucleotide exchange factor (Rho-GEF) domain; a calcium-dependent lipid binding site (pleckstrin homology PH) and a Rac GTPase activating protein (Rac-GAP) domain [48,50]. Based on these structural domains the other P190 and P230 variants of *BCR-ABL1*, more commonly associated with Philadelphia chromosome ALL and chronic neutrophilic leukemia and are rarely seen in CML, have different leukemogenic potential [33,45].

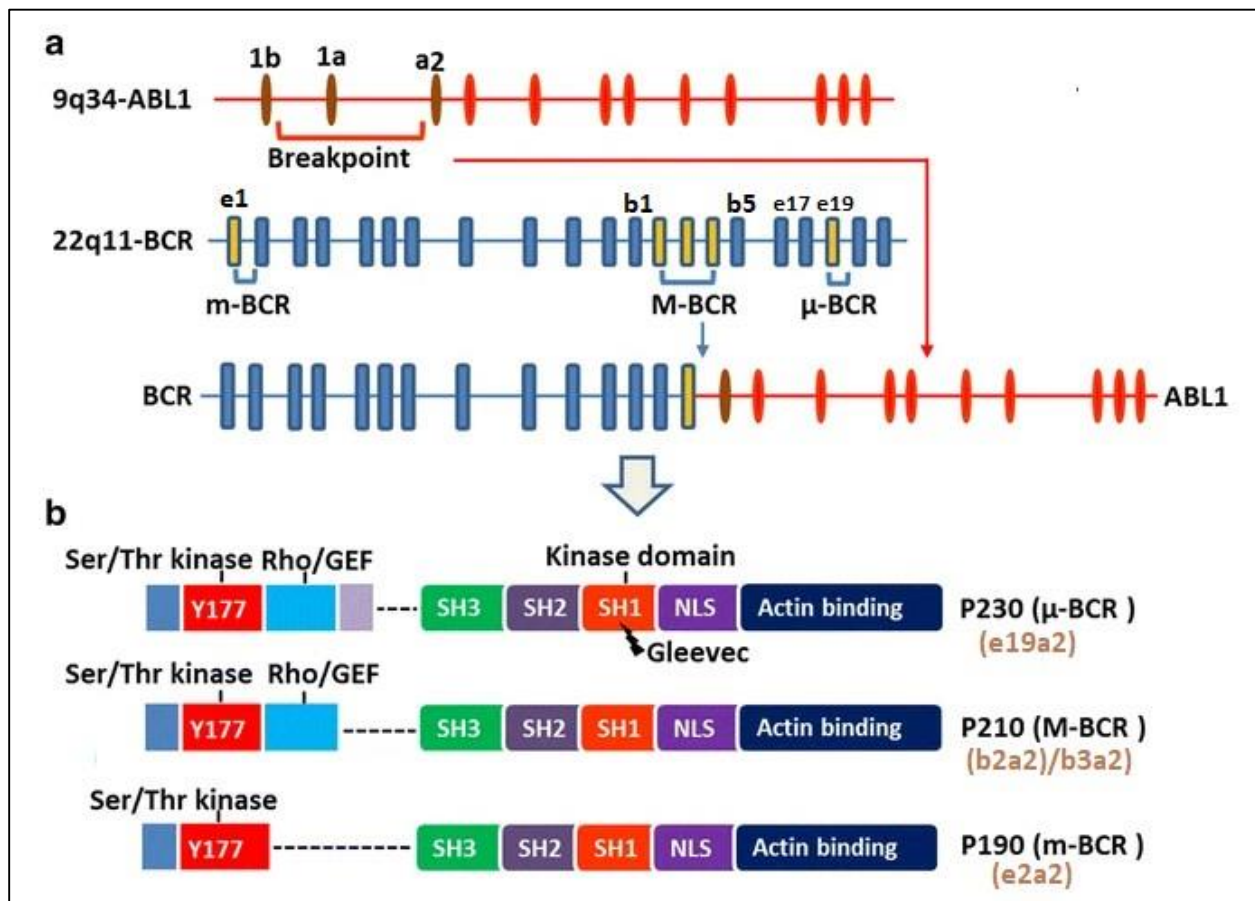


Figure 1.4. Schematic map of the BCR, ABL1, and *BCR-ABL1* fusion variants. a) The *BCR-ABL1* fusion gene consists of the 5' end of the *BCR* gene located at 22q11 and the 3' end of the *ABL1* gene located at 9q34. The breakpoints of the translocation usually involve the intron 13

or 14 of *BCR*, named major breakpoint (M-*BCR*), intron 1 of *BCR*, named minor breakpoint (m-*BCR*), and exon 19 of *BCR*, named μ breakpoint (μ -*BCR*). For *ABL1*, the breakpoint always involves the region between exons 1b and 2. **b)** The BCR-ABL1 protein contains the Ser/Thr kinase domain and the Rho/GEF domain of BCR and the src homology (SH) domains, nuclear localization signal (NLS), and actin-binding domains from ABL1. The SH1 kinase domain is the target of Imatinib. The different breakpoints that generate the p230, p210, and p190 isoforms are shown [51].

1.3.3 Clinical Aspects and CML Treatment

The 20-40% of CML cases are usually diagnosed during a routine blood count. These patients are asymptomatic whereas others 60% of patients experienced symptoms like fatigue due to anemia, weight loss, malaise, night sweats [37]. The gold standard diagnostic observation for CML is the detection of the Ph chromosome, which is found in 95% of CML patients [38]. Bone marrow is aspirated and chromosomal analysis by fluorescence in-situ hybridization (FISH), or reverse transcriptase quantitative polymerase chain reaction (RT-qPCR), are the most frequent method used for detection of BCR-ABL1 mRNA [52]. BCR/ABL1 transcript levels detected by RT-qPCR in peripheral blood is nowadays converted and expressed on an international scale to an international scale (BCR/ABL1^{IS}), in order to obtain comparable values between different laboratories and to monitor with accuracy minimal residual disease (MRD) [53].

Concerning the treatment, traditional therapies for CML before the advent of targeted therapy were α -interferon (α -IFN), and allogeneic bone marrow transplant depending upon the availability of compatible donors [54,55]. The therapeutic revolution came with the introduction of the TKI Imatinib, 40 years after the discovery of the Philadelphia chromosome. TKIs function as molecular mimetics that by competitively binding to the kinase domain of BCR-ABL1, prevent the ATP link [56]. Consequently, BCR-ABL1 fails to phosphorylate itself and downstream proteins thus selectively kills CML cells [57,58]. TKIs have been tremendously successful and nowadays the 80% of patients reached 5-year survival rate, while more than 60% achieved long-term major cytogenetic responses [59,60]. Despite this dramatic success, a limited number of patients who discontinue TKI therapy due to economic or clinical reasons, relapsed [61,62]. Some patients develop TKI resistance due to point mutations localized within the ABL1 kinase domain of the *BCR-ABL1* gene [63,64]. Though, one of the more well-described causes of resistance is

mutations in the ABL1 kinase domain, TKIs resistance is most likely often multifactorial including effects on drug metabolism and efflux from target cells, leaving patients unresponsive [64,65].

These issues have been addressed by the recent development of the second generation of TKIs namely Dasatinib, Nilotinib, Bosutinib and Ponatinib, that are even more effective in killing BCR-ABL1⁺ CML cells, though with significant side effects [66]. Unfortunately, a substantial number of patients fail to respond to all TKIs, because primitive CML stem cell, (CD34⁺CD38⁻), are less sensitive to TKI treatment and can remain quiescent and unharmed by treatment for long periods of time [67,68]. Alternatives to TKI, treatments are needed both with regards to patients that develop TKI resistance and those that respond to treatment but have persisting MRD. Recent studies indicate that CML stem cells respond to cytokine stimulation such as IL-1B and MSTNpp (myostatin pro-peptide) suggesting alternative points for therapeutic targeting [69,70]. The synergistic effect of anti-diabetic drugs glitazons (effect on PPAR γ and resulting decrease in STAT5 activity) together with TKI also reveals effective way to eradicating CML leukemic stem cells [71]. Currently, no antibody-based therapies targeting stem cells are in clinical trials for CML although several promising preclinical studies are available [72,73]. However, any curative therapy will have to invariably target the leukemic stem cell population critical for disease persistence and thus for the relapse of the disease.

In general, close monitoring and adequate use of Imatinib and/or alternatively the second-generation TKIs (e.g. Dasatinib, Nilotinib, Bosutinib and Ponatinib) have resulted in a close to normal life expectancy for chronic phase CML patients [74,75], while patients in accelerated phase or blast crisis are often treated with second generation TKIs and considered for allogeneic stem cell transplantation [75,76]. The high survival rates achieved with the use of TKIs come at the price of one of the side effects, such as myelosuppression, gastrointestinal problems, hepatotoxicity, hyperglycemia, pleural effusion or cardiovascular events [77,78]. Treatment discontinuation trials have been conducted to alleviate these side effects, approximately half of CML patients can stop their treatment after at least two years of TKI therapy if they have achieved and sustained a deep molecular response (defined as BCR-ABL1-IS $\leq 0.0032\%$, also referred to as MR4.5) and not experience a relapse [79]. Some of these patients still harbor very low levels of BCR-ABL1 positive cells that are believed to be held back by the restored immune system [80].

1.3.4 CML Stem Cells

Leukemic cell in CML that first acquires the *BCR-ABL1* rearrangement, is believed to be a hematopoietic stem cell as evidenced by the presence of *BCR-ABL1* in both myeloid and lymphoid cells [81]. The resulting HSCs harboring Ph⁺ chromosome are considered as CML stem cells due to their capability of self-renewal and of generation of the other cell types of the leukemia. *BCR-ABL1* activates a number of signaling pathways e.g. PI3K/AKT, JAK/STAT and WNT/ β -catenin, that protect LSC from apoptosis, enhance self-renewal, aberrant cell proliferation and increased production of myeloid progenitors and mature cells, constituting the bulk of leukemia [31,46,81]. Generally leukemic cells can remodel the microenvironment in bone marrow, including stromal cells, osteoblasts and nerve fibers, to support LSCs compared to healthy HSCs [82]. Connie and Allen Eaves in 1999 describe CD34 as a specific marker on quiescent CML leukemic stem cells which were later specified to be CD34⁺ CD38⁻ [83,84]. The primitive immature CD34⁺ CD38⁻ CML cells specifically expressed IL1RAP (Interleukin 1 Receptor Accessory Protein 1), whereas *BCR-ABL1* negative CD34⁺ CD38⁻ cells without IL1RAP expression has been considered as residual healthy HSCs [85].

IL1RAP has also expressed on primitive AML and MDS stem cells and its absence on normal HSC makes it an interesting target for therapy in myeloid malignancies with potentially limited toxicity [86,87]. T cells engineered with chimeric antigen receptor against IL1RAP reveals a potent anti-leukemic effect *in vitro* against primary CML cells and on model cell lines [88]. So far, no clinical trials have been initiated in myeloid leukemia using anti-IL1RAP antibodies. CD25 is also expressed on a subpopulation of CD34⁺CD38⁻IL1RAP⁺ CML primitive cells as compared to corresponding cells from healthy bone marrow. CD25 is a part of the IL-2 receptor complex, known as Interleukin 2 Receptor Subunit Alpha (IL2RA), is normally expressed on lymphocytes, including B-cells and regulatory T-cells [89,90]. Later reports indicate, that CD25 is even expressed on basophils in CML patients and its expression decreases during treatment with the TKIs Nilotinib or Ponatinib [91]. CD25 also seems to be a significant malignant marker in AML, MDS and *BCR/ABL1* positive ALL. Clinical trials with an anti-CD25 antibody conjugate to pyrrolobenzodiazepine (ADCT-301) are currently conducted in AML and ALL (NCT02588092), as well as lymphomas (NCT04052997 and NCT02432235), and other solid tumors (NCT03621982) [92–94].

Similar to CD25, the CD34⁺ CD38⁻ CML primitive cells also express CD26, a protein with enzymatic activity against CXCL12 leading to reduced chemotaxis, increased niche escape, and spread of disease [95,96]. CD26 known as Dipeptidyl Peptidase 4 (DPP4), cleaves and inactivates CXCL12 (C-X-C motif chemokine 12, also known as SDF1), normally expressed on a variety of cells including subsets of both CD4⁺ and CD8⁺ T-cells and can deliver T-cell activating signals [97]. The relative frequency of CD34⁺ CD38⁻CD26⁺ cells is higher in blood than in bone marrow, because CD26 activity makes CML cells less prone to bone marrow sequestering [98]. FACS index sorting combined with single-cell qPCR analyses of 95 genes identified Lin⁻CD34⁺CD38^{-/low}CD45RA⁻cKIT⁻CD26⁺ cells to persist during TKI treatment and to have a primitive and quiescent expression profile [99]. CD26 has also been reported to be upregulated CLL, mesothelioma and renal clear cell carcinoma [100,101].

1.4 Classic Philadelphia Chromosome-Negative MPNs

1.4.1 Polycythemia Vera (PV)

Polycythemia Vera (PV) is a clonal disease of the hematopoietic stem cell characterized by excessive proliferation of the erythroid lineage, leading to an elevated erythroid cell mass, hemoglobin concentration, and blood viscosity [18].

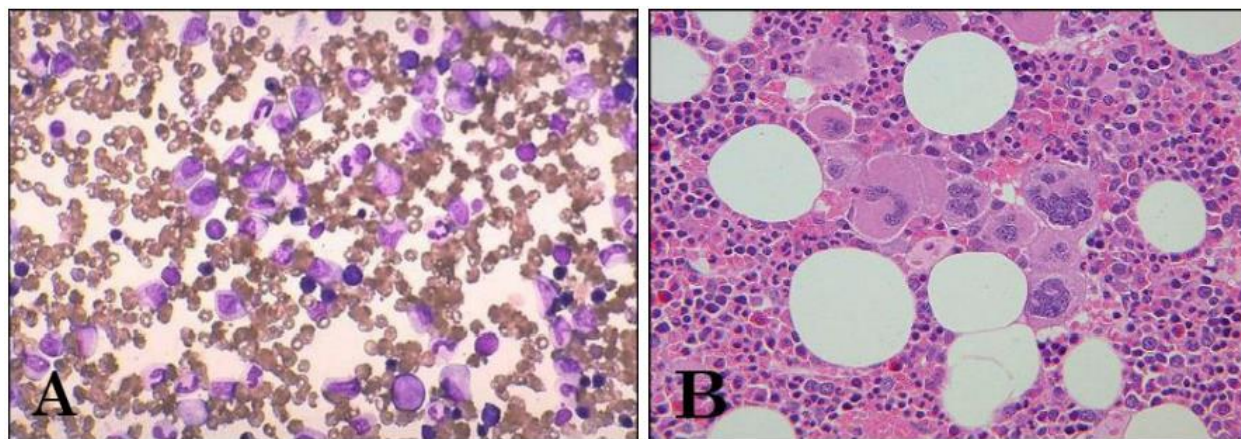


Figure 1.5. Polycythemia Vera vs Healthy subject bone marrow biopsy. Bone marrow core biopsy sample from a patient with polycythemia vera showing a dominating erythropoiesis and large megakaryocytes (B) compared to healthy bone marrow (A) (Hematoxylin and eosin stain (H&E staining x400) [102,103].

This overproduction of red blood cells can lead to vascular and thrombotic complications. In many patients there is also hyperproliferation of other myeloid lineages, resulting in high

platelet and white blood cell counts. The median age at diagnosis is between 55 and 60 years old and the condition affects men and women equally [104]. Symptoms commonly encountered include fatigue, pruritus, headache, sleeping difficulties, blushing, bone pain, thrombosis, and bleeding. The annual incidence rate has been estimated at 0.86 per 100,000 based on pooled data from European studies [105]. The risk for transformation in myelofibrosis (MF) and acute myeloid leukaemia over 10-15 years is 10-15% and 5-10%, respectively [106]. PV is diagnosed according to the World Health Organization (WHO) 2016 classification system (Table 1.1).

Table 1.1: 2016 WHO Diagnostic Criteria for PV. A diagnosis of PV is determined by the presence of all three major criteria or the 1st two major criteria and the minor criteria [17].

POLYCYTHEMIA VERA	
Major criteria	➤ Haemoglobin > 165/160 g/L (men/women) or Haematocrit > 49/48% (men/women) or Elevated red cell mass > 25% above mean normal predicted value ➤ BM biopsy showing hypercellularity for age with trilineage growth (panmyelosis) including prominent erythroid, granulocytic, and megakaryocytic proliferation with pleomorphic, mature megakaryocytes (differences in size). This criterion may not be required in cases with sustained absolute erythrocytosis, haemoglobin levels > 185/165 g/L and haematocrit > 49.5%
Minor criteria	➤ Presence of <i>JAK2V617F</i> or similar mutation ➤ Subnormal serum EPO level

1.4.2 Essential Thrombocythemia (ET)

Essential Thrombocythemia (ET) is characterized by neoplastic proliferation of megakaryocytes resulting in an abnormally high platelet count ($> 450 \times 10^9 /L$). Due to functionally and morphologically altered platelets, ET patients display a higher risk of bleeding and thrombosis [107]. The estimated ET prevalence is about 38-57 per 100,000 individuals, with

a median age ranging between 50-60 years old and with a higher prevalence in females. Clinically, ET is characterized by thrombosis, erythromelalgia, transient ischemic attacks and splenomegaly [108].

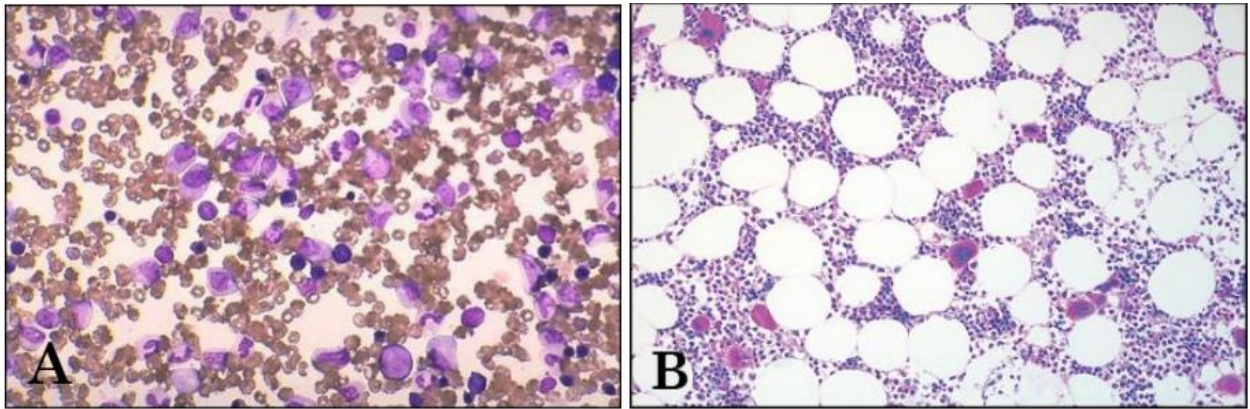


Figure 1.6. Essential Thrombocythemia vs Healthy subject bone marrow biopsy. Bone marrow core biopsy sample from a patient with essential thrombocythemia showing hypercellular bone marrow with increased numbers of megakaryocytes. (A) Normal subject and (B) ET bone marrow sample (Hematoxylin and eosin stain (H&E staining x400) [102,103].

The majority of patients with ET (60%) carries mutations in JAK2(JAK2^{V617F}) 30% harbor CALR while 5-10% of patients present a thrombopoietin receptor (c-MPL) mutation. Recent investigations have reported a differing risk of thrombosis according to the driver mutation. CALR mutated patients have a lower risk of thrombosis than JAK2 and c-MPL mutated patients [22,109]. Risk of progression to myelofibrosis (MF) and transformation to Myelodysplastic Syndromes (MDS) or Acute Myeloid Leukemia (AML) is low in the first decade of life (9.1% and 1.4% respectively) but increases significantly into the second (28.3 and 8.1% respectively), and third decades after diagnosis (58.5% and 24.0% respectively) [110,111]. ET is diagnosed according to WHO criteria as mentioned in table 1.2.

Table 1.2: 2016 WHO Diagnostic Criteria for ET. All four major criteria required for diagnosis or first three major criteria and the minor criteria [17].

ESSENTIAL THROMBOCYTHEMIA	
	➤ Platelet count $\geq 450 \times 10^9$ /L

Major criteria	➤ BM biopsy showing proliferation mainly of the megakaryocyte lineage with increased numbers of enlarged, mature megakaryocytes with hyperlobulated nuclei. No significant increase or left shift in neutrophil granulopoiesis or erythropoiesis and very rarely minor (grade 1) increase in reticulin fibers ➤ Not meeting WHO criteria for CML, PV, MF, MDS or other myeloid neoplasm ➤ Presence of <i>JAK2</i>, <i>CALR</i>, or <i>MPL</i> mutation
Minor criteria	➤ Presence of a clonal marker or absence of evidence for reactive thrombocytosis

1.4.3 Primary Myelofibrosis (PMF)

Primary myelofibrosis (PMF) is the least common non-CML myeloproliferative neoplasm characterized by neoplastic proliferation of megakaryocytes, abnormal cytokine expression and reactive bone marrow fibrosis [112].

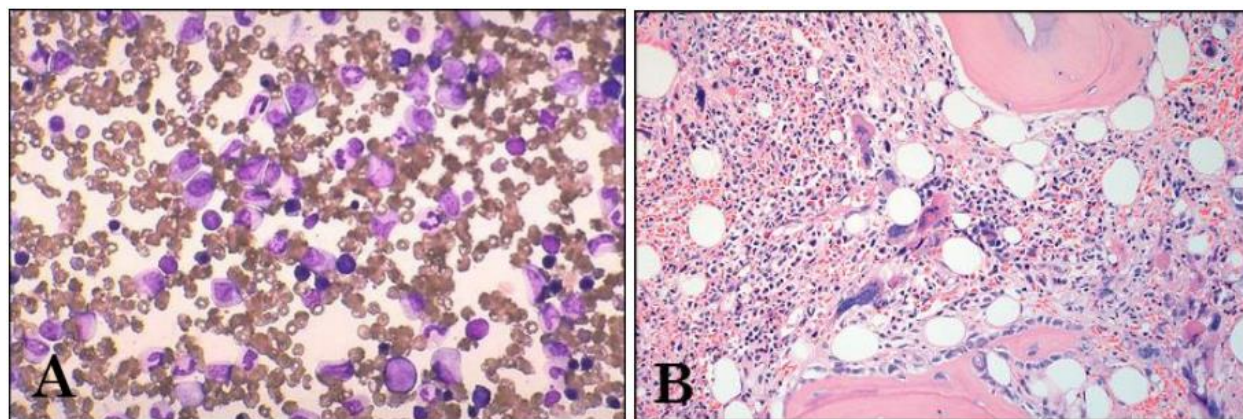


Figure 1.7. Primary Myelofibrosis vs Healthy subject bone marrow biopsy. Bone marrow core biopsy sample from a patient with PMF showing increased marrow fibrosis, decreased hematopoiesis, and clustering of megakaryocytes (B) compared to healthy bone marrow (A) (H&E staining x400) [102,103].

The prevalence of PMF is lower than ET, being 4-6 per 100,000 individuals and prevalence of PMF post-ET and post-PV varies between 0.5-1.1 and 0.3-0.7 per 100,000 respectively [112].

The estimated median age at PMF diagnosis is approximately 65 years old and there are no significant differences between sex. It is the disease with a worse prognostic outcome within all the Ph-negative MPNs and the median survival time after a PMF diagnosis is only 6 years [113]. Increased bone marrow fibrosis leads to ineffective erythropoiesis and consequently results in anemia, hepatosplenomegaly, thrombosis, and bleeding [114]. Constitutional symptoms have a significant negative impact on the quality of life and arise as a result of bone marrow failure, fatigue, night sweats, weight loss, fever and bone pain [105,114]. Blast transformation and progression to AML occurs in up to 25% of MF patients [105,115]. PMF has been subcategorized in prefibrotic (prePMF) and overtly fibrotic PMF, according to 2016 WHO classification [17]. Pre and overt PMF diagnostic criteria are summarized in Table 1.3. Cautious analysis of megakaryocyte characteristics are recommended to distinguish prePMF, from ET since both share a similar mutation profile with 60% *JAK2* mutation, 35% *CALR* mutation, 10% *c-MPL* mutation approximately [112].

Table 1.3: 2016 WHO Diagnostic Criteria for PMF. All four major criteria required for diagnosis or first three major criteria and the minor criteria. Minor criteria to be confirmed twice on consecutive investigations [17].

Primary Myelofibrosis (PMF)		
Major criteria	prePMF	overtPMF
	1. Megakaryocytic proliferation and atypia, without reticulin fibrosis grade 1, accompanied by increased age-adjusted BM cellularity, granulocytic proliferation, and often decreased erythropoiesis	1. Presence of megakaryocytic proliferation and atypia, accompanied by either reticulin and/or collagen fibrosis grades 2 or 3
	2. Not meeting WHO criteria for CML, PV, MDS, or other myeloid neoplasm	
	3. Presence of <i>JAK2</i> , <i>CALR</i> or <i>c-MPL</i> mutation or in the absence of these mutations, the presence of another clonal marker or absence of minor reactive BM reticulin fibrosis	

Minor criteria	i. Leucocytosis: $\geq 11 \times 10^9 /L$ ii. Increased serum LDH level iii. Anemia not attributed to a comorbid condition iv. Palpable splenomegaly v. Leukoerythroblastosis
-----------------------	--

1.5 Acute Myeloid Leukemia

AML is a heterogeneous disease characterized by clonal expansion and accumulation of myeloid precursor cells characterized by a block in their differentiation. This event results in impaired normal hematopoiesis which causes anemia, thrombocytopenia, and granulocytopenia [116,117]. About one-fourth of AML are considered “end-stage point” or secondary to other hematological like myelodysplastic syndrome (MDS), CML as well as MPNs [118]. AML is the most frequent type of myeloid leukemia in adults, accounts for approximately 25% of all blood disorders [117]. The development of AML is a sequential process in which several mutations and chromosomal abnormalities are acquired [119]. It has been proposed that both mutations able to give a proliferative advantage to hematopoietic cells and mutations involving transcription factors crucial for hematopoietic differentiation are pivotal for AML development [120]. In addition to these, tumor suppressors, splicing factors, and genes involved in DNA methylation are also frequently mutated in AML [121]. The variety of mutations identified in AML results in a very heterogeneous group of diseases [116,117]: de novo AML, if originate in a healthy individual, b) therapy-related AML (tAML) if result from cytotoxic agents [118] and secondary AML (sAML) if originating from other hematological conditions such as myelodysplastic/myeloproliferative neoplasms (MDS/MPN) [122].

1.5.1 Clinical Aspects of AML

The AML is the most common leukemia in adults still remains a great clinical challenge with an overall incidence of 3-5 new cases per 100,000 people per year. The incidence is age-dependent, rising from 1.3/100,000 individuals below 65 years old to 12.2/100,000 in those above 65 [123]. Almost three-quarters of cases are diagnosed in individuals with ≥ 60 years of age. It is a heterogeneous disease, occurs even in children (20%) and its incidence strongly increases with age

(>12 in people over 65 years of age). It occurs at all ages and is slightly more common among men than women [117,123].

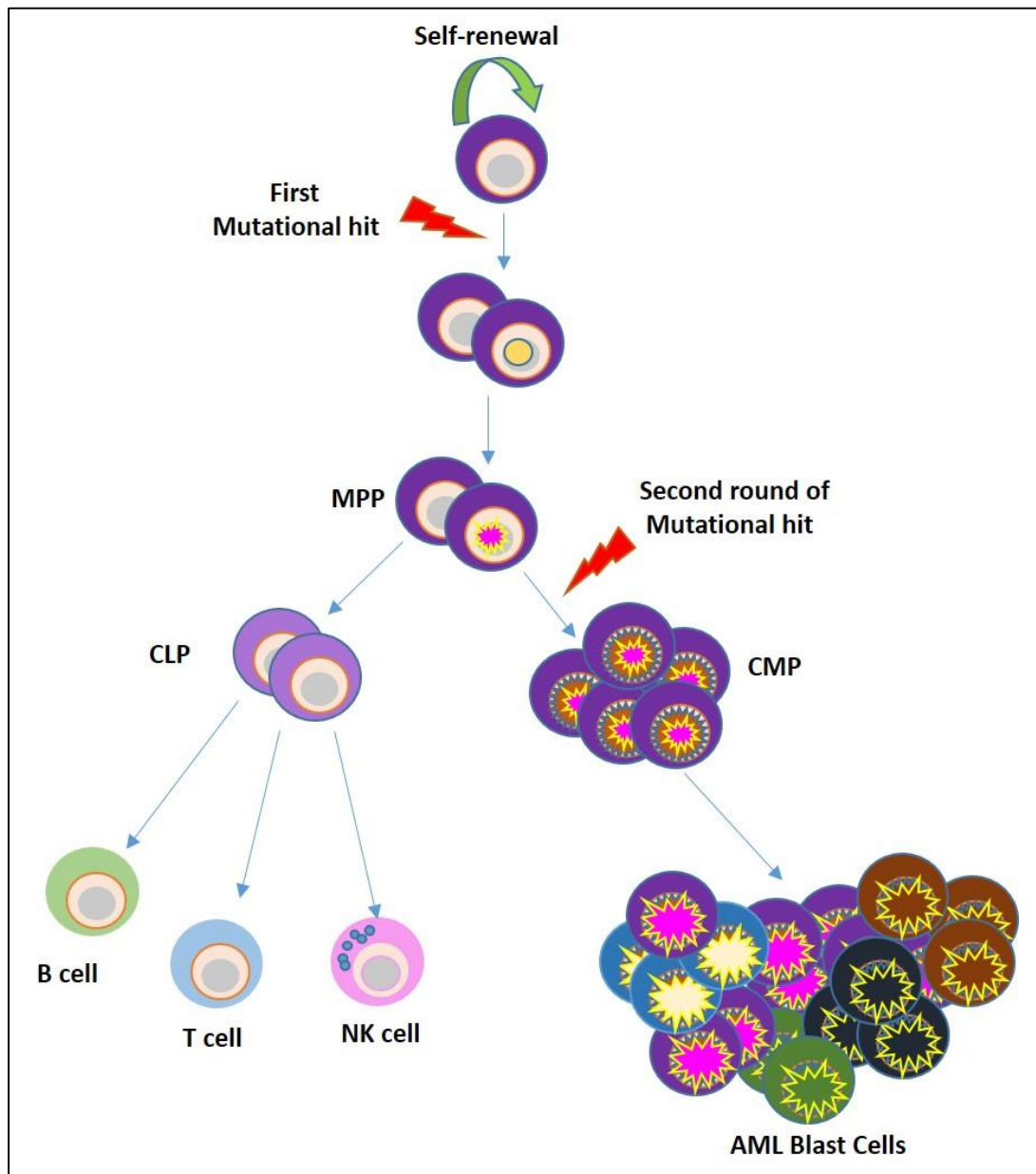


Figure 1.8. Schematic illustration of the development of AML. The first round of mutational hit/s result/s in aberrant expansion in mutated HSC also termed LSC. While second mutational round block differentiation and severe blast expansion in CMP/blast cells with bone marrow infiltration.

AML prognosis is dependent on age, health and genetics aberrations in leukemic cells, cell count, and prior hematopoietic disease. Symptoms at diagnosis of AML can vary from acute bleeding and severe infections to merely fatigue and cachexia. Approximately 40-50% of AML cases, are cytogenetically normal but characterized by distinct DNA mutations in the leukemic cells, with the remaining 50% showing various levels of aneuploidy and cytogenetic alterations such as translocations [124]. Next-generation sequencing (NGS) allowed to define the mutational landscape of myeloid neoplasms. The most common mutations in AML are *NPM1*, *DNMT3A*, *IDH1/2*, and *FLT3* [121]. The *NPM1* mutation is a heterozygous and most frequently detected in about one-third of AML cases with majority insertions of 4 bases found at exon-12 (rarely at exon-9 and exon-11) [121,125]. This insertion results in an encoded protein with a changed C-terminal that restrict NPM1 localization to cytoplasm which impairs the protein's nuclear shuttle function essential for activation and stabilization of tumor suppressors such as p53 and ARF [126]. Mutations in the *FLT3* gene are found internal tandem duplication located within the intracellular domain of this receptor tyrosine kinase (FLT3-ITD), in 15–35% of AML patients. The fragment duplication within exons 14 and 15, that code for the juxtamembrane domain leads to constitutive activation of *FLT3* that results in uncontrolled proliferation of leukemic cells [121,127,128]. Missense mutations occurred in the activation loop domain of the tyrosine kinase of FLT3 (FLT3-TKD) where the codon 835 changed from aspartic acid to tyrosine (D835Y). Other point mutations, deletions, and insertions within codon D835 and its adjacent codons have also been found [129,130]. Many loss-of-function mutations have been described in all exons of *DNMT3A*, including 20–35% with normal karyotype. Most frequently missense point mutation at amino acid R882 occurred, interestingly knockout of *DNMT3A* itself was insufficient to initiate leukemia [131,132]. Mutations in *IDH1/2* gene results in the synthesis of a novel oncometabolite 2-hydroxyglutarate (2-HG) from alpha-ketoglutarate (α -KG), which alters the genome-wide epigenetic pattern by inhibiting the α -ketoglutarate-dependent epigenetic regulators [133].

Mutations in *FLT3*, are associated with adverse risk, while AML with *NPM1* mutations have a favorable risk profile [121,134]. Overexpression of anti-apoptotic protein BCL2 has also been reported in *FLT3* mutated (*FLT3-DupY591*) AML cases [135]. Cytogenetic abnormalities such as t(8;21)(q22;q22), t(15;17)(q22;q12), inv(16)(p13.1;q22) and alterations of 11q23 are the four most common translocations occurring each in 3% to 10% cases of AML, while the frequency of t(16;16)(p13.1;q22), t(9;11)(p22;q23), t(1;22)(p13.3;q13.3) is significantly less. Interestingly,

translocations t(8;21), inversion (16) and t(16;16) constitute the core-binding factor (CBF) leukemias [136]. The *RUNX1-RUNXT1* gene fusion results from t(8;21) and together with inv(16) or t(16;16) with a resulting *CBFB-MYH11* gene fusion, are associated with a favorable prognosis. Deletions of chromosomes 5, 7, 17, inv(3), del(5q), del(17p), a monosomal karyotype, or a complex karyotype defined chromosomal abnormalities, all indicate a poor prognosis [137].

Diagnostic procedures include cell counts and morphology of blood and bone marrow samples, immune-phenotyping, cytogenetics and mutational analyses [138]. AML diagnostic criteria requires $\geq 20\%$ myeloid blasts in peripheral blood or bone marrow or the presence of t(15;17), t(8;21), inv(16) or t(16;16) regardless of blast count [117,138,139]. AML is classified based on lineage/phenotype of the leukemic cells as well as genetic alterations. Based on morphology and cytochemistry the French-American-British (FAB) classification system divides AML into subtypes M0-M7 where, for example, M4-M5 represents myelomonocytic or monocytic AML [140,141]. While M3 (FAB) class of leukemia, acute promyelocytic leukemia (APL), where 97% of the patients have a characteristic translocation resulting in the fusion protein PML-RAR α which blocks the differentiation and drives proliferation at the promyelocytic stage [117,142]. The WHO system, which classifies AML based on genetic aberrations in leukemic cells, is nowadays the most widely used classification system [17]. The 2016 revised WHO classification recognizes the following AML subgroups: AML with recurrent genetic abnormalities, AML with myelodysplasia-related changes, therapy-related myeloid neoplasm, AML not otherwise specified, myeloid sarcoma, myeloid proliferations related to downs syndrome and acute leukemia of ambiguous lineage including mixed phenotype acute leukemia [17,117]. Based on genetic aberrations, AML can be divided into three risk groups according to European Leukemia Net, favourable risk including t(8;21), inv(16), t(16;16), bi-allelic *CEBPA* mutations and *NPM1* mutation without *FLT3-internal tandem duplication (ITD)* or with low allelic ratio, intermediate-risk including mutated *NPM1* with *FLT3-ITD* with high allelic ratio and t(9;11), and high risk for patients with t(6;9), t(v;11), t(9;22), t(3;3), -5, -7, -17, inv(3), del(5q), del(17p), complex karyotype or mutations in *RUNX1*, *ASXL1* or *TP53* [121,137].

Table 1.4: French-American-British and 2016 revised WHO classification of acute myeloid leukemia.

French-American-British classification of acute myeloid leukemias	2016 revised WHO classification of acute myeloid leukemia
M0: Acute myeloblastic leukemia, minimally differentiated (5% of all AML cases)	AML with recurrent genetic abnormalities, AML with t(8:21)(q22;q22); <i>RUNX1-RUNX1T1</i> AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); <i>CBFB-MYH11</i>
M1: Acute myeloblastic leukemia, without maturation (15%)	APL with <i>PML-RARA</i> AML with t(9;11)(p21.3;q23.3); <i>MLLT3-KMT2</i>
M2: Acute myeloblastic leukemia, with granulocytic maturation (25%)	AML with t(6;9)(p23;q34.1); <i>DEK-NUP214</i> AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); <i>GATA2, MECOM</i>
M3: Acute promyelocytic leukemia (10%)	AML (megakaryoblastic) with t(1;22)(p13.3;q13.3); <i>RBM15-MKL1</i>
M4: Acute myelomonocytic leukemia (20%)	AML with <i>BCR-ABL1</i> (provisional entity) AML with mutated <i>NPM1</i> AML with biallelic mutations of <i>CEBPA</i>
M4eos: Acute myelomonocytic leukemia with eosinophilia (5%)	AML with mutated <i>RUNX1</i> (provisional entity)
M5: Acute monoblastic leukemia (10%)	AML with myelodysplasia-related changes, Therapy-related myeloid neoplasm,
M6: Acute erythroid leukemias (5%)	AML with minimal differentiation AML without maturation AML with maturation
M7: Acute megakaryoblastic leukemia (5%)	Acute myelomonocytic leukemia Acute monoblastic/monocytic leukemia Acute erythroid leukemia Pure erythroid leukemia Acute megakaryoblastic leukemia

	Acute basophilic leukemia Acute panmyelosis with myelofibrosis Myeloid sarcoma, Myeloid proliferations related to Downs syndrome Transient abnormal myelopoiesis ML associated with Down syndrome AML not otherwise specified, Acute leukemia of ambiguous lineage including mixed phenotype acute leukemia
--	--

1.5.2 AML Treatment

The standard therapy for AML has not significantly changed in the last decades. The treatment approaches are remarkably similar for all patients except for those with acute promyelocytic leukemia that may benefit from all-trans retinoic acid (ATRA) in a great effect on high overall survival rates [143]. AML is genetically much more diverse, therefore a larger challenge to offer targeted therapies are available. A majority of AML patients are treated in a stepwise manner, first patients receive induction therapy with an anthracycline (DNA alkylator and topoisomerase II inhibitor, thus interfering with both replication and transcription) such as daunorubicin (≥ 60 mg/m²) for three days and cytarabine (a nucleoside analog leading to apoptosis) intravenously (100-200 mg/m²) continuously for seven days. Induction therapy is designed to decrease the amount of the leukemic cell burden several orders of magnitude and complete remission (CR) is achieved in approximately 70% of patients below 60 years and 40-60 % in patients above 60. Mostly relapse unavoidably occurred if the therapy is discontinued due to the presence of leukemic stem cells (LSCs) that contrary to their progeny, are much more resistant to chemotherapy [144–146]. These small numbers of cancer cells that persist in a patient during or after treatment, even though clinical and microscopic examinations confirmed CR, represented the minimal residual disease (MRD). Molecular MRD is based on detecting a disease-specific DNA or messenger RNA (mRNA) sequence through the use of real-time polymerase-chain-reaction (PCR)-based approaches, since cytogenetic techniques such as karyotyping and/or FISH lacks the

sensitivity required for MRD monitoring. Based on the presence of a persisting PCR-positive leukemia-specific signal after therapy, relapse risk can be estimated in individual patients [147].

To minimize the risk of relapse, induction therapy is followed by consolidation therapy to eliminate residual leukemia cells, which can be either additional chemotherapy (additional nucleoside analogs and anthracyclines) or bone marrow transplantation [148]. In patients not fit for intensive chemotherapy, hypomethylating agents such as azacitidine are alternative treatment regimens and for cytoreduction in palliative cases, hydroxyurea or low dose cytarabine can be used. The preferred consolidation therapeutic option, for patients responding poorly to chemotherapy alone or high-risk AML patients, is allogeneic stem cell transplantations. The severe intensity and stress of allogeneic stem cell transplant is poorly tolerated in older individuals, above the age of 70, while advances have significantly improved the prognosis of younger patients with AML. However, only approximately 40% of all younger patients (<60 years old) and 5-15 % of older than 60 years patients are cured from AML [149]. In a large epidemiological study, the reported relative 5 year overall survival rates for patients below 45 was 41%, below 65 was 30%, and above 65 was reported to be only 4% [124].

Allogeneic stem cell transplantations, however, carries a risk of treatment-related mortality of 10–25% due to graft-versus-host disease (GVHD) reflecting that transplanted cells may also attack healthy tissue as the patient's own hematopoietic system needs to be destroyed before donor bone marrow be transplanted [150]. Therefore, the risk/benefit ratio must be carefully assessed before HSCT transplantation. Few targeted therapies in clinical trials including FLT3 inhibitors Sorafenib, Midostaurin, Quizartinib and Crenolanib, IDH1 and IDH2 blockers Ivosidenib and Enasidenib, and BCL-2 inhibitor Venetoclax, Hedgehog inhibitor glasdegib and monoclonal antibodies such Gemtuzumab (targeting CD33) showed good results, particularly in patient with an unfavorable cytogenetic and genetic mutation profile. Their own disadvantage is that they are restricted to patients harboring these particular genetic mutations. Chimeric antigen receptor (CAR) T-cells have proved to be very satisfactory in lymphoblastic leukemias and some lymphomas, while in AML serious side effects (targeting CD33 results in profound myeloid cytopenia, and other appropriate antigens have so far not been identified) have reduced their clinical use[151].

1.6 Ubiquitination

The function and activity of almost every cellular protein can be modulated by posttranslational modifications (PTMs) [152]. In particular, ubiquitination has emerged as one of the most conserved eukaryotic PTMs from yeast to humans. Ubiquitination is associated with almost every cellular process including signal transduction, DNA damage repair, cell cycle regulation, cellular differentiation and autophagic clearance [153]. Ubiquitination is a reversible and energy-dependent enzymatic process by which the ubiquitin is either covalently linked to substrate proteins or alternatively can form free polyubiquitin chains. The ubiquitination process requires three classes of enzymes namely ubiquitin-activating (E1), ubiquitin-conjugating (E2) and ubiquitin ligase (E3) (**Figure. 1.9**). The ubiquitin ligases are the terminal enzymes transferring the ubiquitin to the ϵ -amino group of a lysine residue on the substrate or the amino-terminus of a polypeptide [154]. The E3 ligases often function as multi-subunit complex determining the substrate specificity [155]. In human, the E3 ligase family comprises more than 600 members classified into three sub-families, based on their structural and catalytic features, named RING (Really Interesting New Gene), HECT (Homologous to the E6AP Carboxyl Terminus) and RBR (Ring Between Ring). The HECT and RBR catalyse the ubiquitin transfer to the substrate by forming an E3-ubiquitin thioester intermediate at a catalytic cysteine residue while RING members do not possess direct catalytic activity but rely on the enzymatic activity of E2 to ubiquitinate the substrate [155,156].

The most common sites of ubiquitination are Lys residues, less frequently Ub can be attached to N-terminal Met, Ser/Thr hydroxyl and to Cys thiol groups [157]. This conserved process mostly targets unwanted proteins for degradation either through proteasome-mediated or by directly sorting proteins to the lysosome and thus helps to maintain cellular homeostasis. However, ubiquitination may also play a crucial role in other non-proteolytic regulatory functions such as protein activation, interaction, and translocation [158].

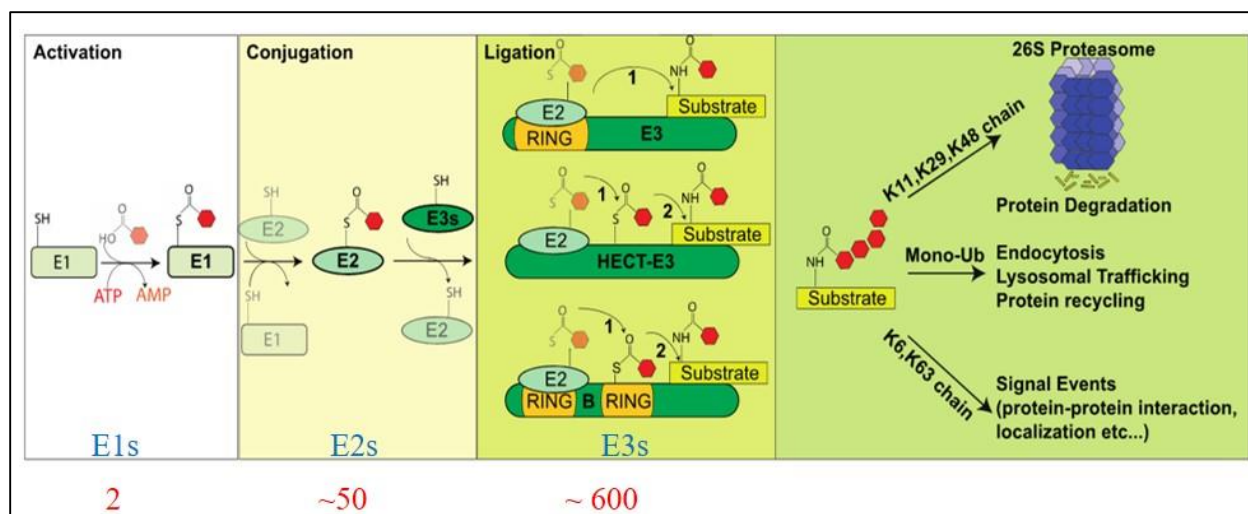


Figure 1.9. The Protein Ubiquitination Pathway. Protein ubiquitination is an ATP-dependent process of a sequential reaction catalyzed by three classes of enzymes. Ubiquitin E1 activating enzyme, through ATP catalysis, form a thioester bond with a cysteine residue (the active-site) of ubiquitin, subsequently activated ubiquitin transferred to a cysteine residue of conjugating enzyme E2 ligase, and ultimately specific E3 ligase catalyze/facilitate the formation of an isopeptide bond between the carboxyl-terminal glycine of ubiquitin and a lysine residue on the substrate protein [153].

The 76-amino acid long ubiquitin-protein has seven intrinsic lysine residues (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48 and Lys63), through one of these ubiquitin can be conjugated directly to a target protein or to itself or in the case of linear head-to-tail poly-Ub chains, through its the N-terminal Met residue [158]. Lysine 48 linked (K48-linked) polyubiquitin chains, in which the carboxyl-terminal glycine residue of one ubiquitin moiety is linked to the K48 of another ubiquitin moiety, direct 26S proteasomal protein degradation [159]. More recent studies have revealed that monoubiquitination and ubiquitin chains conjugated through the other six lysine residues also play important roles in the regulation of numerous cellular processes. K63-linked polyubiquitin chains, primarily results in an altered function and in general does not trigger proteasomal degradation of the modified protein, for example, has been reported to control DNA repair, NF κ B activation, translational regulation, and endocytosis [160,161]. Monoubiquitination or multi-monoubiquitination of proteins, on the other hand, has been shown to regulate endocytosis and trafficking of plasma membrane proteins. Another additional layer of ubiquitin signal regulation/diversification is represented by PTMs affecting ubiquitin itself, including acetylation and phosphorylation [162,163].

Similar to phosphorylation or many other protein posttranslational modifications, ubiquitination is a reversible process that can be reversed by the action of deubiquitinases (DUBs). Most of DUBs catalyse a proteolytic reaction between the isopeptide bond formed between the ϵ -lysine amino group of the substrate and the carboxyl group of the ubiquitin C-terminus. Such dynamic regulation allows the protein ubiquitination procedure to respond to various signals to regulate the fast-changing cellular processes, along with the maintenance of monomeric pools of ubiquitin [164].

E3 ubiquitin ligases regulate many biological processes through timely ubiquitination and degradation of many cellular proteins responsible for gene transcription/repression, receptor endocytosis, intracellular trafficking, signal transduction, cell cycle progression, DNA damage recognition and repair, autophagic clearance, differentiation and apoptosis [165,166]. For these reasons, dysregulation of E3 ligases often lead to the development of several forms of diseases [156] such as central nervous system disorders, inflammation, metabolic dysfunctions and several cancers [167,168] including hematologic malignancies [169]. Remarkably, several ubiquitin ligases are mutated, overexpressed, or deleted in the hematologic malignancies, contributing to the pathogenesis of the diseases through accumulation, or excessive degradation, of their substrates that may in turn acquire critical functions during the hematopoietic cell growth and differentiation [169]. The overexpression of E3 ligase MDM2, has been reported in many leukemia patients, involved in ubiquitination and degradation of the p53 [170]. Similarly, the mutations in the E3 ligase *CBL* gene has been identified in acute myelogenous leukemia. CBL (Casitas B-cell lymphoma) is involved in the ubiquitination and subsequent internalization and degradation of the activated FLT3 (fms-related tyrosine kinase 3) receptor. The mutations in *Cbl* affect its activity, which results in a halt in the FLT3 receptor ubiquitination and thereby it is no longer inactivated, resulting in a constitutively active form of the receptor [171]. Accumulating shreds of evidence are showing that HERC family proteins are key components of a wide range of cellular functions including pivotal roles in cancer-related pathways and the assessment of their transcript amount reveals that they might act as effective prognostic factors (proteinsatlas.org) [172]. However, evidence concerning the non-solid tumors, and especially myeloid related neoplasms, are currently lacking. In the present study I aimed to assess the potential association of the gene expression of *HERC1*, a giant HERC E3 ubiquitin ligase family member, in myeloid related disorders, namely CML, MPNs and AML and then investigate the potential functions of *HERC1* in blood cells.

1.6.1 HERC Protein Family

The human HERC protein family belongs to the superfamily of HECT E3 ubiquitin ligases. HERC family consists of six protein members, which are characterized by having one or more RCC1 (regulator of chromosome condensation)-like domains, and a C-terminal HECT (homologous to E6-associated protein carboxyl terminus domain) domain act as ubiquitin ligases [173]. Sequence analysis makes it possible to classify HERC family members into two groups: the large and the small HERCs [174]. Despite their similarities in terms of both structure and domains, these subfamilies are evolutionarily very distant which result from the convergent evolution of ancestors belonging to distant families [175]. Large HERCs, including HERC1 and HERC2, encoded in chromosome 15, are giant proteins containing close to 5000 amino acid residues and more than one RLD, other known sequence motifs in addition to the HECT, whereas small HERCs (HERC3, HERC5, and HERC6) encoded on chromosome 4, while HERC4 encoded on chromosome 10, are less than one-fourth the size of their larger counterparts have only one RLD and the HECT domain [174]. Structurally, small HERC proteins contain characteristic two domains, whereas, functionally, they have been related to ubiquitination and ISGylation processes associated with membrane traffic and the immune response. Structural differences were also observed between the RLDs in Large and Small HERCs [176]. Although not much is known about the cellular functions of all these proteins, their molecular characterization so far points to a role in intracellular trafficking.

1.6.2 Domain Organization of HERC1

The human *HERC1* gene designated *p619*, (GenBank GeneID: 8925) spans 225 kb on chromosome 15q22 and consists of 78 exons, that encodes a polypeptide of 4861 amino acid residues. HERC1 is the largest family member (532 kDa) and is the founding member of HERC E3 ubiquitin ligases, contains a number of conserved sequence features which are supposed to play different roles in the overall function(s) of the protein [177]. It possesses two RLDs (RLD1 and RLD2) which have been implicated in different cellular tasks, RLD1 stimulates guanine nucleotide dissociation on several ARF and Rab family GTPases and also has an affinity for several membrane phospholipids including all monophosphate phosphoinositides as well as PI(3,5)P₂, PI(4,5)P₂, RLD2 forms a ternary complex with clathrin heavy chain and the chaperone Hsp70 [178,179]. This fact, together with HERC1's localization in inner cell membranes such as the Golgi

apparatus and the cytosol [177], have led to the suggestion that this giant protein has an important function in intracellular membrane trafficking. In addition to its RLDs, the most relevant conserved regions in HERC1 include a SPRY (repeats in splA and RyR) domain, a WD-40 domain and BH3 domain, this type of domain has been found in proteins implicated in distinct cellular functions and is believed to play a role in protein-protein interactions [180]. It also has three proline-rich motifs, seven polar amino acid-rich regions consisting preferentially of acidic residues, a putative leucine zipper, several potential phosphorylation sites for cAMP-dependent protein kinases, also several tyrosine phosphorylation and a carboxy-terminal HECT domain characteristic of E3 ubiquitin-protein ligases [181,182].

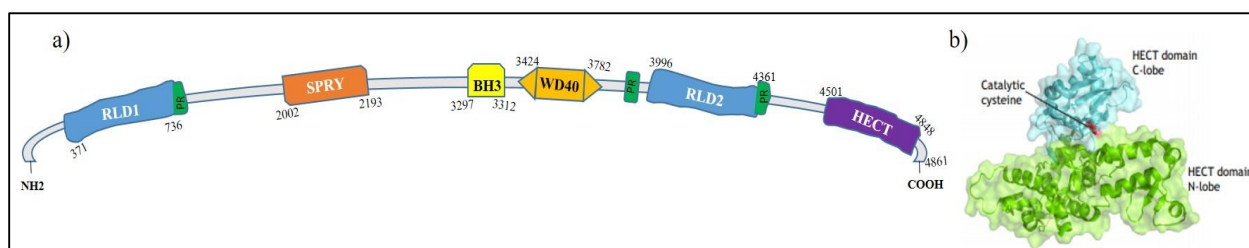


Figure 1.10. Structural features of human large HERC1. **a)** Overview of the approximate relative position of domains organization. **b)** Crystal structure of the HECT domain consists of two lobes the N-terminal lobe and the C-terminal lobe (PDB) [181,182]. Domain abbreviations used are as follows: *RLD*; RCC-like domain, *WD40*; W-D dipeptide repeat domain, *BH3*; BCL-2 homology 3 domain, *HECT*; homologous to E6AP C-terminus, *SPRY*; SPIA and ryanodine receptor domain.

It has been reported that the HECT domain of HERC1, has a physical interaction with the M2 isoform of glycolytic enzyme pyruvate kinase (M2-PK). Nevertheless, the physiological significance of this interaction could not be pinpointed [183]. In E6 protein expression cells, the HECT domain of HERC1 interact with BAK, a pro-apoptotic BCL-2 protein family member that functions ultimately permeabilize the mitochondrial outer membrane, thereby committing a cell to death, a process involving changes in BAK phosphorylation and conformation. HERC1 has been reported to mediate proteasomal degradation of BAK while knockdown of the HERC1 ligase robustly restored levels of BAK to E6-expressing cells [182].

Each RLD region is followed by a proline-rich domain whose amino acid sequence matches that of the class II consensus SH3 binding sites [184]. These domains likely mediate the

interaction of HERC1 with SH3-containing adaptor proteins that may facilitate the access of the RLDs to other p619 substrates. The HERC1 carboxy-terminal HECT domain, like other HECT domains, consists of two lobes the N-terminal lobe and the C-terminal lobe, most likely catalyze the transfer of ubiquitin to specific proteins in a fashion similar to that reported for other HECT domain-containing proteins such as E6-AP, in which N-terminal lobe first interact with a charged E2–ubiquitin and the C-terminal lobe, by its conserved catalytic cysteine residue form E3-ubiquitin thioester intermediate during ubiquitylation activity [181]. It is possible that some of the above protein-protein interaction motifs may serve to bring putative ubiquitination substrate(s) to HERC1.

1.6.3 *HERC1* Gene Variants (SNP and Mutations)

A few *HERC1* polymorphisms in coding exons reveal pronounced differences in allele and haplotype frequencies between East Asian populations (Chinese and Japanese) and Africans and Caucasians populations [185]. While recent in vivo studies have implicated mutations in *HERC1* cause neurological disorders. Evidence obtained with the tambaleante mutant mouse, which carries a spontaneous Gly483Glu mutation in the amino-terminal RCC1-like domain of the HERC1 protein. This result in misfolding in the RCC1 domain and an increase of mutated protein levels, which reduced the growth and lifespan of mice, and an ataxic syndrome caused by the almost complete loss of cerebellar Purkinje cells as well as in other neurons during adult life these mice [186–188]. Retardation in growth might be caused by an abnormal HERC1-TSC1/TSC2 interaction and that HERC1 function impairment might occur through the mTORC1[189].

Biallelic mutations in human *HERC1* have been associated with overgrowth and intellectual disability [190,191]. Mutations in HERC1 have been also reported in breast cancers [192,193], non-melanoma skin [182], and in different hematologic malignancies [169,194]. Recent studies reported that HERC1 deletion in acute lymphoblastic leukemia (ALL) cells affect the DNA mismatch repair enzyme MSH2 levels [195]. In addition to that, an atypical *HERC1-PML* transcript fusion mRNA was reported in acute promyelocytic leukemia [196].

1.6.4 HERC1 Interactome and its Function

HERC1, ubiquitously expressed with higher levels in brain and testis, and the lowest level in liver, has been implicated in membrane trafficking, cell proliferation/growth and apoptosis through its interaction with ARF, Rab, Clathrin, M2-pyruvate kinase, TSC2 and BAK proteins [174]. HERC1 has been reported to interact with tuberous sclerosis 2 (TSC2, tuberin), and results in its destabilization which suppresses cell growth [189]. The C-RAF autophosphorylation at Serine 621 stabilizes C-RAF, inhibiting its degradation by the proteasome. Interestingly, in the absence of Serine 621 phosphorylation, C-RAF is degraded by the proteasome by mechanisms that involves an unknown E3 ubiquitin ligase [197]. Indeed, recent findings revealed that HERC1 finely controls the ERK signaling by regulating the C-RAF stability via ubiquitination [198].

Membrane trafficking is believed to be regulated by a series of small (20- 25 kDa) GTP binding proteins that confer specificity and directionality [199]. One of these families of proteins, designated as ARFs, act as co-factors required for ADP-ribosylation of the trimeric G protein, involved in intracellular vesicular transport, actin polymerization, phospholipid metabolism. ARFI, the best characterized member of this protein family, appears to be localized primarily on coated vesicles and Golgi membranes [200]. Another group of small GTP binding proteins involved in membrane trafficking is the Rab family [201]. HERC1 has been reported to act as GEF for ARFI (and possibly for certain Rab proteins), which regulates secretory trafficking across Golgi membranes through interaction with vesicle coat proteins, and thus plays an important role in the regulation of membrane trafficking [177]. RLD1 domain of HERC1 also has an affinity for several membrane phospholipids including all monophosphate phosphoinositides as well as PI(3,5)P₂, PI(4,5)P₂ and its activity as guanine release factor (GRF) depends on the presence of bound PI(4,5)P₂ on its RLD1 domain [178,202]. Cellular levels of PI(4,5)P₂ are controlled by the relative activities of two sets of enzymes, namely phosphoinositide kinases and inositol phosphatases, which are in charge of its synthesis and degradation, respectively. One of the former enzymes, PI(4)P 5-kinase, has been shown to be one of the major downstream targets of active ARFs [203,204]. IQGAP1 (IQ-motif-containing GTPase activating protein 1) a scaffolding protein involved in the regulation of actin and microtubule organization, cell migration and cell proliferation, also associate with HERC1 and promotes ARF6 activation [205]. More recent work

uncovered the role of HERC1 in the regulation of cell migration through RAF-dependent MKK3/p38 signalling [206].

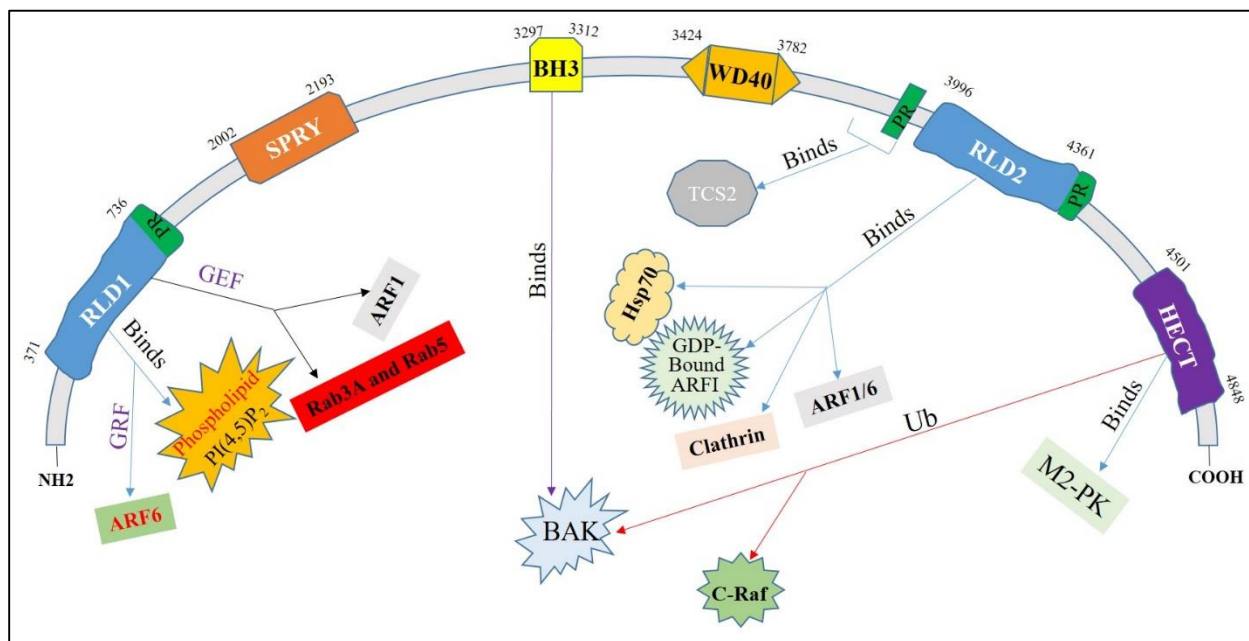


Figure 1.11. Structural domains of HERC1 and HERC1 Interactome. Schematic indication of different binding partners of HERC1 and their interaction with specific domains.

RLD2 domain of HERC1 interacts with ARF1 and forms a ternary complex with clathrin and the heat shock protein 70 (Hsp70) [179]. This indicates the multi-domain structure of HERC1 makes it a suitable candidate to act as a scaffolding protein by interacting simultaneously with many other proteins, thus bringing them together, in a way similar to the manner in which the protein paxillin works [207]. In the last few years, some efforts have been made to identify large HERCs interacting partners with the aim to gain insight into the cellular functions of these proteins [181]. The close family member, the giant HERC2 has been reported to interact with a dozens of binding partners while no more than a bunch have been experimentally ascertained for HERC1 [208]. Thus it derived my attention to investigate the potential binding partners of HERC1 in order to clarify/shed some light on its functional role in regulating pivotal cellular pathways.

AIMS OF THE STUDY

Besides the roles played by HERC1 in the nervous system of higher organisms like mammals, in the past few years it has emerged that few hematological neoplasms harbor somatic mutations affecting the HERC1 locus in different kind leukemia. However, the roles played by HERC1 in blood cells, under physiological and pathological conditions, currently remain unknown. Hence, the aims of this study were to

- Assess whether *HERC1* might be, or not, associated with myeloid related neoplasms, namely Acute Myeloid Leukemia (AML), Myeloproliferative neoplasms (MPNs) and Chronic Myeloid Leukemia (CML).
- Investigate the interaction of HERC1 and BCR/ABL1 (as binding-partner) in the pathogenesis of chronic myeloid leukemia.
- Identify the potential physical binding partners of HERC1 through co-immunoprecipitation (co-IP) under physiological condition.
- Investigate the potential role/involvement of HERC1 in blood cells differentiation and hematopoiesis.

2. MATERIALS AND METHODS

2.1 Patient Cohorts and Cell Lines

2.1.1 Clinical Samples

This study was performed on cells derived from bone marrow and peripheral blood samples of patients affected by myeloproliferative neoplasms (MPN) including polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF), chronic myeloid leukemia (CML) and acute myeloid leukemia (AML). All these samples were collected and processed in the Laboratory of Molecular Medicine and Oncology, Department of Clinical and Biological Sciences of the Hospital San Luigi Gonzaga Orbassano (TO), after written informed consent (local ethic committee S. Luigi Gonzaga Hospital 201/2014). In addition, peripheral blood (PB, n=34) and bone marrow (BM, n=16) specimens were also collected from healthy donors. Among CML samples, 37 were PB and 35 BM (**Annex Table A2.1**), among AML specimens, 80 were BM and 17 were PB (**Annex Table A2.2**) while MPNs specimens were exclusively PB (**Annex Table A2.3**). In CML cases, samples were also collected at relapse (BM, n=08) and 54 at remission (BM, n=09 and PB, n=45). The research project was done in accordance with the Helsinki Declaration of 1975, which was revised in 1983.

2.1.2 Cell Lines

Three myelogenous leukemia cell lines, K-562, HL60 and HEL, were used throughout this investigation. Additionally, a human embryonic kidney cell line known as HEK-293T was also used. The K-562 cells were cultured in complete RPMI-1640 medium (Euroclone, #ECB9006L), HEL and HEK-293T cell lines in Dulbecco's modified Eagle's medium and HL60 in ISCOVE's medium. All cell lines media were supplemented with heat-inactivated 10% fetal bovine serum (FBS, Euroclone, #ECS0180L), 1% glutamine (Euroclone, #ECB3000D-20) and 1% penicillin/streptomycin (Euroclone, #ECB3001D). Primary CML cells derived from patients' bone marrow were cultured in ISCOVE's medium, supplemented with heat-inactivated 20% FBS. All cell lines and primary cells were incubated at 37°C and 5% CO₂ in a humidified incubator and seeded upon confluency every 2-3 days.

2.1.3 Freezing and Thawing Cells

The exponentially growing cells were centrifuged at 1200 rpm for 5 minutes and the cell pellet was re-suspended in 1mL FBS containing 10% dimethyl sulfoxide (DMSO). The cell suspension was transferred into cryovials and stored initially at -80 °C and for a long period of storage in liquid nitrogen. For cell thawing, cryovials were incubated in a water bath at 37 °C to rapidly defrost. Then, cells were centrifuged at 1200 rpm for 5 minutes, after suspension in a pre-warmed complete medium with 20% FBS. The supernatant was discarded and the cell pellet was re-suspended in fresh media and transferred to a new cell culture flask. Subsequently, cells were cultured in optimal conditions.

2.1.4 Cell Counting and Trypan Blue Exclusion

In order to seed the appropriate number of cells during the experiments, viable cells were counted using haemocytometers after staining with trypan blue exclusion buffer. Trypan Blue (Sigma-Aldrich T6146) was used to calculate a dead-viable cell ratio within the cell suspension. Under light microscopy, dead cells were recognized as blue due to the internalization of trypan in dead cells. To perform cell counting and trypan blue exclusion assay 10µl of cell dilution was resuspended in 10µl of trypan blue. Then 20µl of the final solution was loaded into the haemocytometer. The number of living cells in each square corner of the haemocytometer chamber were counted and averaged. Subsequently, calculations were performed to obtain the number of living cells per µl in order to seed the adequate number of live cells required for specific experiments.

2.1.5 General Reagents

The composition of chemical solutions, buffers, and reagents that were used in this study are given in table 2.1. Unless otherwise stated, all chemical reagents were purchased from Sigma-Aldrich.

Table 2.1: Composition of Solutions and Buffers.

Reagents/Solution	Composition
	10mM Tris-HCl pH 7.5, 100mM NaCl, 0.3% CHAPS, 50mM NaF, 0.5mM EDTA, 1mM sodium vanadate, 1mM phenylmethylsulfonyl fluoride, 5µg/mL leupeptin,

CHAPS Buffer (1×) pH 7.2.	5µg/mL aprotinin, 1µg/mL pepstatin A, 50mM β-glycerophosphate, 100µg/mL benzamidine, and 1µM E-64.
LDS sample buffer (4×)	250mM Tris-HCl pH 8.5, 2%(w/v) LDS (Lithium dodecyl sulfate), 100mM DTT, 0.4mM EDTA, 10%(v/v) glycerol, 0.2mM phenol red, and 0.2mM Coomassie Brilliant Blue (CBB) G.
Running Buffer (1×) pH 8.2	50mM tricine, 50mM Tris, 0.1%SDS, 1.3mM sodium bisulfite.
Running Buffer (1×) pH 7.2.	20%methanol, 25mM bicine, 25mM Bis-Tris, 1mM EDTA, and 1.3mM sodium bisulfite.
Phosphate buffered saline (PBS)	137 mM NaCl, 2.7 mM KCl, 10 mM Na ₂ HPO ₄ , 1.8 mM KH ₂ PO ₄
Tris-buffered saline (TBS) pH 7.5	50 mM Tris-Cl, 150 mM NaCl
TBS-Tween buffer	0.3% Tween20 in TBS
FACS buffer	1% Foetal bovine serum (FBS), 1mM EDTA, 0.1% sodium azide in PBS
FACS Blocking buffer	Equal volumes of FACS buffer and FBS
Tris-acitrate-EDTA (TAE) buffer	40 mM TAE, 1 mM EDTA (pH 8.2 - 8.4)
Benzidine solution	0.5% glacial acetic acid, 0.2% benxidine, 30% H ₂ O ₂
Complete medium	10% FCS, penicillin (100units/ml), streptomycin (100units/ml), L-Glutamine (2mM) in RPMI/DMEM/ISCOVE's.
Red Blood cell (RBC) lysis buffer	10% (v/v) 10x RBC lysis soln (Miltenye Biotech, Bergisch Gladbach, Germany) in dH ₂ O
4% Paraformaldehyde (PFA) solution	4% (v/w) PFA in PBS. pH 6.9 with 1N NaOH. Stored at -20C
Cryopreservation media	90% FCS, 10% Dimethyl Sulphoxide (DMSO)
<i>In-vitro</i> Kinase Buffer	50 mM HEPES, 150 mM NaCl, 1 mM MgCl ₂ , 1 mM MnCl ₂ , 10 mM NaF, 1 mM NaVO ₃ , 5% glycerol, 1% NP-40, 1 mM DTT, 1 mM PMSF.
ACD Buffer	0.5% of bovine serum albumin (BSA) and 2mM EDTA in PBS pH 7.2

2.2 Separation of Cellular Fractions

2.2.1 Samples Preparation from Patients' PB and BM

Mononuclear cells (MNCs) were isolated from blood samples via centrifugation in density gradient Lymphoprep™ (Pharmacia Biotech, Piscataway, NJ, density 1.077 ± 0.001 g/ml). To optimize the procedure and to avoid the sedimentation of groups of cells, whole blood was diluted 1:2 with phosphate buffered saline (PBS) 1X. Two volumes of diluted blood were layered on a volume of Ficoll and subjected to centrifugation at 1500 rpm without brakes for 30 minutes at room temperature. In this way, various blood cells are stratified according to their density on the density gradient created by the Ficoll: erythrocytes, with greater density, through the Ficoll layer, were deposited on the bottom, the polymorphonuclear (PMN) were stratify forming a thin whitish coating over of red blood cells, while mononuclear cells of peripheral blood, being less dense than Ficoll, were arranged in the interface between the plasma and the polymer forming a whitish ring, consisting mainly of lymphocytes and monocytes. Mononuclear cells were washed with 1X PBS to remove the residual plasma and Ficoll reagent. Cells were counted using a disposable hemocytometer and then pelleted down for downstream experiments. The granulocytes, obtained from the previous centrifugation on a density gradient, however, were additionally treated with a red blood cell (RBC) lysis buffer (Table 2.1), to remove the residual red blood cells, for 10minutes at room temperature. The granulocytes were, then, pelleted by centrifugation at 2800 rpm for 10minutes after washing with 1X PBS.

2.2.2 CD34⁺ Cells Separation

The isolation of CD34⁺ cell fraction, was achieved through the direct CD34 Progenitor Cell Isolation Kit (Miltenyi Biotec Inc., Auburn, CA, USA), following the manufacturer's instructions. 10^8 cells were re-suspended in 300μl of ACD-Buffer and incubated with 100μl of FcR Blocking Reagent and 100μl of positive CD34 MicroBeads for 30 minutes at 4°C. Magnetic separations of CD34⁺ fraction was performed by using the MACS separator with appropriate MACS (MS) Column after its rehydration with an appropriate volume of ACD-buffer. Subsequently, the cell suspension was deposited on the column, filtered and washed three times with ACD-buffer washes. Flow through, containing unlabeled CD34 negative (CD34⁻) cells, were also collected. Positively labeled cells (CD34⁺), which remain magnetically linked to the column, were flushed out by

firmly pushing the plunger into the column with an appropriate amount of buffer in a collection tube. The CD34⁺ cells were confirmed through FACS analysis.

2.2.3 CD3⁺ and CD19⁺ Cells Separation

The isolation of CD3⁺ and CD19⁺ cell fraction was achieved through the direct CD3 and CD19 Cell Isolation Kit (Miltenyi Biotec Inc., Auburn, CA, USA), following the manufacturer's instructions as described in the previous section. Briefly, MNC cells (10^8), were re-suspended in 100uL of ACD buffer, and incubated for 15minutes at 4°C with 40μl of CD3 or CD19 Microbeads. The subsequent magnetic separation, was performed following the same protocol used to elute the bound CD34⁺ cells.

2.2.4 *In Vitro* Treatment

Primary CML cells derived from patients' bone marrow were cultured in ISCOVE's medium, supplemented with heat inactivated 20% FBS. Cells were maintained for 5 hours in the medium in incubator prior to drug treatment with Imatinib (2.5uM) for 48 hours. Similarly, the K-562 cells were also treated with Imatinib (2.5uM) and geldanamycin (10uM) for 48hrs in different experiments. Additionally, HEK-293T normal and transfected cells with overexpressing *BCR-ABL1*-vector were treated with geldanamycin (10uM) and Imatinib (15uM) for 48hrs respectively.

2.2.5 Cytospin Preparation

K-562, BM and PB cells from CML patients and healthy donor were counted, washed and resuspended at a concentration of 0.5×10^6 cells/ml in 1X PBS. Glass slides (VWR International, Radnor, USA) were appropriately labeled and adjusted into the metal slide holder, followed by a cytopsin filter and a cuvette. 100μl of cell suspension was added to the cuvette and placed in the cytopsin centrifuge. The cell suspension was centrifuged at 500 rpm for 5minutes. The slide was carefully removed from the metal slide holder and left to air dry. Slides were fixed in 4% paraformaldehyde (PFA) for 10minutes, followed by immunofluorescence.

2.2.6 Immunofluorescence

Cytospins were prepared using K562, BM and PB cells from CML patients and healthy donor cells. HEK-293T cells were directly grown on coverslips placed on the bottom of a 24-well plate, and subsequently fixed with 4% paraformaldehyde (PFA). After fixation cells were

permeabilized with PBS 0.1% Triton X-100 for 5 minutes. After two rapid washes with 1X PBS, the cells were saturated with blocking solution (10% Fetal Bovine Serum +5% BSA + 1% fish gelatin) for 45 minutes followed by washing for 3 times with 1X PBS and then incubated for 2 hours at room temperature or overnight at 4 °C with the primary antibody of interest diluted 1:100 in blocking solution [antibodies used: anti-HERC1 anti-BCR (Table 2.4)], and then washed 3 times with PBS for 5 minutes. The antigen-antibody complex was detected by incubation for 45 minutes with appropriate secondary antibody (dilution 1:1000) goat anti-mouse/rabbit Alexa Fluor488, labeled with fluorescein (FITC) or rhodamine. The cells were then washed in PBS for 5 minutes and the nuclei were stained with DAPI. Slides were analyzed by fluorescence confocal microscopy (LSM5110; Carl Zeiss MicroImaging, Inc.); the images are captured using the 63X objective. The intensity of the signal from approximately 80 cells from each condition, were evaluated through the use of the ImageJ software. For each sample, repeated in triplicate, were performed three independent measurements and each measurement was normalized on background noise. The mean intensity $\pm$ standard deviation δ (ds), expressed as fluorescence intensity/number of pixels, was calculated and plotted using the program GraphPad Prism.

2.3 Analysis of Gene Expression

2.3.1 Isolation of RNA

Total RNA was extracted using TRIzol Reagent (Ambion, Thermo Fisher Scientific, Waltham, Massachusetts, MA, USA), according to the manufacturer's instructions. The RNA obtained were re-suspended in RNase-free water (Sigma-Aldrich) and quantified using Nanodrop (ThermoFisher Scientific). A 260/280nm ratio was used as a parameter to evaluate the purity with a ratio of 1.80 as regarded highly pure.

2.3.2 Reverse Transcription and Gene Expression Analysis by qPCR

Complementary DNA (cDNA) was synthesized from 1 μ g of total RNA using the Applied Biosystem high capacity cDNA reverse transcription kit (Catalog number: 4368814 Applied Biosystems, Foster City, CA) according to manufacturer's instructions. The reagents for the RT master mix and the PCR program conditions are shown in table 2.2. cDNA samples were stored at -20°C until use. Real-Time quantitative PCR (qPCR) was performed by using the C1000 Thermal Cycler CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA). The RT-qPCR allows the

determination of relative gene expression in a sample. All PCR reactions were performed in triplicates, by using TaqMan Gene expression mastermix (Applied Biosystems). The qPCR cycle condition was described in Table 2.3. The *GUSB* was used as an internal control. For *HERC1* mRNA quantification, specific assays (assay ID for *GUSB* Hs00939627_m1, and Hs01032486_m1 for *HERC1*-Applied Biosystems, Thermo Fisher Scientific, Massachusetts, MA, USA) were used. The TaqMan qPCR system uses a fluorescent probe, specific to the gene of interest, in addition to the primers. The *HERC1* Ct values obtained by RT-qPCR were normalized with respect to the Ct counterparts of *GUSB* and expressed as $2^{-\Delta\Delta C_t}$. A universal human references RNA (Stratagene, San Diego, CA, USA) was used to calibrate the assay.

Table 2.2: Reagents and PCR Programs used for cDNA Synthesis

Mastermix for cDNA synthesis	Stock concentration	Per sample (μl)
RT-PCR Buffer II	10x	2
MgCl ₂	25mM	4
DTT	0.1M	2
dNTP Mix	10mM	2
Random Hexamers	50μM	2.5
RNase Inhibitor	20U/μl	1
MuL-V Reverse Transcriptase	50U/μl	2
RNase free water	---	7.5
RNA	1μg/μl	1
PCR programs	Temperature	Time
	1. 25°C	5 min
	2. 42°C	60 min
	3. 80°C	5 min
	4. 4°C	Hold

Table 2.3: Reagents and PCR Programs used for Gene Expression Analysis by qPCR

Mastermix for qPCR	Stock concentration	Per sample (µl)
TaqMan Buffer	2x	5
HERC1 TaqMan assay/ control	20x	0.5
RNase free water		2
cDNA		2.5
PCR programs	Temperature	Time
	1. 50°C	2 min
	2. 95°C	10 min
	3. 95°C	15 sec
	4. 60°C	60 sec
	Repeat steps 3-4 for 40 cycles	
	5. 4°C	Indefinitely

2.3.3 Isolation of Protein from Cells

Proteins were extracted from suspension or adherent cells. Cells were counted and between 1×10^6 - 1×10^8 cells were used to obtain lysates. Suspended cells were washed twice in 1X PBS and pelleted down by centrifugation. Adherent cell cultures were washed in the flask twice then trypsinised, and washed again. Subsequently, cells were pelleted and lysed in 100-200 µl of lysis buffer (CHAPS buffer with Protease and Phosphatase inhibitor cocktail, (Table 2.1) through vortexing. The lysate was incubated on ice for 20 minutes to complete lysis and vortexed again. Lysates were cleared of debris by centrifugation at 15,000g for 15minutes at 4°C. The supernatant containing total cell lysates were carefully transferred to a new 1.5 mL conical tube, and concentration was determined using the Bradford assay, to ensure the use of equal amounts of proteins among samples.

2.3.4 Bradford Assay

Use of even amounts of protein is necessary for experiments such as Western Blotting. Therefore, calculations based on the results obtained from the Bradford assay quantification were performed. The working solution was prepared by diluting 200µl of protein assay dye reagent (Cat #5000006 Bio-Rad) into 800µl of distilled water. 2µl of protein sample was added into 1ml of working solution in semi-micro cuvettes. As a control, 2µl of CHAPS buffer was used for spectrophotometer calibration. Duplicates of each protein sample were prepared in order to obtain a final average of protein concentration. Using the spectrophotometer cuvettes were read at 595 nm. BSA assay standards were created by preparing a bovine serum albumin (BSA) solution at 2 mg/ml in PBS. Serial dilutions were made from 2mg/ml down to 25µg/ml to generate 8 standard dilution points. A standard curve was plotted using standard measurements and sample values were generated extrapolating values from standard curves.

2.3.5 SDS Gel Electrophoresis

Proteins were separated using SDS gel electrophoresis. One hundred micrograms (100µg) of protein samples were thawed and incubated with 4X LDS sample loading buffer (Table 2.1) at 75°C for 10minutes. Commercially available 4-14% Criterion™ TGX Stain-Free™ Precast gel was loaded in an electrophoresis tank. The tank was filled with running buffer (Table 2.1) as per manufacturer's instructions. 100µg of sample was carefully loaded into each well using extra-long gel loading tips. Protein size marker (Dual Color Standards #161-0374, Bio-Rad, Hercules, CA, USA) was loaded in one well. Electrophoresis was run at 150 volts for 70 minutes until the protein bands had run approximately 95% length of the gel. After the electrophoresis, the gel was removed from its cassette and transferred to the Polyvinylidene fluoride (PVDF) membrane (Immun-Blot #162017, Bio-Rad Hercules, CA, USA) using specific transfer buffer (Table 2.1) in Trans-Blot™ (BioRad, #5678083) wet protein transfer system according to manufacturer's instructions.

2.3.6 Antibody Binding and Visualization

The blots were blocked in block buffer (TBS plus 5% non-fatty acid milk) for 1 hour at room temperature on a plate shaker. Primary antibodies (Table 2.4) of interest (diluted 1:1000 in TBS-0.3% tween buffer) was added and the blot was incubated at 4°C overnight, with gentle continuous agitation, in a sealed container to prevent evaporation. The blot was then washed in TBS-Tween

for 10 minutes for 3 times and HRP linked secondary antibody (Santa Cruz Biotechnology, Dallas, Texas, USA) was added (diluted 1:4000-10,000 in TBS-0.3% tween buffer) for two hours at room temperature on a plate shaker. The blot was then washed in TBST (10 minutes for 3 times). The binding of the antibody was detected by an HRP mediated chemiluminescent reaction using an enhanced chemiluminescence (ECL) system (Clarity Western ECL Substrate #170-5061, Bio-Rad, Hercules, California, CA, USA). Blots were optimized to an exposure time of 1minute where possible, however, in some cases, different exposure times between 10seconds to 10minutes were used.

2.3.7 Estimation of Protein Loading

The blot was stripped off from primary antibody used by using Western Blot stripping buffer (0.4N NaOH/KOH). Blot was washed in TBS-Tween buffer (5minutes) and then incubated with Western stripping buffer (7-15minutes), washed again in TBS-Tween for 5 minutes and blocked in block buffer (TBS plus 5% non-fatty acid milk) for one hour at room temperature on a plate shaker. After blocking, the blot was stained with loading control antibody (Vinculin, Actin or Tubulin) as mention in previous section, in order to ensure an equal amount of protein in each sample.

2.3.8 Antibodies for Western Blotting and Flow Cytometry

In this study, commercially available antibodies were used for Western Blot assays and FACS analysis of different protein of interest.

Table 2.4: Antibodies

Antibody	Cat.no	Manufacturer	Dilution
HERC1	A301-904A	Bethyl Laboratories Inc.	1:1000
phospho-Tyr	sc-7020	Santa Cruz Biotechnology.	1:1000
α -Tubulin	sc-23948	Santa Cruz Biotechnology.	1:1000
β -Actin	sc-47778	Santa Cruz Biotechnology.	1:1000
c-Abl	sc-23	Santa Cruz Biotechnology.	1:1000
BCR	sc-20707	Santa Cruz Biotechnology.	1:1000
Vinculin	MA5-11690	Invitrogen.	1:1000

p44/42 MAPK (Erk1/2)	4695 (137F5)	Cell signaling.	1:1000
P-p44/42 MAPK (pErk1/2)	4377 (197G2)	Cell signaling.	1:500
Akt (pan)	4685 (11E7)	Cell signaling.	1:1000
Phospho-Akt (Ser473)	9271	Cell signaling.	1:500
Anti-Human CD11b-APC	D12	Miltenyi Biotec	1:50
Anti-Human CD61b-PE	VI-PL2	BioLegend	1:50
Anti-Human Annexin V-FITC	ANXVFK-500T	BioLegend	1:50

2.3.9 Immunoprecipitation

For immunoprecipitation (IP) clarified total cell lysate (1500µg of total protein) was used. Briefly, the lysates were incubated with Protein A/G Sepharose for 45 minutes at 4°C on a rotator to remove unspecific bound protein from total cell lysate. After incubation, the cell lysates were centrifuged at 2500rpm for 3 minutes and the cleared cell lysates were transferred to a new conical tube and beads were discarded. The cleared lysates were incubated overnight at 4°C with 5µg of appropriate antibody and subsequently Protein A/G-PLUS-Agarose beads (SC-2001#J1510, Sanza Cruz Biotechnology) were added and incubated for 2 hours at 4°C with rotation. The immune-complex Sepharose beads were then washed three times with cold CHAPS buffer and absorbed proteins were eluted by boiling for 10 minutes in LDS-sample buffer followed by SDS-PAGE and analysis of the results using Western blotting.

2.3.10 *In-vitro* Kinase Assay

The kinase assay was performed with immune-precipitated HERC1 substrate and purified full-length c-ABL1 (Invitrogen, #P3049). Briefly, the HEK-293T cells were lysed in CHAPS buffer and endogenous HERC1 was immune-precipitated as mentioned above in section 2.3.6. Purified full-length ABL (Invitrogen, #P3049) and immune-precipitated endogenous HERC1 were re-suspended in kinase buffer (Table 2.1) and 10 mM ATP (Sigma). After 30 minutes at 37°C, reaction was stopped by the addition of SDS-PAGE buffer, followed by boiling for 10 minutes at 100 °C. Proteins samples were separated on 4-14% gradient precast gel (Criterion™ TGX Stain-Free™, #5678083, Bio-Rad, Hercules, California, CA, USA) electrophoresis and transferred to PVDF (Bio-Rad, Hercules, California, CA, USA) membranes, and incubated with anti-HERC1

and anti-phosphotyrosine antibodies. Membranes were then washed and incubated with appropriate peroxidase-linked secondary antibody (Santa Cruz Biotechnology, Dallas, Texas, USA). Specific binding was detected using an enhanced chemiluminescence system (Clarity Western ECL Substrate #170-5061, Bio-Rad, Hercules, California, CA, USA).

2.3.11 Plasmids Amplification

For DNA plasmid amplification 1µg of recombinant ampicillin resistant vector of interest was added to 25µl of competent *E.coli* (DH5a) cells (Thermo Fisher Scientific). Cells were gently mixed and kept on ice for 30 minutes. After incubation, samples were heat-pulsed in a 42°C water bath for 45 seconds and immediately transferred to ice for 2 minutes. 250-500µl of sterile Luria-Bertani (LB) broth was added to the bacteria and samples were incubated for 1 hour at 37°C under shaking conditions. Cells were plated on LB agar plates (100µg/ml ampicillin) and were incubated aerobically overnight at 37°C for selection of the transformed cells. The following day, colonies were picked and inoculated into 3ml of LB (100µg/ml ampicillin) for selection. Then, samples were incubated overnight at 37°C whilst shaking for further bacterial growth. Following the growth of selective bacteria containing the plasmid of interest, QIApre Spin Midiprep kit (Qiagen) was used as per manufacturer's guidelines to isolate the DNA plasmid. Finally, DNA concentration was measured using NanoDrop spectrophotometer and samples were kept at -20°C until use in further experiments.

2.3.12 Plasmid Construction and Transfection

The p210 *BCR-ABL* was cloned into pcDNA-3.1 (Invitrogen, Grand Island, NY, USA, #V790-20, a gift from Prof. A. Morotti); and this vector was used for transient transfection of HEK-293T cell line by X-treme GeneHP (Roche, Basel, Switzerland) according to the manufacturer's instructions. Briefly, before transfection HEK-293T cells were grown up to 70-80% confluency in 10cm petri plate (Sigma). 2µg of the DNA plasmids of interest were diluted in cell growth medium without serum proteins or antibiotics in a total volume of 100µl in a sterile 1.5ml conical tube. Afterwards 15µl of X-treme Transfection Reagent (Roche, Basel, Switzerland) was added dropwise to the DNA solution. Samples were incubated at room temperature for 20-30 minutes. Meanwhile, the cell growth medium was replaced with pre-warmed complete medium

without antibiotics after two time washing of adherent cells with PBS. After incubation, the solution was added drop by drop over the cell plate. The following day medium was removed, and cells were washed twice with PBS in order to avoid cytotoxicity from the reagent. Finally, fresh complete medium was added to the cells and incubated for another 24hours for further experiments. Additionally, the simultaneous transfection with pEGFP-C2 vector alone was used as a control to check the transfection efficiency by using fluorescent microscope (Leica DM2000 LED, Leica microsystem) after 48 hours.

2.4. Differentiation Analysis

2.4.1 Induction of Differentiation and Flow Cytometry

To induce differentiation, exponentially grown HL60, K-562 and HEL cells (5×10^5 cells/ml) were seeded in 10ml of respective growth medium and exposed to: HL60 with 250 nM phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich # P8139) or 10uM All Trans Retinoic Acid (ATRA, Sigma-Aldrich #302-79-4) for 48h and with 1.5% dimethyl sulfoxide (DMSO, Sigma-Aldrich #D2650) for 7 days as described elsewhere [209,210], HEL with PMA and K-562 with 20U of erythropoietin (EPO; Pfizer, Europe MA EEIG 1050 Bruxelles.) and PMA for 48h [211,212]. Morphological changes of cells were observed using a phase-contrast microscope. Following treatment cells were collected by centrifugation (500g for 3minutes) and each group was then washed in FACS buffer (PBS, 1 mM EDTA, 2% FBS, 0.1% sodium azide) and then re-suspended in 500µl blocking buffer (equal volumes of FACS buffer and FBS) for 10 minutes at room temperature. The cells were again washed with FACS buffer for two time and incubated for 20-30 minutes in the dark with fluorescently conjugated antibodies. Antibodies used were APC Mouse Anti-Human CD11b (D12, BioLegend) and PE Mouse Anti-Human CD61 (VI-PL2, BioLegend). Subsequently, cells were washed twice with 1X PBS, resuspended in FACS buffer and analyzed by using a fluorescence-activated cell sorter (FACS) BD flow cytometer to detect the percentages of differentiated cells. Results were analyzed on FlowJo[®] software version 10.5.3.

2.4.2 Benzidine Staining

Erythroid differentiation was evaluated by counting blue benzidine-positive cells (hemoglobin expressing cells) by benzidine staining described elsewhere [212]. Briefly, the EPO treated K-562 cells were suspended in a benzidine staining solution containing 0.2% benzidine

(Sigma-Aldrich #B3503-5G) in 10% H₂O₂ (Sigma-Aldrich) and 0.5 M glacial acetic acid (Sigma-Aldrich). Following 2 minutes of staining, the differentiated cells were stained blue and images were captured by phase contrast microscopy. The percentage of benzidine-positive cells was the number of blue cells divided by the total number of cells.

2.4.3 Apoptosis Assay

Apoptosis was assessed by flow cytometry of all drug-treated cells by measuring annexin staining. Briefly, 1×10^6 cells were washed once with PBS(1X) and then incubated in 1X annexin binding buffer (0.1 M HEPES, pH 7.4; 1.4 M NaCl; 25 mM CaCl₂) for 5 minutes. After centrifugation cells were resuspended with fluorescein isothiocyanate (FITC)-conjugated annexin V and propidium iodide and incubated at room temperature for 15 minutes (Annexin V-FITC Apoptosis Detection Kit ANXVFK-500T, Immunostep). After incubation, cells were analyzed by flow cytometry. Results were analysed on FlowJo[®] software version 10.5.3.

2.5 Statistical Analysis

Graphs are presented with error bars representing standard error calculation. Paired t-test or one-way ANOVA were used for non-parametric statistical analysis using GraphPad Prism v5.0 (GraphPad Software, Inc., La Jolla, CA, USA). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

3. RESULTS

3.1. *HERC1* Gene Expression is Aberrantly Regulated in Myeloid Related Disorders and Leukemias

Despite the mutational status of the genes encoding for the large HERCs has been scrutinized in different myeloid and lymphoid blood tumors [195,213,214], their expression levels in normal Bone Marrow (BM) and Peripheral Blood (PB) from healthy subjects has never been assessed. Hence, initially, we determined the *HERC1* gene expression in a cohort of healthy subjects by comparing its mRNA abundance in PB and BM specimens. Interestingly, PB and BM displayed different levels of *HERC1* mRNA being the amount in PB significantly higher than in BM. Indeed, PB showed values that were approximately twice when compared to those found in BM (PB median=4.01 vs BM median=2.20) (**Figure 3.1A**). Since our interest was related to myeloid related disorders we then assessed the *HERC1* gene expression in a panel of myeloid malignancies including AML (n=97), MPNs (n=78.) and CML (n=72).

When compared to the pool of PB and BM healthy specimens, the *HERC1* gene expression was sharply down-regulated at diagnosis both in acute (median value approximately of 1.0) and in chronic myeloid (median value approximately 0.93) leukemia. Differently, in MPNs specimens, we observed that the *HERC1* gene expression level did not significantly differ from that of the healthy subjects, albeit the values appeared rather disperse within a window ranging from 0.40 to 12.65 with a median value of 3.33 (**Figure 3.1B**). On the whole, *HERC1* gene expression is differentially regulated in healthy PB and in BM, markedly downregulated both in AML and CML and expressed with a rather scattered pattern in the MPNs group.

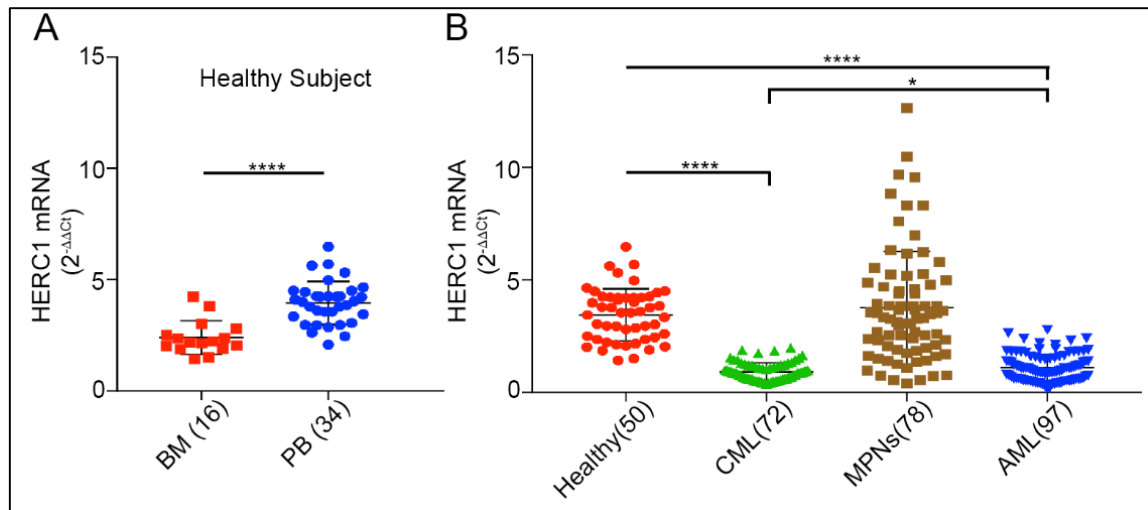


Figure 3.1. The *HERC1* gene expression differently regulated in BM and PB and it is dysregulated in haematological malignancies. A) The expression of the *HERC1* gene is differently regulated in healthy blood specimens. PB samples exhibited higher *HERC1* amount (median=4.0) when compared to the BM (median=2.2). **B)** *HERC1* transcript was assayed in healthy donors (PB=34, BM=16) and in a panel of myeloid related disorders at diagnosis, namely Chronic Myeloid Leukemia (CML), (PB=37, BM=35), Acute Myeloid Leukemia (AML), (BM=80, PB=17), MyeloProliferative Neoplasms (MPNs) (PB=78), by RT-qPCR and compared with normal healthy specimens. The *HERC1* mRNA quantity was expressed as $2^{-\Delta\Delta C_t}$ after normalization against *GUSB*. *HERC1* gene expression was significantly down-regulated in newly diagnosed CML and AML, with median values of 0.9 and 1 respectively, when compared to healthy specimens (median=4.01). In MPNs the *HERC1* expression fluctuated from 0.4 to 12.65 with a median value of 3.3 and thus not significantly differing from healthy subjects. *P*-values were calculated on the differences in *HERC1* gene expression observed between patients and the healthy control specimens (* $P < 0.05$, **** $P < 0.0001$).

3.2. Newly Diagnosed AML Display *HERC1* Downregulation Independently from the Genetic Alterations

The AML is the most common type of leukemia in adults and it is characterized by a high degree of genetic heterogeneity, including inactivation of oncosuppressors [121]. Hence, it is among the most challenging myeloid neoplasm to be treated [215]. We examined the gene expression of *HERC1* in this type of leukemia accordingly to the most popular genetic alterations. The *HERC1* expression level was assessed in newly diagnosed AML (80 were BM and 17 PB specimens). Interestingly, both the BM and the PB specimens collected from newly diagnosed AML patients exhibited comparable *HERC1* transcript amount (BM median=0.97 vs PB

median=1.12), revealing that the expression levels were distinctly decreased in both BM and PB patient's samples when compared to healthy subjects ($p<0.0001$) (**Figure 3.2A**).

Since AML displays a quite broad genetic diversity we stratified the BM specimens collected at diagnosis according to the most common cytogenetic and genetic variants. By comparing the *HERC1* transcript among the different categories we did not observe any major difference (**Figure 3.2B**). However, the only significant difference detected regarded the *IDH2* mutated specimens, in which the *HERC1* gene expression was significantly higher when compared to all the other AML subgroups (ANOVA, $p=0.03$) displaying a median level (1.9) similar to that of the healthy donors ($p=0.27$).

These results indicated that in AML patients the *HERC1* gene expression level was significantly lower at diagnosis and not associated to any of the different cytogenetic/genetic group with the interesting exception of the 5 AML patients harbouring the *IDH2* R140Q mutation, which displayed *HERC1* levels indistinguishable from the control group.

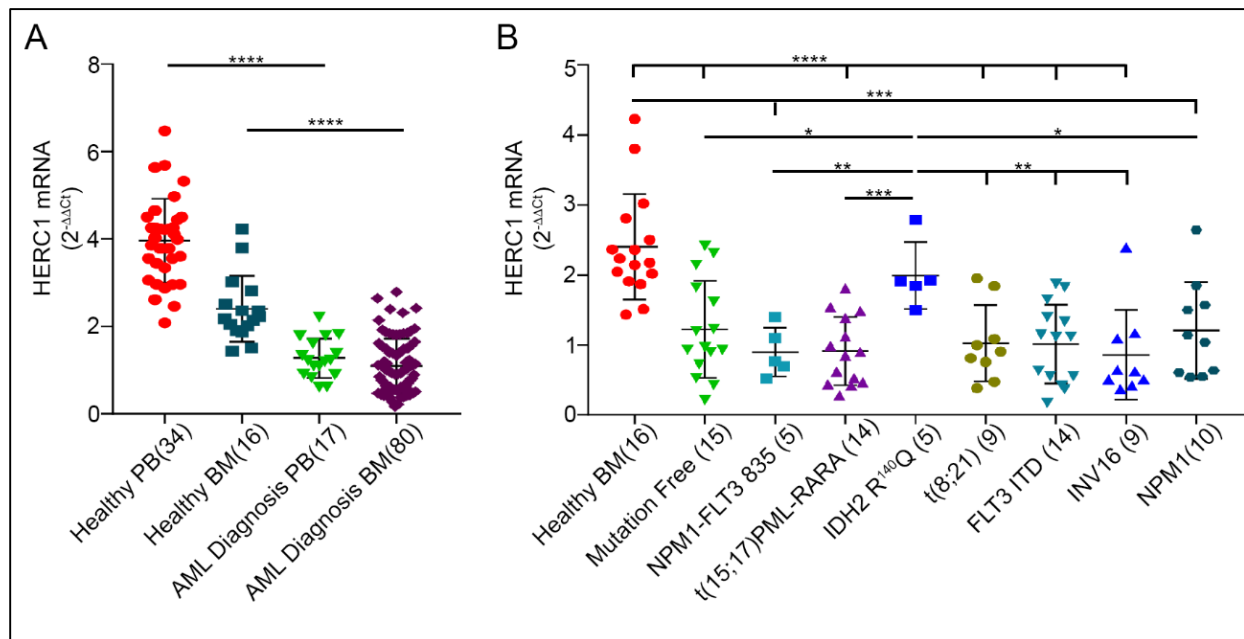


Figure 3.2. The *HERC1* transcript is severely downmodulated in acute myeloid leukemia independently from genetic alteration. A low level of *HERC1* expression was observed in newly diagnosed AML patients, when compared to healthy control. **A)** *HERC1* level is significantly downregulated in both PB (n=17) and BM (n=80) specimens of AML at diagnosis. **B)** The stratification of the AML samples according to the different cytogenetic categories and to the most

common genetic variants displayed that the *HERC1* transcript downregulation is a common feature shared by almost all the different groups with the exception of the *IDH2* mutated cohort, in which its gene expression is comparable to that of the controls ($P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$).

3.3. MPNs Display a Peculiar Pattern of *HERC1* mRNA Abundance

Philadelphia chromosome-negative myeloproliferative neoplasms (MPNs) are hematopoietic stem-cell disorders characterized by an abnormal proliferation of one or more terminal myeloid cells. They include Essential Thrombocythemia (ET), Polycythemia Vera (PV) and Primary Myelofibrosis (PMF). The PV and ET are chronic diseases associated with a long life expectancy, while PMF is a more aggressive disease characterized by harsher symptoms. As opposed to the closest related myeloid neoplasm, namely CML, the MPNs are characterized by different somatic genetic mutations involving *JAK2*, Calreticulin (*CALR*) and the thrombopoietin receptor (*MPL*) encoding genes [216].

The expression pattern of *HERC1* in MPNs appeared rather scattered and different in each category. Among the three different MPNs, the PB samples from ET patients (n=39) were those displaying the most variable *HERC1* gene expression levels (ranging from 1.4 to 12.6). Interestingly, while either the ET specimens harboring the *JAK2V617F* mutation or those non-mutated displayed *HERC1* levels similar to those of the controls, those mutated for *CALR* exhibited significantly higher *HERC1* mRNA abundance (median 7.3) (**Figure 3.3A**). Conversely, the non-mutated PMF patients (n=11) displayed very low amount of *HERC1* transcript with values very similar to those observed in AML and CML specimens (median=0.8) (**Figure 3.3B**). Worthy of note, the PMF *JAK2V617F* mutated patients showed a slightly, but significantly ($p=0.0001$) higher level of *HERC1* mRNA (median=2.3) when compared to the non-mutated group. Eventually, specimens from PV patients showed a moderate, though significant, lowering in *HERC1* gene expression level (**Figure 3.3C**).

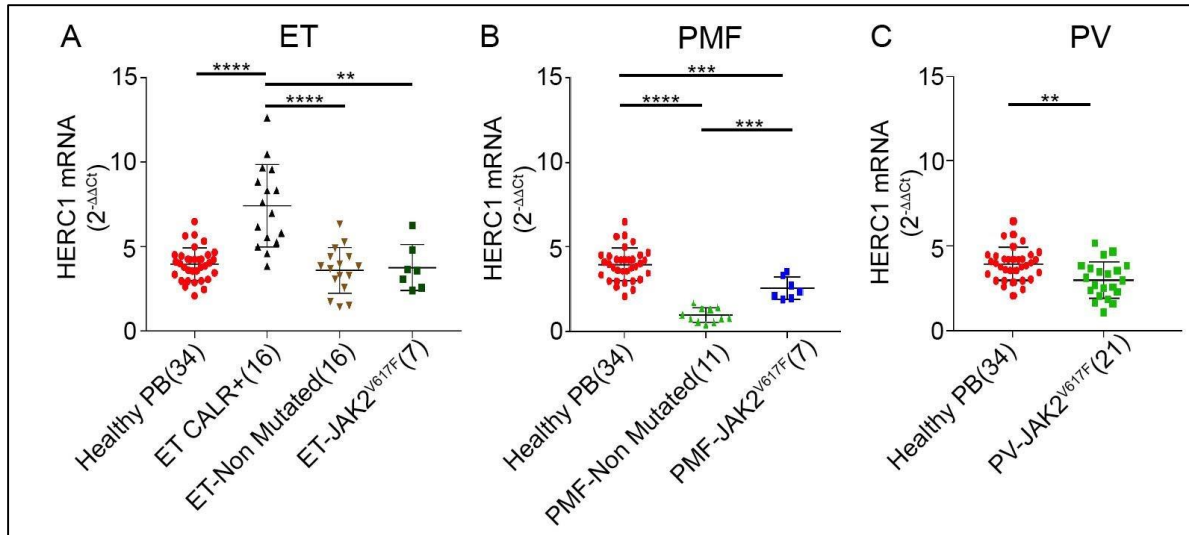


Figure 3.3. *HERC1* mRNA is differentially and peculiarly expressed in MPNs patients. On the whole, the expression pattern of *HERC1* in MPNs appeared to be rather scattered and variable. Among MPNs, when compared to the healthy subjects the *CALR* mutated Essential Thrombocythemia (ET) specimens (**A**) displayed a significantly higher amount of *HERC1* transcript, ($P \leq 0.0001$). On the contrary, the *JAK2* mutated, and non-mutated, specimens showed levels comparable to the control counterpart. **B**) Differently, the Primary Myelofibrosis (PMF) patients displayed a rather steep downregulation of *HERC1* gene expression. However, the *JAK2*^{V617F} mutated samples showed higher *HERC1* levels in comparison to the mutation-free cohort. **C**) In Polycythemia Vera (PV) patients the *HERC1* mRNA level was slightly, but significantly, lower than that of the healthy subjects (** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

3.4. The *HERC1* Expression is Sensitive to the BCR-ABL1 Tyrosine Kinase Activity

Among the myeloid related disorders, the CML is the only one in which there is an unambiguous correlation between the disease and the genotype. Indeed, the reciprocal chromosomal translocation, occurring between the chromosomes 9 and 22 and giving rise to the fusion oncogenic tyrosine kinase BCR-ABL1, is the CML primary molecular-genetic defect [217].

By measuring the transcript level of *HERC1* in CML patients at diagnosis we noticed that it was decreased in both PB (median=1.03) and BM (median=0.66) specimens. We then also asked whether *HERC1* expression might be affected by the different CML clinical stages, including diagnosis, remission and relapse. Our analysis unveiled that after the molecular remission ($\leq 0.1\%$ BCR-ABL1^{IS}) the level of the *HERC1* transcript sharply increased, reaching values (median=3.5) comparable to those observed in the control subjects (**Figure 3.4A**). Indeed, an inverse correlation

between the *HERC1* and the *BCR-ABL1* gene expression was observed at the different time points of therapy (3, 6, 12 and 24 months) with tyrosine kinase inhibitor (TKI) Imatinib®, the gold standard for treatment of chronic myeloid leukemia (**Figure 3.4B**), indicating that might be with the eradication of sick cells/clone and re-appearance of normal cells/polyclonal hematopoiesis the expression of *HERC1* upregulate gradually. Ultimately, when patients relapsed again a reduction in *HERC1* gene expression occurred. Due to the very large size of the HERC1 protein, in human primary cells, we encountered technical difficulties in blotting it. Hence, we decided to determine the HERC1 protein levels in CML specimens collected at different clinical stages (diagnosis, remission and relapse) by immunofluorescence. Consistently with gene expression results, the HERC1 protein amount was low at diagnosis but increased during remission and decline again when patients relapsed (**Figure 3.4C**). Surprisingly similar expression-pattern was also observed in a very close HERC family member, the *HERC2* gene (**Figure A1**), but there was a significant difference in the mRNA levels of *HERC1* and *HERC2* through all the disease phases. For instance, the overall median value at diagnosis of *HERC2* was lower (BM median=0.30 compared to the median value of *HERC1* at diagnosis (BM median=0.66).

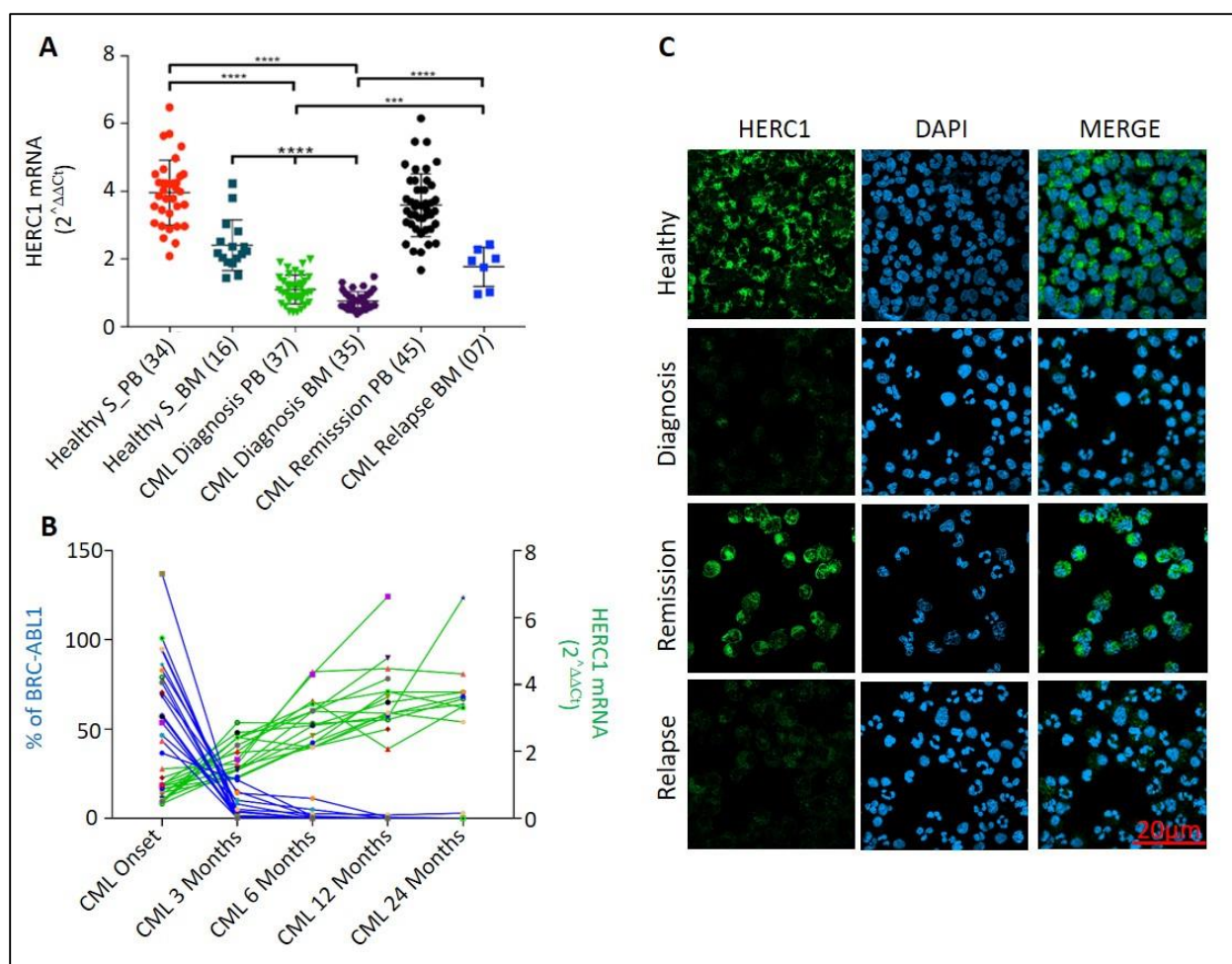


Figure 3.4. *HERC1* gene expression is differently regulated in CML patients during the evolution of the disease. **A)** *HERC1* gene was differentially expressed during the different CML phases being severely down-regulated at diagnosis, both in BM and PB CML specimens, while its transcript level arose at remission and declined again at the onset of relapse. **B)** CML patients showed an inverse correlation between *HERC1* and *BCR-ABL* gene expression at the different time points of disease. **C)** Similarly, to the mRNA, in primary CML cells the *HERC1* protein amount was barely detectable at diagnosis and at the relapse onset. On the contrary, at remission, the protein level was comparable to that of healthy control. Immunofluorescence was performed on cytopun cells by using the specific rabbit polyclonal anti *HERC1* antibody. DAPI staining (blue) indicated the cell's nuclei. The green signal, corresponding to *HERC1* protein.

3.5. *HERC1* is Severely Downmodulated in Myeloid Cell Population of CML Patients

In the light of the above results, in order to uncover which cell population is mostly affected by *HERC1* downregulation in CML, we decided to measure *HERC1* gene expression on different cell types. Consequently, we first separated the mononuclear cells through gradient centrifugation

from both healthy and CML bone marrow blood specimens. The various cell populations were then enriched through an affinity separation with magnetic conjugated beads. We proceeded with the separation of T and B lymphocytes by using CD3 and CD19 antibodies, respectively. The remaining cell population was considered as myeloid lineage (monocytes), from which we further isolate CD34 cells, a most immature component. The analysis was carried out at the protein level but, given the small cell numbers, it was not possible to perform an analysis by Western Blot. We, therefore, evaluated and quantified the expression by immunofluorescence by measuring the integrated intensities of HERC1 signal of each sample (**Figure A2**). Our results indicate that, in the CD34+ progenitor cells from CML patient samples, the levels of expression of the HERC1 protein is relatively low compared to healthy CD34+ cells. While the expression of HERC1 in CD3+ and CD19+ cells (B and T lymphocytes respectively) is relatively similar in both healthy and CML cell populations. Surprisingly, a severe downregulation of HERC1 protein level was observed in myeloid cells (monocytes) population compared to the corresponding healthy counterpart (**Figure 3.5A & B**). Remarkably, these results are in line with the *HERC1* mRNA expression levels identified in different types of normal blood population (biogps.org, and BloodSpot database) (**Figure A3**). This further confirmed that the down-modulation of HERC1 is severely affected in the myeloid compartment of CML, which represent the almost totality of the pathological population.

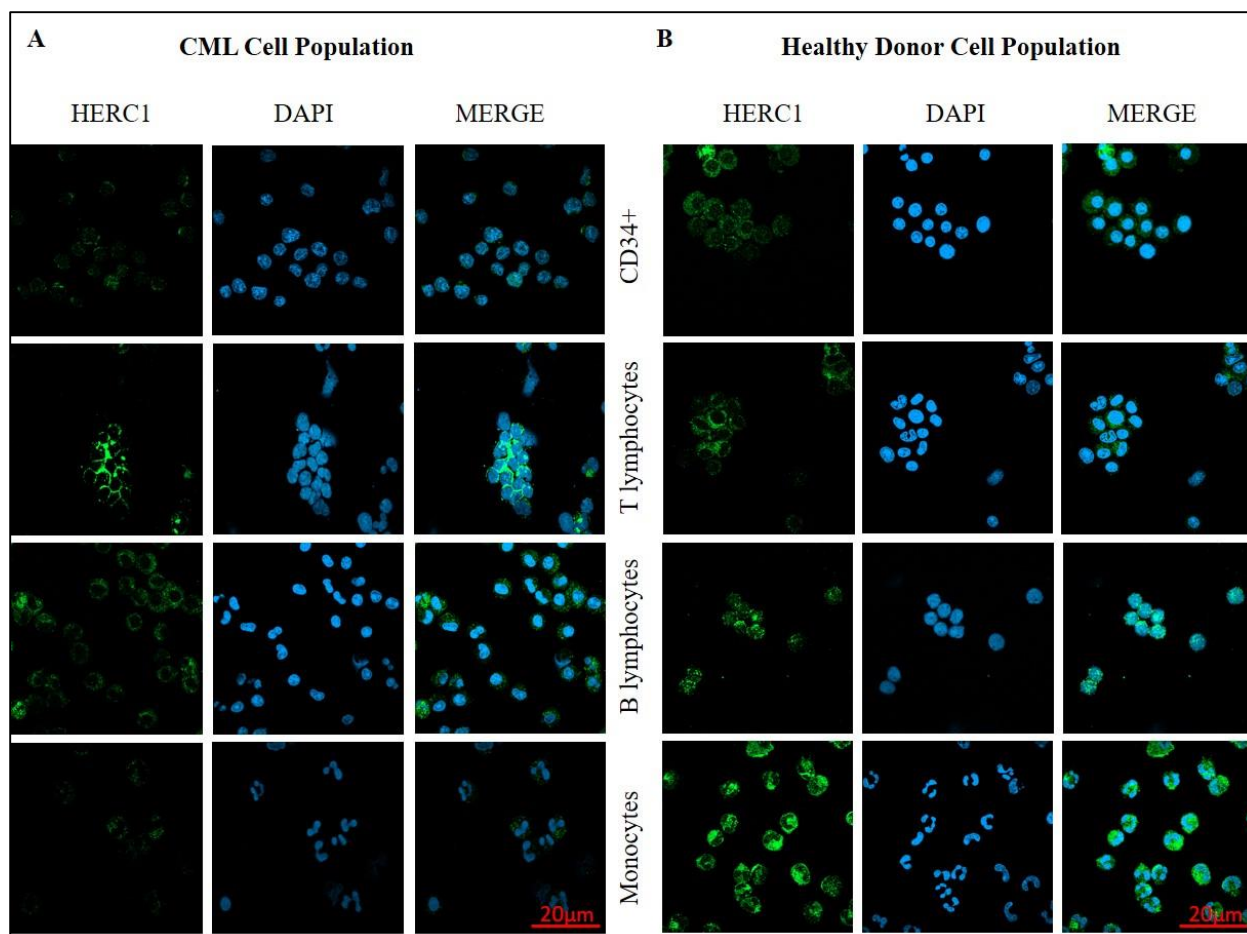


Figure 3.5. HERC1 is mainly downregulated in the myeloid lineage. Immunofluorescence staining assay was performed on different cell population after magnetic isolation from a healthy donor and of CML bone marrow (BM) samples. The green signal corresponds to HERC1 while blue DAPI is used to stain nuclei. **A)** HERC1 protein expression in different cells separated from CML BM specimen. **B)** HERC1 protein expression in healthy donor cell population. HERC1 protein expression is relatively low in CD34+ and mononuclear cells (mainly Myeloid cells population) derived from CML BM specimen compared with corresponding cell population of the healthy donor control. While the HERC1 protein level in T and B-lymphocytes is comparable in sick and healthy cell population and it displays an intense and cytosolic localization.

3.6. *HERC1* Gene Expression is Linked to Imatinib Treatment in CML Leukemia Cells

In CML patients, remission is usually achieved in response to tyrosine kinase inhibitors (*e.g.* Imatinib®) monotherapy. Therefore, we assessed the *HERC1* expression at both mRNA and protein levels in 5 primary BM samples collected at diagnosis, treated *in-vitro* for 48 hours with Imatinib. In parallel, Ph+ K-562, a human CML cell line, was treated with Imatinib. Interesting,

leukemic cells (**Figure 3.6A & C**) as well as Ph⁺ K-562 (**Figure 3.6B & D**), showed a clear increase in *HERC1* levels after TKI treatment, suggesting that inhibiting BCR-ABL1, the oncogenic driver in CML, may restore *HERC1* level, reaching values similar to healthy donors or remission condition.

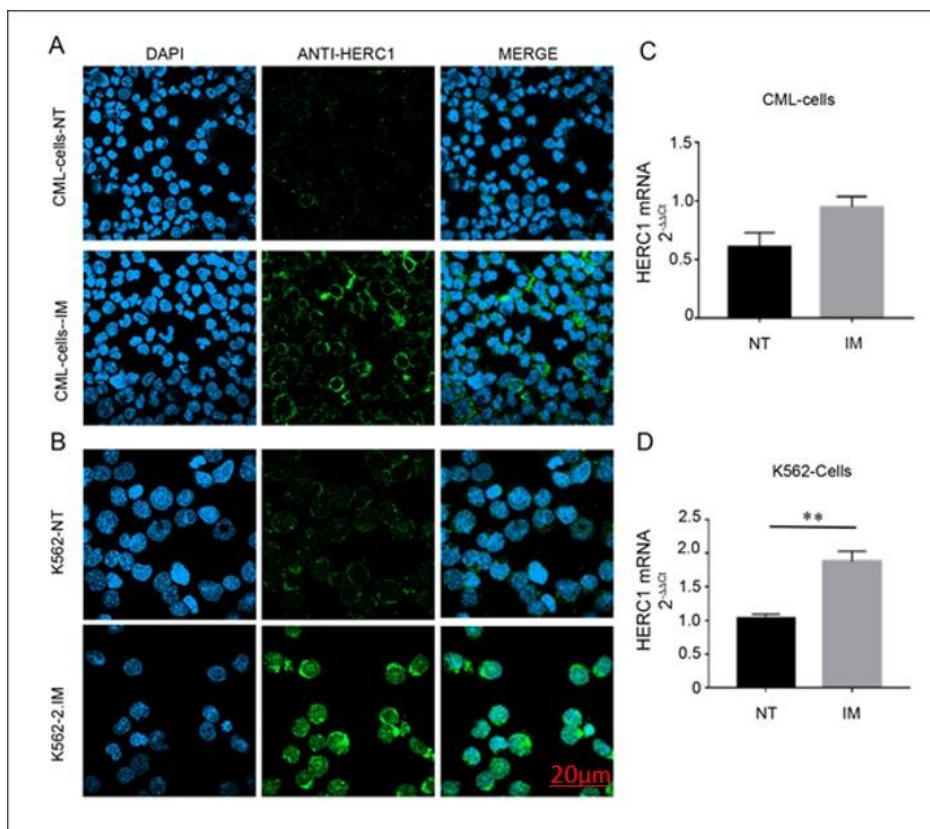


Figure 3.6. Effect of Imatinib treatment on *HERC1* gene expression in CML primary and K562 (Ph⁺) cells. *HERC1* gene expression following Imatinib (2.5 μ M) treatment was determined at mRNA and protein expression by RT-qPCR and immunofluorescence assay. Immunofluorescence was performed by using rabbit polyclonal anti *HERC1* antibody after treating, or not (NT), the primary CML leukemic cells derived at diagnosis from bone marrow and K-562 cell line with Imatinib (2.5 μ M) for 48 hours. **A & C**) Imatinib treated primary CML leukemic cells showed an increase in *HERC1* expression both at protein and mRNA compared to non-treated cells. **B & D**). Similarly, K-562 cells also showed an increase in *HERC1* gene expression after Imatinib treatment both at protein and mRNA levels.

These results prompted us to ascertain whether the BCR-ABL1 tyrosine kinase might affect the *HERC1* expression. Hence, we transfected the HEK-293T cell line with the p210 *BCR-ABL1*

overexpressing plasmid. We observed a significant decrease in the HERC1 protein and mRNA levels in *BCR-ABL1*-transiently transfected HEK-293T cells (**Figure 3.7**), when compared to HEK-293T cells transfected with a control plasmid. These results are in agreement with the findings in CML specimens at diagnosis.

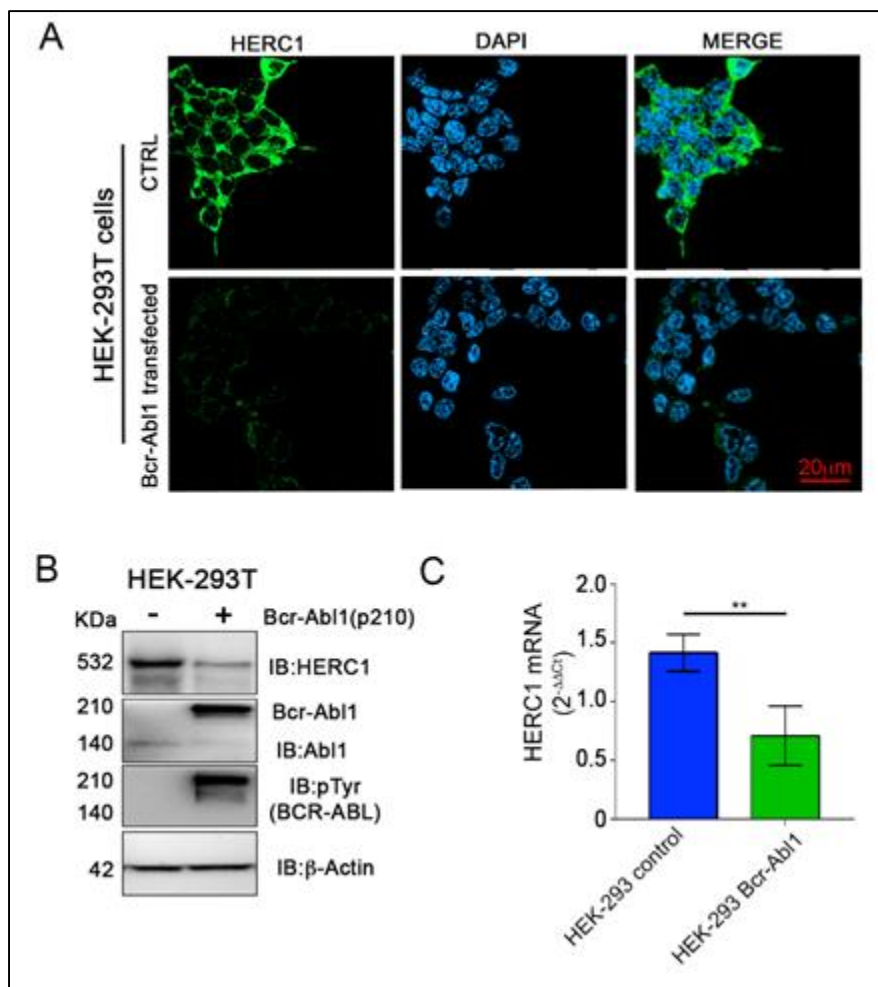


Figure 3.7. HERC1 mRNA and protein amounts are regulated by the BCR-ABL1 tyrosine kinase activity. BCR-ABL1(p210) over-expressing vector was transiently transfected into HEK-293T cells. Immunofluorescence and Western Blot along with qPCR were performed to determine both the HERC1 protein and mRNA level after transfection. HEK-293T cells transiently and exogenously expressing the constitutively active p210 BCR-ABL1 tyrosine kinase exhibited a severe down-modulation of either the HERC1 protein (**A & B**) and, to a significant extent, the *HERC1* transcript **C**). DAPI staining (blue) indicated the nuclei of the cells while the green signal was corresponding to HERC1 protein. β -Actin was used as a loading control in Western blot.

3.7. The HERC1 is a Novel Binding Partner and a Substrate of BCR-ABL1

Besides the HECT and the RLD domains, and despite of their very large size, the HERC E3 Ub-ligases harbor only few recognizable structural regions [174]. Noticeably, many somatic mutations associated to different tumors fall within unknown structural constrained amino acid stretches [172]. In the last years, extensive efforts have been made to discover HERCs interacting partners with the aim to dissect insight in the cellular functions of these proteins [181]. While currently the HERC2 accounts for dozens of binding partners [208] but no more than a bunch have been experimentally ascertained for HERC1.

By analyzing and comparing the HERC1 and BCR-ABL1 subcellular localization in K-562 cells, we observed that both proteins displayed a diffuse pattern and mostly they co-localized (**Figure 3.8A**). Noteworthy, HERC1 showed a more spotted pattern, when compared to BCR-ABL1, with “*speckle-like*” structures mainly dispersed within the cytoplasmic compartment. These observations prompted us to investigate whether the two proteins might also interact. For this purpose, we attempted to pull-down HERC1, in K-562 Ph-positive cells, by co-immunoprecipitation experiment using as a bait BCR-ABL. As revealed by the intense and sharp HERC1 Western Blot band, obtained after BCR-ABL1 immunoprecipitation, the two proteins not only co-localized but they also interacted with each other (**Figure 3.8B**).

Interestingly, the HERC1 turns out to be even tyrosine phosphorylated (**Figure 3.8B**). Since BCR-ABL1 displays constitutive tyrosine kinase activity and phosphorylates a very broad spectrum of different effectors we next asked whether the HERC1 tyrosine phosphorylation status might be affected by BCR-ABL1 itself. Hence, we determined the HERC1 tyrosine phosphorylation status in K-562 cells. Immunoprecipitation of K-562 lysates with an anti-phosphotyrosine specific antibody and the subsequent immunoblotting with anti-HERC1 antibody unveiled that HERC1 is one among many proteins heavily phosphorylated in this CML cell line (**Figure A4**). An *in-silico* search (www.cbs.dtu.dk/services/NetPhos) revealed that HERC1 protein harbors several putative tyrosine phosphorylation sites and at least three of them (Y905, Y3867 and Y4452) appeared to be *bona-fide* BCR-ABL1 phosphorylation consensus motif [218]. Altogether, these bodies of evidence suggested that BCR-ABL1 may directly phosphorylate HERC1.

To validate this surmise, we performed an “*in-vitro*” kinase assay by mixing the purified c-ABL1 kinase with the putative HERC1 substrate. c-ABL1 directly phosphorylated HERC1 on tyrosine residues, and this phosphorylation was significantly inhibited by treatment with Imatinib (**Figure 3.8C**). Furthermore, this phenomenon was noticed in HEK-293T BCR-ABL1 (P210) transfected cells. We observe a clear HERC1 phosphorylation in the presence of BCR-ABL, which disappeared upon Imatinib treatment (**Figure 3.8D**). On the whole, these findings indicate that HERC1 is a novel binding partner and a substrate of BCR-ABL1.

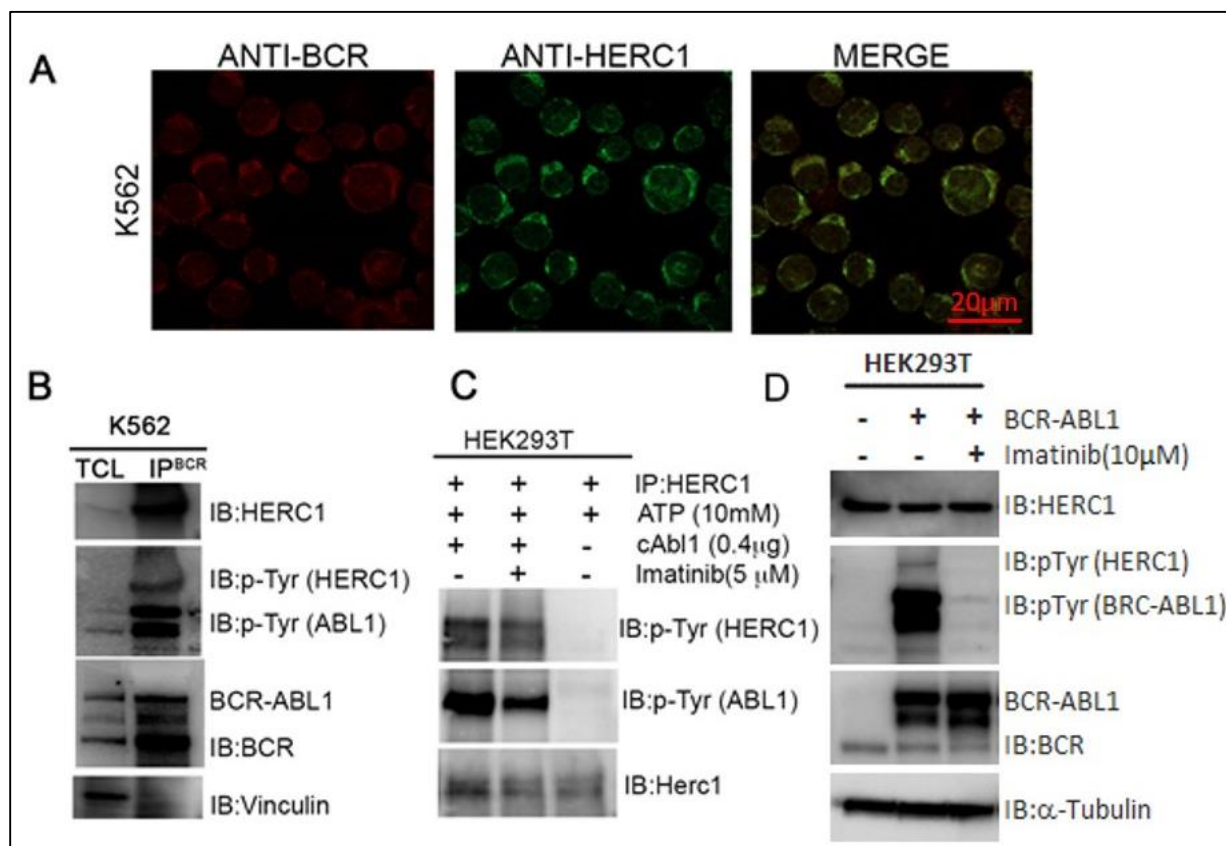


Figure 3.8. HERC1 is a novel interactor and a potential substrate of BCR-ABL1. **A)** The subcellular localization of either the HERC1 or BCR-ABL1 (p210) proteins were assessed by staining cytopun K-562 cells with anti-HERC1 and anti-Bcr specific antibodies. The immunofluorescence assay revealed that the localization of the two proteins was mostly restricted to the cytoplasmic compartment and they co-localized. **B)** In K-562 cells BCR-ABL1 and HERC1 proteins form a complex. The immunoprecipitated BCR-ABL1 was employed to pull-down HERC1 from K-562 total cell lysate. The presence of HERC1 in the immunoprecipitated sample was determined by immunoblotting the membrane with the HERC1 antibody (upper panel). Subsequently probing with a tyrosine phospho-specific antibody proved the phosphorylated HERC1 status. Vinculin and α -tubulin were used as loading control (lower panel). **C)** In vitro kinase assay with purified c-ABL and HERC1 substrate, obtained after HERC1

immunoprecipitation on 293T, performed in the absence or presence of 5 μ M Imatinib treatment for 30 minutes. **D)** HERC1 phosphorylation in transiently transfected HEK293T cells disappeared upon Imatinib treatment. Imatinib impaired the activity of BCR-ABL1 which in turn is not able to phosphorylate its potential substrate, in this case, the HERC1.

3.8. *HERC1* Gene Expression is Associated to Differentiation Arrest in Myeloid Leukemia Cells

Generally, leukemia is a blood or bone marrow disorder, which is characterized by increased numbers of abnormal immature white blood cells. The abnormality of leukemic cells lies in their inhibited differentiation and increased proliferation rate [219]. Our findings indicated a severe downregulation of *HERC1* gene expression in myeloid related leukemia such as AML, where a long-held tenet is that multiple genetic events culminate with the block in the differentiation of myeloid compartment. To validate either the *HERC1* gene expression could have any association with blood cell maturation, we conducted differentiation experiments on the HL60 acute myeloid leukemia cell line model. After treatment with phorbol 12 myristate 13-acetate (PMA) and All Trans-Retinoic Acid (ATRA), the differentiation was confirmed by quantifying CD11b neutrophil cell surface marker with flow cytometry (FACS) analysis. Cell treated with PMA showed an increase by approximately 75% of CD11b, associated with morphological changes in the form of cell aggregation, which tightly attached to the substratum, while with ATRA differentiating marker CD11b raised approximately by 30%, with enlarged/elongated cell morphology and weakly attached to the substratum (**Figure 3.9A & B**). To investigate either the *HERC1* gene expression following PMA and ATRA treatment could play a pivotal role in differentiation, the total RNAs and proteins were extracted and *HERC1* mRNA and protein expression were analyzed by RT-qPCR and Western Blots. Interesting, *HERC1* gene expression both at mRNA and protein level was upregulated after differentiation induction when compared to non-treated cells (**Figure 3.9C & D**). To further confirm these observations we also conducted differentiation experiments by incubating the HL60 cells with dimethyl sulfoxide (DMSO) for 7 days which is well known to induce neutrophilic differentiation in HL60 cells [210]. Interestingly, DMSO induced morphological changes and neutrophils specific cell surface marker CD11b enrichment in HL60 differentiating cells (**Figure 3.9E & F**). Moreover, differentiated cells showed an up-regulation of *HERC1* gene expression both at mRNA and protein levels when compared to non-differentiated cells (**Figure 3.9G & H**), which was in alignment with our results.

Remarkably, the phosphorylation of Erk1/2 was severely impaired in differentiated cells and is tightly linked with the HERC1 protein level when compared to undifferentiated control. These findings revealed that the downregulated *HERC1* gene expression might be linked to differentiation arrest at the onset of myelogenous leukemia. From our preliminary observations, we can hypothesize that HERC1 is potentially involved in blood cell differentiation process.

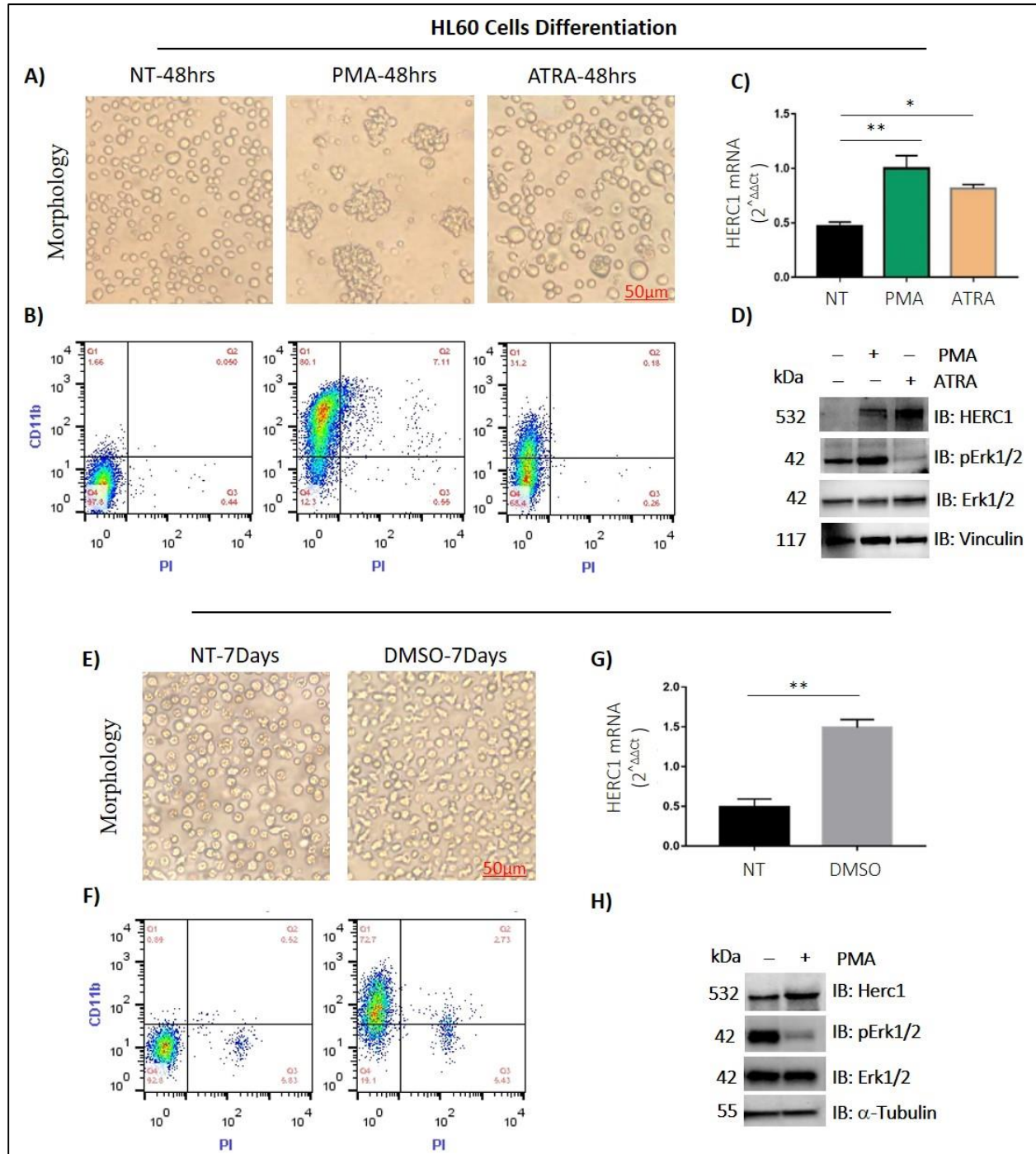


Figure 3.9. *HERC1* gene expression modulated upon HL60 cell differentiation, a tight association between the HL-60 leukemic cell differentiation and *HERC1* gene expression. Upon incubation with differentiation agents the HL-60 cells change the morphology and adhesion properties, being capable to adhere to a substratum. **A & B)** Morphological changes and CD11b differentiation-specific cell surface marker expression was observed after incubation of HL60 cells with phorbol 12 myristate 13-acetate (PMA) and All Trans-Retinoic Acid (ATRA). **C & D)** *HERC1* gene expression both at mRNA and protein level upregulated in differentiated cells.

HERC1 mRNA and protein amount were up-modulated upon DMSO differentiation in HL-60. **E & F)** The morphological changes in HL60 cells upon DMSO differentiation were coupled with an enrichment of the cell surface marker CD11b indicating that cells underwent a differentiation process towards a neutrophil-like lineage. Eventually, on the enriched population both the HERC1 mRNA and protein expression were analyzed by RT-qPCR and Western Blots. **G & H)** Interestingly, in either case, the levels were up-regulated when compared to non-differentiated cells. The pErk1/2 downregulation indicates the differentiating cells stopped to proliferate. The vinculin and α -tubulin were used as loading control in Western Blot.

3.9. *HERC1* Gene Expression Linked to the Differentiation of Ph⁺(K562) Myeloid Leukemia Cells with Erythropoietin (EPO) and PMA

The differential *HERC1* mRNA expression at different phases of CML patients prompted us to investigate either *HERC1* gene expression is linked to blood cells differentiation. Previously reported data revealed that CML cells underwent erythroid differentiation upon Imatinib treatment prior to undergoing apoptosis [220]. We attempted to differentiate Philadelphia positive K562 myeloid leukemia cell line with phorbol 12 myristate 13-acetate (PMA) and Erythropoietin (EPO). It is known that erythropoietin induces K562 cell line toward erythroid cells [211], while PMA toward megakaryocytic-like cells in a dose-dependent manner [212]. In our experiments after EPO treatment K562 cell underwent enlarged erythroid-like cells, compared to untreated cells, confirmed by benzidine test (**Figure 3.10A & B**). More in detail, this test is specific for the determination of blood in a sample, by exploiting the ability of benzidine to link directly the hemoglobin, thereby producing a blue reaction. EPO induces in K562 an increase of erythroid differentiation by approximately 35% (benzidine-positive cells) with respect to control (**Figure 3.10B**). While PMA treated cells underwent phenotypic and morphologic changes that are associated with megakaryocytic-like elongated cells, which strongly attached to the substratum. PMA-induced megakaryocytic differentiation was confirmed by a significant increase of megakaryocyte specific cell surface marker CD61 (**Figure 3.10C & D**). Subsequently, total RNAs and proteins were extracted and *HERC1* mRNA and protein expression was analyzed by RT-qPCR and Western Blots. Interestingly, *HERC1* gene expression both at mRNA and protein was upregulated in EPO induced cells (**Figure 3.10E & F**). The increase in protein expression during EPO-induced differentiation was even confirmed by immunofluorescence microscopy. After 48h of differentiation, HERC1 protein levels were increased and HERC1 protein was found to localize predominantly in the cytoplasm (**Figure 3.10G**). In contrast, HERC1 mRNA and protein levels

were downregulated in PMA treatment compared to non-treated cells, suggesting that *HERC1* expression and modulation may be cell specific (**Figure 3.10E & F**)

To validate the cell-specific *HERC1* gene expression upon differentiation, we attempted to differentiate Human Erythroleukemia (HEL) by using phorbol 12-myristate 13-acetate (PMA) toward megakaryocytic-like cells. After 48 hours the differentiation cell surface marker (CD61) specific to the megakaryocytic-like cells population was increased by 43% (**Figure 3.10I**) and these differentiated cells underwent phenotypic and morphologic changes that were associated with megakaryocytic like elongated cells, which strongly attached to the substratum (**Figure 3.10H**). To confirm the cell-specific *HERC1* gene expression following PMA treatment total RNAs and proteins were extracted and *HERC1* mRNA and protein expression was analyzed by RT-qPCR and Western Blots. As was expected the *HERC1* gene expression both at mRNA and protein level was downregulated compared to non-treated cells (**Figure 3.10J & K**). Thus, our findings suggest that *HERC1* gene expression is cell-specific and it might be a novel differentiation regulator associated with blood malignancies. Remarkably, we also observed a variation in the phosphorylation of Erk1/2 pattern which was tightly linked with the *HERC1* protein level in differentiated cells while the phosphorylation of BCR-ABL1 was severely impaired in PMA induced K-562 cells when compared to undifferentiated control.

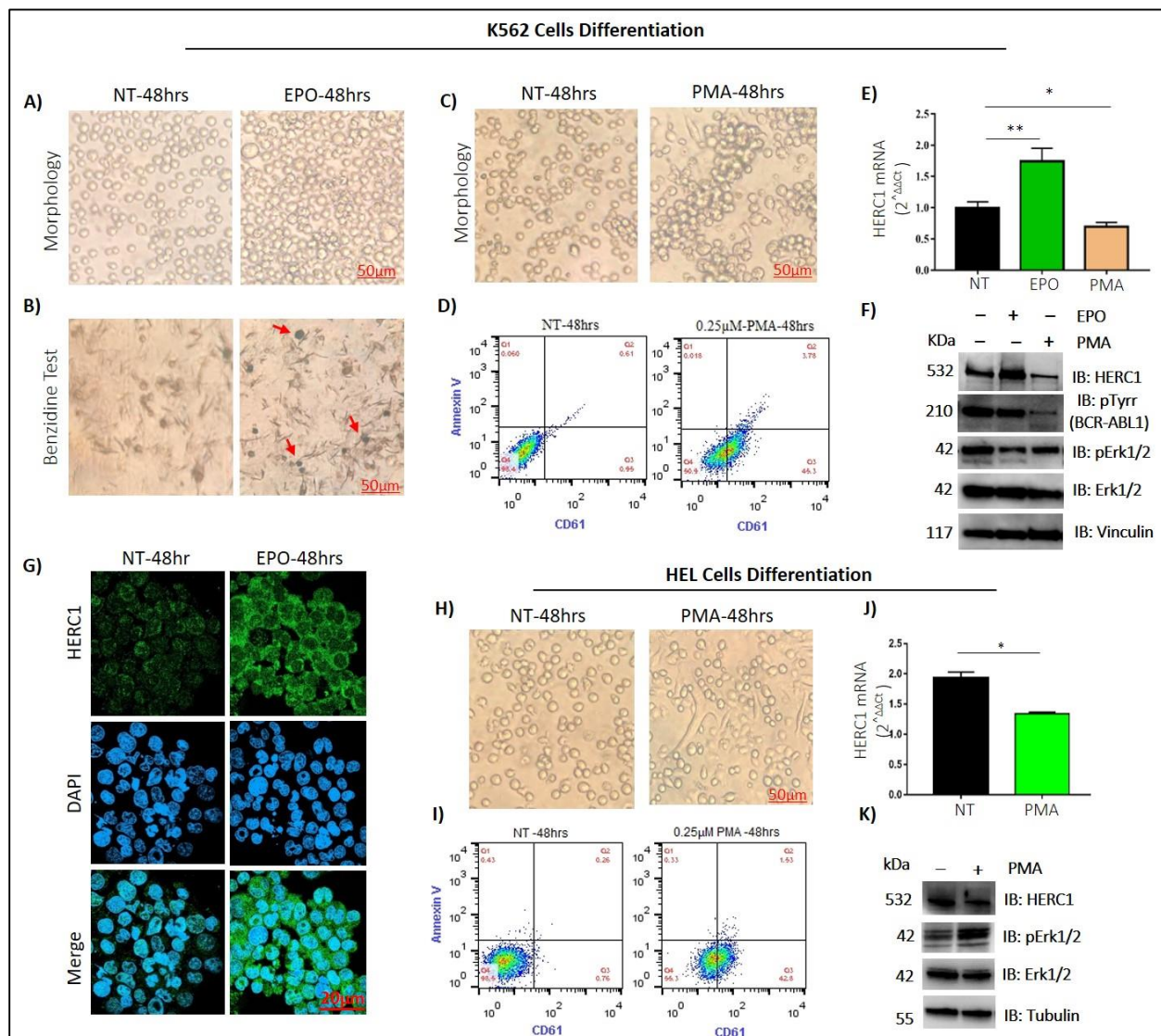


Figure 3.10. Effect of *HERC1* gene expression on the differentiation of Ph⁺(K562) myeloid leukemia cell line with PMA and EPO. Differentiation was performed by incubating Ph⁺ K562 myeloid leukemia cell lines with 250nM phorbol 12 myristate 13-acetate (PMA) and 20U/ml Erythropoietin (EPO). **A)** Representative pictures of the morphology of un-induced control and differentiated cells **B)** K562 cells were harvested after 48 hours with or without EPO induction and stained with benzidine. Benzidine-positive cells turned blue indicated with red arrows. Untreated K562 cells served as a control. **C & D)** Representative cell morphology images and flow cytometry analysis of K562 cells stained using anti-CD61 megakaryocyte specific cell surface marker of undifferentiated and differentiated cells. **E & F)** Expressional changes in *HERC1* gene at mRNA and protein level in non-treated, EPO and PMA induced K562 cells. **G)** Representative images of immunofluorescence microscopy revealing the EPO-induced differentiation of K562 cells resulted in an increase of HERC1 protein levels. **H & I)** Representative cell morphology images and flow cytometry analysis of HEL cells stained using anti-CD61 megakaryocyte specific

cell surface marker of undifferentiated and differentiated HEL-cells, after 48hours in the presence of PMA (250nM). **J & K)** *HERC1* gene expression both at mRNA and protein in undifferentiated and differentiated HEL cells. The α -tubulin and vinculin were used as loading controls in Western Blots.

3.10. HERC1 as a Novel and Emerging Binding Partner of mTORC2 Complex

HERC1 contains a number of conserved domains which are supposed to play different roles in the overall function(s) of the protein. Along with its RLDs and HECT domains, the most conserved regions in HERC1 include a SPRY (repeats in splA and RyR), WD-40 and BH3 domains. These domains play a pivotal role in protein-protein interactions and are directly implicated in distinct cellular functions [184,221,222]. The HERC1 has been involved in membrane trafficking, cell proliferation/growth and apoptosis through its interaction with ARF, Rab, Clathrin, M2-pyruvate kinase, TSC2 and BAK proteins, [179,182–184,221,222]. The HERC1 interaction with tuberous sclerosis 2 (TSC2, tuberin) results in its destabilization which suppresses cell growth [189]. The recent finding showed that HERC1 is a regulator of ERK signaling by interacting with RAF proteins. Specifically, HERC1 modulates C-RAF stability, and it controls cellular proliferation by regulating C-RAF protein levels through ubiquitylation [198]. In our previous work, we identified a large *Dictyostelium* HECT member (HectPH1), exhibiting many structural similarities with the mammalian HERC1, as a suppressor of the mTORC2-PKB/AKT signaling axis [223]. In addition, based on the findings in *Dictyostelium* that HectPH1 interact with Pia/Rictor and Hsp90, we also attempted to assess whether HERC1 in mammalian cells has any physical association with mTORC2 complex through co-immunoprecipitation experiments by using Rictor as a bait. Interestingly, we identified a direct physical interaction of HERC1 with the components of mTORC2 complex, along with the well-known mTORC2 substrate Akt. Surprisingly, the close large HERC family member HERC2 did not participate in the interacting complex (**Figure 3.11B**). Similarly, we also identified the Hsp90 as a binding partner of HERC1 via co-immunoprecipitation experiment by using Hsp90 as a bait (**Figure 3.11C**). To further investigate deep into the role of Hsp90 interaction with HERC1-mTORC2 supramolecular complex, we treated HEK-293T cells with Hsp90 inhibitors geldanamycin. Interestingly our results revealed that upon geldanamycin treatment the HERC1 protein downregulated compared to the non-treated control (**Figure 3.11E**). Astonishingly, the

phosphorylation of Akt at serine 473 also impaired which indicate that the stable HERC1 along with active Hsp90 might mediate mTORC2 to phosphorylate the Akt. These preliminary results might help us to speculate that Hsp90 not only interact with HERC1 but also involve in the HERC1 proper folding/stability and might be mediating directly or indirectly the proper activation of Akt through mTORC2 complex (**Figure 3.11D**). To validate these findings, we need further investigations in detail to prove this speculation.

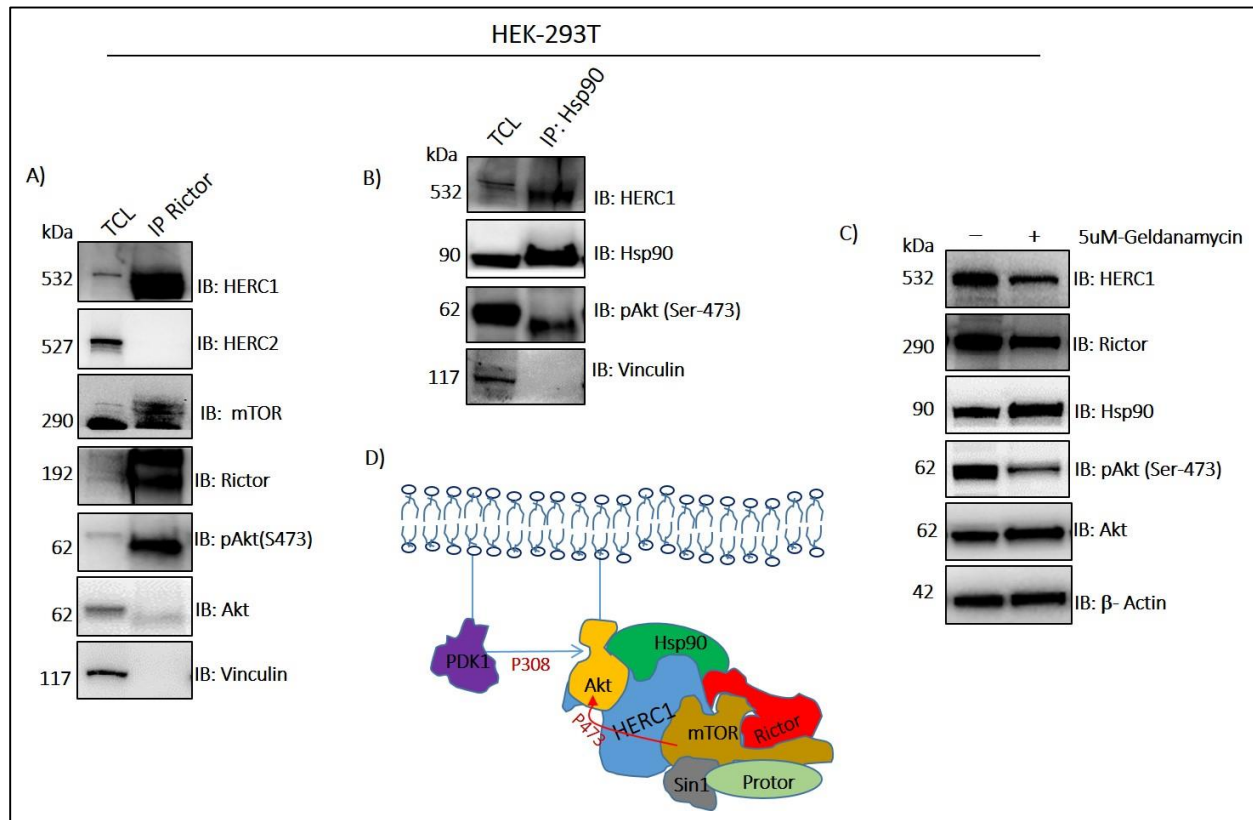


Figure 3.11. HERC1 mediate Akt activation by establishing a complex with Akt and the components of mTORC2 along with Hsp90. Lysates prepared from HEK-293T cells were immunoprecipitated (IP) using anti-Rictor and anti-Hsp90 antibodies and analyzed by immunoblotting with antibodies against the indicated proteins. **A)** Representative diagram showing the physical association of HERC1 with the component of mTORC2 complex. The close relative member, the HERC2 did not interact with the mTORC2 complex. **B)** HERC1 interacts with Hsp90. **C)** HERC1 and rictor proteins were downregulated upon treatment of HEK-293T cells with Hsp90 inhibitor geldanamycin, along with impaired activation of Akt. **D)** Schematic representation of HERC1 and Hsp90 as novel mediators in the activation/phosphorylation of Akt(Ser473) through mTORC2 complex. Vinculin and β -Actin were used as loading controls in Western Blots.

4. DISCUSSION

To overcome stresses and to properly balance the entire proteome, every eukaryotic cell relies on the ubiquitin-proteasome system (UPS). Damaged, or malfunctioning, proteins are ubiquitin tagged and then degraded by the 26S proteasome. However, the ubiquitination process is not exclusively engaged under stress conditions. Indeed, ubiquitin moieties can serve as a docking site for protein-protein interaction. Eventually, as important as the ubiquitin conjugates the cell survival and proliferation rely also on the free ubiquitin chains and the free ubiquitin pool [224]. Alterations in the UPS are often associated with severe diseases and numerous E3 ubiquitin ligases are aberrantly regulated in different cancers [225,226].

Despite the HECT sub-family members represent a small proportion of the E3 ubiquitin ligase superfamily many of them have been found to be either aberrantly regulated or mutated in several tumors [167,227,228]. Among HECT proteins, the HERC subfamily contains two giant members, HERC1 and HERC2. Mutations in either gene have been reported in some kind of lymphoid leukemia (*e.g.* acute lymphoblastic and pro-lymphocytic leukemia) and solid tumors (*e.g.* breast cancers, non-melanoma skin and gastric carcinoma) [172].

However, currently, specific inquiries aiming to assess the potential role of the giant HERCs in myeloid related neoplasia are lacking. We initially started by comparing the *HERC1* transcript amount between the PB and the BM of healthy subjects. Afterwards, we moved to analyze a panel of myeloid related disorders including AML, MPNs and CML. Interestingly, our findings revealed that in healthy control specimens the *HERC1* is differentially transcribed when comparing PB and BM, displaying significantly lower levels in the BM (**Figure 3.1A**). Furthermore, by determining the quantitative expression of the *HERC1* gene in a panel of myeloid related disorders we found a steep decline in those samples collected from patients with newly diagnosed AML, CML and PMF (**Figure 3.1B**). Interestingly, we also observed a downregulation of HERC1 protein level in myeloid cells (monocytes) population of leukemic specimens, compared to the corresponding healthy counterpart (**Figure 3.5A & B**). Remarkably, these results were in line with publically available datasets in which the *HERC1* mRNA expression levels were assessed in different types of normal blood population (biogps.org, and proteinatlas.org). Based on our observations, HERC1 levels being higher in normal blood, we cannot exclude that HERC1 might be involved in blood cell differentiation thus contributing to the proper hematopoiesis.

Future investigations, aiming to explore the functional role of *HERC1* in blood cells, are required. In AML, being a heterogeneous disease, we observed the *HERC1* transcript levels were regulated independently from the different cytogenetic categories and genetic variants. Remarkably, we additionally perceived that those AML patients harboring the *IDH2-R140Q* mutation showed higher *HERC1* transcript levels when compared to all the other cytogenetic categories and genetic variants we screened (**Figure 3.2B**). The mutated IDH2 enzyme is characterized by a peculiar neomorphic activity resultant in the synthesis of a novel oncometabolite, the 2-hydroxyglutarate, which in turns inhibits the α -ketoglutarate-dependent epigenetic regulators (e.g. histone demethylase TETs). As a consequence, the genomic DNA results in hypermethylated thus contributing to profoundly impair the cellular differentiation [133,229]. Our results advise to argue that, likely, the *IDH2-R140Q*-dependent genomic DNA hypermethylation leads to the transcriptional repression of an, by now, unknown transcriptional repressor and thus maintaining unaltered the *HERC1* mRNA levels. Further research will be required to ascertain this hypothesis.

Conversely from PMF, the other two MPNs showed higher *HERC1* levels that were either quite similar to those of the healthy subjects, as it occurred for PV, or even higher as it was in the case of the *CALR* mutated ET samples (**Figure 3.3A**). Unambiguously, our findings indicate that in MPNs the *HERC1* transcript inversely correlates with the severity of the disease, being the PMF specimens those with the lowest level of *HERC1*, but not with the *JAK2* mutational status in PV and ET. Furthermore, among MPNs the *CALR* mutated ET patients, displaying a favorable prognosis, exhibited the highest *HERC1* gene expression level, with a median value of 7.3 that was significantly higher than that of healthy controls. The recurrent *CALR* somatic mutations cause a +1 frameshift in the reading frame leading to mutant specific *CALR* carboxyl-terminus that lacks the Endoplasmic Reticulum (ER)-retention signal (KDEL). Calreticulin is a lectin chaperone that, alongside GRP78 and calnexin, promotes and facilitates the proper folding and maturation of the ER luminal proteins. Damaged or improperly folded proteins usually escape the calreticulin cycle and enter the ER Associated Degradation (ERAD). Otherwise, proteins can continue their journey and when required move to Golgi Apparatus. *CALR* deficient cells exhibit accumulation of misfolded proteins and an increase in oxidative DNA damage has been reported in *CALR* mutated leukemia cell (K562) due to the downmodulation of OXR1 (Oxidation Resistance Protein 1) [22]. However, a significant increase in ubiquitin-proteasome activity has been detected as a result of a compensatory mechanism for cell survival [230]. Furthermore, it is well known that ER stress

leads to activation of both the Unfolded Protein Response (UPR) and autophagy processes that in turn tightly depend on the ubiquitination process. A transcriptomic analysis carried-out in colon cancer cell line treated with ER stressors (*e.g.* brefeldin A and tunicamycin) revealed an up-regulation of a large group of autophagy-related genes, including *HERC1* [231]. These data, together with the *HERC1* subcellular localization in inner membranes (*e.g.* Golgi Apparatus) suggest that the ubiquitin-proteasome system (UPS) might be a novel potential, and by now uninvestigated player in the pathogenesis of the ET *CALR* mutated and contribute to the clonal advantage in hematopoietic cells by downregulating pro-apoptotic, or pro-differentiating, signaling processes. Furthermore, among the MPNs group those patients expressing either the highest or the lowest, *HERC1* transcript levels define two novel subsets that overlap with ET *CALR* mutated and PMF groups thus leading to wonder whether the *HERC1* transcript levels might represent a novel additional diagnostic marker along with the ones currently available.

Differently from its closest relatives, namely ET, PV and PMF, the CML is characterized by a single genetic alteration giving rise to a constitutively active tyrosine kinase, the BCR-ABL1 fusion protein. This peculiar molecular landmark confers unique properties to be feasibly investigated from the very basic biological point of view by simply overexpressing, or pharmacologically inhibiting (*e.g.* Imatinib), the BCR-ABL1 protein. Our findings indicate that upon effective Imatinib treatment the *HERC1* mRNA levels raised sharply to levels similar to that of the control specimens but decreased again when the patients relapsed (**Figure 3.4A**). Consistently, cells overexpressing BCR-ABL1 displayed lower *HERC1* transcript when compared to the control (**Figure 3.7**). Though the genotype is different, the newly diagnosed AML, PMF and CML displayed low amounts of *HERC1* transcript when compared to the healthy control. Nonetheless, BCR-ABL1 appears to trigger a decline in *HERC1* gene expression. Hence, it is conceivable to hypothesize that: a) in AML, PMF and CML *HERC1* might act as oncosuppressor, [198] and b) though genotypically different all these myeloid disorders share a common *HERC1* transcriptional repressor mechanism that in CML it is positively regulated by BCR-ABL1. The different structural motifs characterizing the BCR-ABL1 fusion protein allows it to interact with dozens of binding partners [232]. Amongst them there are also few UPS components, comprising E3 Ubiquitin-ligases and deubiquitylating enzymes (*e.g.* Cbl, USP1) [233,234], that are fundamental to control the BCR-ABL1 stability and as a consequence its oncogenic potential. Our data show that *HERC1* represents both a novel BCR-ABL1 interacting partner and substrate.

However, the functional meaning of the interaction and tyrosine phosphorylation remain presently to be ascertained. Currently, it cannot be excluded that HERC1 might act as BCR-ABL1 negative regulator and the tyrosine phosphorylation might control its enzymatic activity. Consistently, the enzymatic activity of Nedd4, a HECT E3 Ubiquitin-ligase member, is tightly regulated by the phosphorylation of a few tyrosine residues [235]. Additional studies are needed to dissect the functional relevance of the *HERC1* aberrant expression in myeloid-related disorders. Indeed, though our data suggest that HERC1 plausibly plays a role as an oncosuppressor in the majority of myeloid malignancies (e.g. AML, CML and PMF) by now we cannot rule out that in those ET calreticulin mutated it might display tumor-promoting properties, thus exerting different properties depending on the context. Determining the most relevant HERC1 substrates might represent a useful hint for novel druggable molecular targets.

A common and prominent feature shared by all leukaemia, as well as many other tumors including solid ones, is that they display impaired cell differentiation. Basically, the differentiation defects featuring leukemic cells, though to different extent, are common to every leukemia form including the myeloid related disorder. The functional role of HERC1 has been recently associated to cellular proliferation. However, with the exception of few observations, restricted to the central nervous system in which HERC1 seems to be important for the axonal myelination and the control of the presynaptic membrane dynamics of central synapses [236], the role of this giant ubiquitin-ligase during cell-differentiation remains currently poorly investigated. Nonetheless, it is well known that the UPS plays an important role during differentiation in almost every cell type, including blood cells [237]. In this regard red blood cells differentiation requires the remodeling of their proteome that takes place by the selective degradation of pre-existing proteins through the UPS. Hence, we asked whether HERC1 might be a potential player involved in blood cell differentiation. To test this working hypothesis we took advantage of leukemic cell lines and we attempted to de-differentiate towards different lineages.

We initially attempted to de-differentiate the HL60 acute myeloid leukemia cell line model. The HL60 cells have been shown to de-differentiate upon treatment with PMA, ATRA and DMSO [209,210]. All of which induce the de-differentiation of HL60 cells towards granulocytes/neutrophils-like cells. Interestingly, after differentiation, we observed an increase of both HERC1 transcript and protein levels compared to non-treated cells (**Figure 3.9**). Surprisingly, the HERC1 protein amount was comparatively higher in ATRA and DMSO induced HL60 cells

when compared to PMA treated cells, while we did not observe any significant difference in mRNA levels in these differentiated cells. Further research work is required to dissect the meaning of this discrepancy in between transcript and protein levels.

Analogous results were also obtained in Ph⁺ K-562 cell line when differentiated either towards the erythroid and megakaryocytic-like lineage by EPO and PMA treatments, respectively. Interestingly, the upregulation of *HERC1* gene expression was observed in EPO treated cells, while PMA induced K-562 differentiate toward megakaryocytic-like cells showed a decline in *HERC1* gene expression. A comparable downregulation of *HERC1* mRNA and protein level were also observed when another cell line, the HEL, was differentiated toward megakaryocytic-like cells by using PMA. On the whole, these findings suggest that *HERC1* protein expression is associated with the de-differentiation process of few leukemic cell lines towards different lineages, ranging from neutrophils- to erythroid-like cells. Since the *HERC1* protein expression pattern changes in a way tightly linked to the terminal cell-fate it is also reasonable to assume that its role is deeply cell specific. Remarkably, in all differentiated cells, the phosphorylation status of Erk1/2 was tightly linked with the *HERC1* protein level, phosphorylation of Erk1/2 declines with an increase in *HERC1* protein level, and this pattern was in agreement with previous findings [198]. Moreover, all the cell lines used in differentiation experiments were either *TP53* mutated to a different extent (K562 and HEL) or *TP53* null (HL60) due to its locus deletion. Based on our findings, which display differential *HERC1* transcript and protein amounts among the three cell lines, we believe that *TP53* plays a negligible, if any, role in regulating *HERC1* gene expression. Interestingly active involvement of E3 ubiquitin ligases in sustaining leukemic cells survival, proliferation and blood cells differentiation have been reported for a couple of members named WWP1 and TRIAD1, respectively. The WWP1 E3 ubiquitin ligase is significantly overexpressed in AML and contributes to sustaining AML growth by limiting the stability of p27^{Kip} and the autophagy signaling, while its inactivation severely impairs the growth of primary AML blasts [238]. Another E3 ubiquitin ligase TRIAD1 has been reported to involved in the ubiquitination of the fibroblast growth factor receptor 1 (Fgf-R1) during the termination of granulopoiesis and its expression increases during myelopoiesis [239]. Also, TRIAD1 has been reported to act as a leukemia suppressor in 11q23-AML [240]. Further research work is needed to investigate the functional role of *HERC1* in the regulation of blood cell differentiation and what are the *HERC1* gene expression regulators.

Previously, our group by making use of a simple model organism *Dictyostelium* reported the possibility to rescue a cell strain displaying mTORC2 deficient phenotype by inactivation of a gene encoding for a large HECT E3 ubiquitin ligase, Hect-PH1, that is close relative to mammalian HERC1 [223]. The mTOR complexes assembly is tightly regulated by the ubiquitination-deubiquitination process through an ubiquitin-proteasome independent way. Indeed, the K63-linked polyubiquitination status of G β L/LST8 edicts the homeostasis of mTORC2 formation and activation. Systematically, the E3 ubiquitin ligase TRAF2 promotes K63-linked polyubiquitination of G β L/LST8, which causes interaction interferences with the other mTORC2 component SIN1, to favour mTORC1 assembly. Meanwhile, the deubiquitinase OTUD7B removes polyubiquitin chains from G β L/LST8 to promote G β L interaction with SIN1 thus facilitating mTORC2 formation. [241]. Therefore, by making use of the Hect-PH1 HECT domain as a bait we attempted to identify putative binding partners by a proteomic approach coupled to mass-spectrometry (unpublished data). Among the different interacting proteins identified there were Pia/Rictor and heat-shock protein 90 (Hsp90). Hence, in the last part of my thesis, we also attempted to validate what we found in *Dictyostelium*. Consistent with the findings achieved with *Dictyostelium* cells, Hsp90 and Rictor physically interact with the mammalian HERC1, we identified the mammalian large HERC1 as a novel interactor of mTORC2 components along with its association with Akt (**Figure 3.11A**). Previously reported studies indicate, Akt and mTORC2 component SIN1, through their PH domains, and HERC1, through its RLD1 domain, have an affinity for cellular membrane phospholipids [202,242,243], which might bring them closer to interact with each other. These preliminary observations might open up new doors to understanding in detail the PI3K-PKB/AKT-mTOR signaling pathway but future investigations, aiming to explore the functional role of HERC1 association with mTORC2, are required. Interestingly, we also unveiled Hsp90 as an interactor of HERC1 along with Akt. As it is well known that Hsp90 acts as a ubiquitous molecular chaperone for stabilization and conformational maturation/folding of many signaling proteins (more than 100) which are mostly dysregulated in different pathological conditions [244]. To properly dissect the meaning of this interaction of Hsp90 with HERC1 under the physiological conditions we treated human embryonic kidney (HEK-293T) cells with Hsp90 inhibitor, geldanamycin. Surprisingly we observed a significant downregulation of HERC1 and Rictor, the pivotal component of the mTORC2, upon the inhibition of Hsp90 activity (**Figure 3.11C**). These preliminary findings help us to hypothesize that Hsp90

might stabilize this complex by controlling the stability of HERC1 which might act as a scaffold to mediate the activation of Akt through the mTORC2 complex (**Figure 3.11D**). Future investigations are required to validate this working hypothesis through all possible ways and especially by mapping the interacting regions of these binding partners.

5. CONCLUSION AND FUTURE PERSPECTIVES

In conclusion, for the first time, we assessed the expression levels of the gene encoding for the giant HECT E3 Ubiquitin ligase, HERC1, in white blood cells and in a panel of myeloid-related disorders including AML, MPNs and CML. Though, in myeloid leukemia and related neoplasms, the HERC1 transcript levels were mostly down-regulated, we noticed a few remarkable exceptions. Hence, we believe it is plausible to suggest that HERC1 might either act as an oncosuppressor or oncogene depending on the cell context. The constitutively active BCR-ABL1 tyrosine kinase contributes significantly in lowering the *HERC1* gene expression. Into the bargain, the HERC1 protein represents a novel BCR-ABL1 interacting partner and substrate. We also observed a differential *HERC1* gene expression when leukemic cell lines were induced to differentiate. These preliminary observations highlighted the pivotal role of HERC1 E3 ligase in the regulation of blood cell differentiation. Additionally, we also have very preliminary data in which we identified Hsp90 and the components of mTORC2 complex (Rictor, mTOR), and Akt as novel binding partners of the HERC1 and this very large supramolecular complex seems to be involved in the regulation of Akt activation. On the whole, future research work is needed to dissect in detail the involvement of HERC1 in blood cell differentiation, or its role to act as oncosuppressor or oncogene and to assess to what extent HERC1 is involved in regulating the mTORC2/Akt axis.

5. REFERENCES

1. Doulatov, S.; Notta, F.; Laurenti, E.; Dick, J.E. Hematopoiesis: A human perspective. *Cell Stem Cell* **2012**, *10*, 120–136.
2. Laurenti, E.; Göttgens, B. From haematopoietic stem cells to complex differentiation landscapes. *Nature* **2018**, *553*, 418–426.
3. Notta, F.; Zandi, S.; Takayama, N.; Dobson, S.; Gan, O.I.; Wilson, G.; Kaufmann, K.B.; McLeod, J.; Laurenti, E.; Dunant, C.F.; et al. Distinct routes of lineage development reshape the human blood hierarchy across ontogeny. *Science* (80-.). **2016**, *351*, doi:10.1126/science.aab2116.
4. Galy, A.; Travis, M.; Cen, D.; Chen, B. *Human T, B, Natural Killer, and Dendritic Cells Arise from a Common Bone Marrow Progenitor Cell Subset*; **1995**; Vol. 3;.
5. Pucella, J.N.; Upadhaya, S.; Reizis, B. The Source and Dynamics of Adult Hematopoiesis: Insights from Lineage Tracing. **2020**, doi:10.1146/annurev-cellbio-020520.
6. Notta, F.; Doulatov, S.; Laurenti, E.; Poepl, A.; Jurisica, I.; Dick, J.E. Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment. *Science* (80-.). **2011**, *333*, 218–221, doi:10.1126/science.1201219.
7. Catlin, S.N.; Busque, L.; Gale, R.E.; Gutter, P.; Abkowitz, J.L. The replication rate of human hematopoietic stem cells in vivo. *Blood* **2011**, *117*, 4460–4466, doi:10.1182/blood-2010-08-303537.
8. Takizawa, H.; Boettcher, S.; Manz, M.G. Demand-adapted regulation of early hematopoiesis in infection and inflammation. *Blood* **2012**, *119*, 2991–3002.
9. Shlush, L.I.; Zandi, S.; Itzkovitz, S.; Schuh, A.C. Aging, clonal hematopoiesis and preleukemia: not just bad luck? *Int. J. Hematol.* **2015**, *102*, 513–522.
10. Yang, X.; Chen, D.; Long, H.; Zhu, B. The mechanisms of pathological extramedullary hematopoiesis in diseases. *Cell. Mol. Life Sci.* **2020**, *77*, 2723–2738.
11. Cedar, H.; Bergman, Y. Epigenetics of haematopoietic cell development. *Nat. Rev. Immunol.* **2011**, *11*, 478–488.
12. Salk, J.J.; Fox, E.J.; Loeb, L.A. Mutational Heterogeneity in Human Cancers: Origin and Consequences. *Annu. Rev. Pathol. Mech. Dis.* **2010**, *5*, 51–75, doi:10.1146/annurev-pathol-121808-102113.
13. Hanahan, D.; Weinberg, R.A. Hallmarks of cancer: The next generation. *Cell* **2011**, *144*, 646–674.
14. Dameshek, W. Some speculations on the myeloproliferative syndromes. *Blood* **1951**, *6*, 372–375.
15. Nangalia, J.; Green, A.R. Myeloproliferative neoplasms: from origins to outcomes. *Hematol. 2014, Am. Soc. Hematol. Educ. Progr. B.* **2017**, *2017*, 470–479.
16. Barbui, T.; Thiele, J.; Gisslinger, H.; Kvasnicka, H.M.; Vannucchi, A.M.; Guglielmelli, P.;

- Orazi, A.; Tefferi, A. The 2016 WHO classification and diagnostic criteria for myeloproliferative neoplasms: document summary and in-depth discussion. *Blood Cancer J.* **2018**, *8*, 1–11.
17. Arber, D.A.; Orazi, A.; Hasserjian, R.; Thiele, J.; Borowitz, M.J.; Le Beau, M.M.; Bloomfield, C.D.; Cazzola, M.; Vardiman, J.W. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood* **2016**, *127*, 2391–2405.
 18. Sangiorgio, V.; Orazi, A. Update on the classification of myeloid neoplasms: The 2016 revised World Health Organization classification of hematopoietic and lymphoid neoplasms. *Adv. CELL GENE Ther.* **2020**, *3*, doi:10.1002/acg2.78.
 19. Cervantes, F.; Dupriez, B.; Pereira, A.; Passamonti, F.; Reilly, J.T.; Morra, E.; Vannucchi, A.M.; Mesa, R.A.; Demory, J.-L.; Barosi, G. New prognostic scoring system for primary myelofibrosis based on a study of the International Working Group for Myelofibrosis Research and Treatment. *Blood* **2009**, *113*, 2895–2901.
 20. Lasho, T.L.; Mudireddy, M.; Finke, C.M.; Hanson, C.A.; Ketterling, R.P.; Szuber, N.; Begna, K.H.; Patnaik, M.M.; Gangat, N.; Pardanani, A. Targeted next-generation sequencing in blast phase myeloproliferative neoplasms. *Blood Adv.* **2018**, *2*, 370–380.
 21. Brecqueville, M.; Rey, J.; Bertucci, F.; Coppin, E.; Finetti, P.; Carbuccia, N.; Cervera, N.; Gelsi-Boyer, V.; Arnoulet, C.; Gisserot, O. Mutation analysis of ASXL1, CBL, DNMT3A, IDH1, IDH2, JAK2, MPL, NF1, SF3B1, SUZ12, and TET2 in myeloproliferative neoplasms. *Genes, Chromosom. Cancer* **2012**, *51*, 743–755.
 22. Salati, S.; Genovese, E.; Carretta, C.; Zini, R.; Bartalucci, N.; Prudente, Z.; Pennucci, V.; Ruberti, S.; Rossi, C.; Rontautoli, S. Calreticulin Ins5 and Del52 mutations impair unfolded protein and oxidative stress responses in K562 cells expressing CALR mutants. *Sci. Rep.* **2019**, *9*, 1–14.
 23. Vainchenker, W.; Kralovics, R. Genetic basis and molecular pathophysiology of classical myeloproliferative neoplasms. *Blood, J. Am. Soc. Hematol.* **2017**, *129*, 667–679.
 24. Tefferi, A. Myeloproliferative neoplasms: a decade of discoveries and treatment advances. *Am. J. Hematol.* **2016**, *91*, 50–58.
 25. Kralovics, R.; Passamonti, F.; Buser, A.S.; Teo, S.-S.; Tiedt, R.; Passweg, J.R.; Tichelli, A.; Cazzola, M.; Skoda, R.C. A gain-of-function mutation of JAK2 in myeloproliferative disorders. *N. Engl. J. Med.* **2005**, *352*, 1779–1790.
 26. Scott, L.M.; Tong, W.; Levine, R.L.; Scott, M.A.; Beer, P.A.; Stratton, M.R.; Futreal, P.A.; Erber, W.N.; McMullin, M.F.; Harrison, C.N. JAK2 exon 12 mutations in polycythemia vera and idiopathic erythrocytosis. *N. Engl. J. Med.* **2007**, *356*, 459–468.
 27. Perner, F.; Perner, C.; Ernst, T.; Heidel, F.H. Roles of JAK2 in aging, inflammation, hematopoiesis and malignant transformation. *Cells* **2019**, *8*, 854.
 28. Passamonti, F.; Elena, C.; Schnittger, S.; Skoda, R.C.; Green, A.R.; Girodon, F.; Kiladjian, J.-J.; McMullin, M.F.; Ruggeri, M.; Besses, C. Molecular and clinical features of the myeloproliferative neoplasm associated with JAK2 exon 12 mutations. *Blood, J. Am. Soc.*

- Hematol.* **2011**, *117*, 2813–2816.
29. Bose, P.; Verstovsek, S. JAK2 inhibitors for myeloproliferative neoplasms: What is next? *Blood* **2017**, *130*, 115–125, doi:10.1182/blood-2017-04-742288.
 30. Mascarenhas, J.; Baer, M.R.; Kessler, C.; Hexner, E.; Tremblay, D.; Price, L.; Sandy, L.; Weinberg, R.; Pahl, H.; Silverman, L.R.; et al. Phase II trial of Lestaurtinib, a JAK2 inhibitor, in patients with myelofibrosis. *Leuk. Lymphoma* **2019**, *60*, 1343–1345, doi:10.1080/10428194.2018.1532509.
 31. Holyoake, T.L.; Vetrie, D. The chronic myeloid leukemia stem cell: stemming the tide of persistence. *Blood* **2017**, *129*, 1595–1606.
 32. Collins, S.; Coleman, H.; Groudine, M. Expression of bcr and bcr-abl fusion transcripts in normal and leukemic cells. *Mol. Cell. Biol.* **1987**, *7*, 2870.
 33. Kurzrock, R.; Gutterman, J.U.; Talpaz, M. The molecular genetics of Philadelphia chromosome-positive leukemias. *N. Engl. J. Med.* **1988**, *319*, 990–998.
 34. Hungerford, D.A.; Nowell, P.C. A minute chromosome in human chronic granulocytic leukemia. *Science (80-.)*. **1960**, *132*, 1497–1499.
 35. Rowley, J.D. A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. *Nature* **1973**, *243*, 290–293.
 36. Heisterkamp, N.; Stephenson, J.R.; Groffen, J.; Hansen, P.F.; de Klein, A.; Bartram, C.R.; Grosveld, G. Localization of the c-abl oncogene adjacent to a translocation break point in chronic myelocytic leukaemia. *Nature* **1983**, *306*, 239–242.
 37. Deininger, M.W.N.; Goldman, J.M.; Melo, J. V The molecular biology of chronic myeloid leukemia. *Blood, J. Am. Soc. Hematol.* **2000**, *96*, 3343–3356.
 38. Jabbour, E.; Kantarjian, H. Chronic myeloid leukemia: 2018 update on diagnosis, therapy and monitoring. *Am. J. Hematol.* **2018**, *93*, 442–459, doi:10.1002/ajh.25011.
 39. Perrotti, D.; Jamieson, C.; Goldman, J.; Skorski, T. Chronic myeloid leukemia: mechanisms of blastic transformation. *J. Clin. Invest.* **2010**, *120*, 2254–2264.
 40. Calabretta, B.; Perrotti, D. The biology of CML blast crisis. *Blood* **2004**, *103*, 4010–4022.
 41. Spiers, A.S.; Baiki, A.G. Cytogenetic evolution and clonal proliferation in acute transformation of chronic granulocytic leukaemia. *Br. J. Cancer* **1968**, *22*, 192.
 42. Rowley, J.D. Nonrandom chromosomal abnormalities in hematologic disorders of man. *Proc. Natl. Acad. Sci.* **1975**, *72*, 152–156.
 43. Branford, S.; Wang, P.; Yeung, D.T.; Thomson, D.; Purins, A.; Wadham, C.; Shahrin, N.H.; Marum, J.E.; Nataren, N.; Parker, W.T. Integrative genomic analysis reveals cancer-associated mutations at diagnosis of CML in patients with high-risk disease. *Blood* **2018**, *132*, 948–961.
 44. Giotopoulos, G.; van der Weyden, L.; Osaki, H.; Rust, A.G.; Gallipoli, P.; Meduri, E.;

- Horton, S.J.; Chan, W.-I.; Foster, D.; Prinjha, R.K. A novel mouse model identifies cooperating mutations and therapeutic targets critical for chronic myeloid leukemia progression. *J. Exp. Med.* **2015**, *212*, 1551–1569.
45. Li, S.; Ilaria Jr, R.L.; Million, R.P.; Daley, G.Q.; Van Etten, R.A. The P190, P210, and P230 forms of the BCR/ABL oncogene induce a similar chronic myeloid leukemia–like syndrome in mice but have different lymphoid leukemogenic activity. *J. Exp. Med.* **1999**, *189*, 1399–1412.
 46. Puil, L.; Liu, J.; Gish, G.; Mbamalu, G.; Bowtell, D.; Pelicci, P.G.; Arlinghaus, R.; Pawson, T. Bcr-Abl oncoproteins bind directly to activators of the Ras signalling pathway. *EMBO J.* **1994**, *13*, 764–773.
 47. Feller, S.M.; Ren, R.; Hanafusa, H.; Baltimore, D. SH2 and SH3 domains as molecular adhesives: the interactions of Crk and Abl. *Trends Biochem. Sci.* **1994**, *19*, 453–458.
 48. McWHIRTER, J.R.; Galasso, D.L.; Wang, J.Y. A coiled-coil oligomerization domain of Bcr is essential for the transforming function of Bcr-Abl oncoproteins. *Mol. Cell. Biol.* **1993**, *13*, 7587–7595.
 49. Lugo, T.G.; Pendergast, A.-M.; Muller, A.J.; Witte, O.N. Tyrosine kinase activity and transformation potency of bcr-abl oncogene products. *Science (80-.)*. **1990**, *247*, 1079–1082.
 50. Reuther, G.W.; Fu, H.; Cripe, L.D.; Collier, R.J.; Pendergast, A.M. Association of the protein kinases c-Bcr and Bcr-Abl with proteins of the 14-3-3 family. *Science (80-.)*. **1994**, *266*, 129–133.
 51. Kang, Z.-J.; Liu, Y.-F.; Xu, L.-Z.; Long, Z.-J.; Huang, D.; Yang, Y.; Liu, B.; Feng, J.-X.; Pan, Y.-J.; Yan, J.-S. The Philadelphia chromosome in leukemogenesis. *Chin. J. Cancer* **2016**, *35*, 48.
 52. Egan, D.; Radich, J. Prognosis and molecular monitoring in chronic myeloid leukemia. *Clin. Lymphoma Myeloma Leuk.* **2015**, *15*, S109–S113.
 53. Shanmuganathan, N.; Hughes, T.P. Molecular monitoring in CML: how deep? How often? How should it influence therapy? *Hematol. 2014, Am. Soc. Hematol. Educ. Progr. B.* **2018**, *2018*, 168–176.
 54. Talpaz, M.; McCredie, K.; Jian, H.K.; Trujillo, J.; Keating, M.; Gutterman, J. Chronic myelogenous leukaemia: haematological remissions with alpha interferon. *Br. J. Haematol.* **1986**, *64*, 87–95.
 55. Pavlů, J.; Szydło, R.M.; Goldman, J.M.; Apperley, J.F. Three decades of transplantation for chronic myeloid leukemia: what have we learned? *Blood, J. Am. Soc. Hematol.* **2011**, *117*, 755–763.
 56. Druker, B.J. Translation of the Philadelphia chromosome into therapy for CML. *Blood* **2008**, *112*, 4808–4817.
 57. Druker, B.J.; Tamura, S.; Buchdunger, E.; Ohno, S.; Segal, G.M.; Fanning, S.; Zimmermann, J.; Lydon, N.B. Effects of a selective inhibitor of the Abl tyrosine kinase on

- the growth of Bcr–Abl positive cells. *Nat. Med.* **1996**, 2, 561–566.
58. Druker, B.J.; Talpaz, M.; Resta, D.J.; Peng, B.; Buchdunger, E.; Ford, J.M.; Lydon, N.B.; Kantarjian, H.; Capdeville, R.; Ohno-Jones, S. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *N. Engl. J. Med.* **2001**, 344, 1031–1037.
 59. Druker, B.J.; Guilhot, F.; O'Brien, S.G.; Gathmann, I.; Kantarjian, H.; Gattermann, N.; Deininger, M.W.N.; Silver, R.T.; Goldman, J.M.; Stone, R.M. Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia. *N. Engl. J. Med.* **2006**, 355, 2408–2417.
 60. Hochhaus, A.; O'Brien, S.G.; Guilhot, F.; Druker, B.J.; Branford, S.; Foroni, L.; Goldman, J.M.; Müller, M.C.; Radich, J.P.; Rudoltz, M. Six-year follow-up of patients receiving imatinib for the first-line treatment of chronic myeloid leukemia. *Leukemia* **2009**, 23, 1054–1061.
 61. Mahon, F.X.; Réa, D.; Guilhot, J.; Guilhot, F.; Huguet, F.; Nicolini, F.; Legros, L.; Charbonnier, A.; Guerci, A.; Varet, B.; et al. Discontinuation of imatinib in patients with chronic myeloid leukaemia who have maintained complete molecular remission for at least 2 years: the prospective, multicentre Stop Imatinib (STIM) trial. *Lancet Oncol.* **2010**, 11, 1029–1035, doi:10.1016/S1470-2045(10)70233-3.
 62. Saussele, S.; Richter, J.; Guilhot, J.; Gruber, F.X.; Hjorth-Hansen, H.; Almeida, A.; Janssen, J.J.W.M.; Mayer, J.; Koskenvesa, P.; Panayiotidis, P.; et al. Discontinuation of tyrosine kinase inhibitor therapy in chronic myeloid leukaemia (EURO-SKI): a prespecified interim analysis of a prospective, multicentre, non-randomised, trial. *Lancet Oncol.* **2018**, 19, 747–757, doi:10.1016/S1470-2045(18)30192-X.
 63. Jabbour, E.; Kantarjian, H.; Jones, D.; Talpaz, M.; Bekele, N.; O'Brien, S.; Zhou, X.; Luthra, R.; Garcia-Manero, G.; Giles, F. Frequency and clinical significance of BCR-ABL mutations in patients with chronic myeloid leukemia treated with imatinib mesylate. *Leukemia* **2006**, 20, 1767–1773.
 64. Shah, N.P.; Skaggs, B.J.; Branford, S.; Hughes, T.P.; Nicoll, J.M.; Paquette, R.L.; Sawyers, C.L. Sequential ABL kinase inhibitor therapy selects for compound drug-resistant BCR-ABL mutations with altered oncogenic potency. *J. Clin. Invest.* **2007**, 117, 2562–2569.
 65. Apperley, J.F. Part I: mechanisms of resistance to imatinib in chronic myeloid leukaemia. *Lancet Oncol.* **2007**, 8, 1018–1029.
 66. Gover-Proaktor, A.; Granot, G.; Pasmanik-Chor, M.; Pasvolsky, O.; Shapira, S.; Raz, O.; Raanani, P.; Leader, A. Bosutinib, dasatinib, imatinib, nilotinib, and ponatinib differentially affect the vascular molecular pathways and functionality of human endothelial cells. *Leuk. Lymphoma* **2019**, 60, 189–199.
 67. Corbin, A.S.; Agarwal, A.; Loriaux, M.; Cortes, J.; Deininger, M.W.; Druker, B.J. Human chronic myeloid leukemia stem cells are insensitive to imatinib despite inhibition of BCR-ABL activity. *J. Clin. Invest.* **2011**, 121, 396–409.
 68. Hamilton, A.; Helgason, G.V.; Schemionek, M.; Zhang, B.; Myssina, S.; Allan, E.K.;

- Nicolini, F.E.; Müller-Tidow, C.; Bhatia, R.; Brunton, V.G. Chronic myeloid leukemia stem cells are not dependent on Bcr-Abl kinase activity for their survival. *Blood, J. Am. Soc. Hematol.* **2012**, *119*, 1501–1510.
69. Zhang, B.; Chu, S.; Agarwal, P.; Campbell, V.L.; Hopcroft, L.; Jørgensen, H.G.; Lin, A.; Gaal, K.; Holyoake, T.L.; Bhatia, R. Inhibition of interleukin-1 signaling enhances elimination of tyrosine kinase inhibitor-treated CML stem cells. *Blood, J. Am. Soc. Hematol.* **2016**, *128*, 2671–2682.
 70. von Palffy, S.; Landberg, N.; Sandén, C.; Zacharaki, D.; Shah, M.; Nakamichi, N.; Hansen, N.; Askmyr, M.; Lilljebjörn, H.; Rissler, M. A high-content cytokine screen identifies myostatin propeptide as a positive regulator of primitive chronic myeloid leukemia cells. *Haematologica* **2020**, *105*, 2095.
 71. Prost, S.; Relouzat, F.; Spentchian, M.; Ouzegdouh, Y.; Saliba, J.; Massonnet, G.; Beressi, J.-P.; Verhoeven, E.; Raggueneau, V.; Maneglier, B. Erosion of the chronic myeloid leukaemia stem cell pool by PPAR γ agonists. *Nature* **2015**, *525*, 380–383.
 72. Nievergall, E.; Ramshaw, H.S.; Yong, A.S.M.; Biondo, M.; Busfield, S.J.; Vairo, G.; Lopez, A.F.; Hughes, T.P.; White, D.L.; Hiwase, D.K. Monoclonal antibody targeting of IL-3 receptor α with CSL362 effectively depletes CML progenitor and stem cells. *Blood, J. Am. Soc. Hematol.* **2014**, *123*, 1218–1228.
 73. Landberg, N.; von Palffy, S.; Askmyr, M.; Lilljebjörn, H.; Sandén, C.; Rissler, M.; Mustjoki, S.; Hjorth-Hansen, H.; Richter, J.; Ågerstam, H. CD36 defines primitive chronic myeloid leukemia cells less responsive to imatinib but vulnerable to antibody-based therapeutic targeting. *Haematologica* **2018**, *103*, 447–455.
 74. Hehlmann, R.; Lauseker, M.; Sauße, S.; Pfirrmann, M.; Krause, S.; Kolb, H.J.; Neubauer, A.; Hossfeld, D.K.; Nerl, C.; Gratwohl, A. Assessment of imatinib as first-line treatment of chronic myeloid leukemia: 10-year survival results of the randomized CML study IV and impact of non-CML determinants. *Leukemia* **2017**, *31*, 2398–2406.
 75. Saglio, G.; Kim, D.-W.; Issaragrisil, S.; Le Coutre, P.; Etienne, G.; Lobo, C.; Pasquini, R.; Clark, R.E.; Hochhaus, A.; Hughes, T.P. Nilotinib versus imatinib for newly diagnosed chronic myeloid leukemia. *N. Engl. J. Med.* **2010**, *362*, 2251–2259.
 76. Baccarani, M.; Deininger, M.W.; Rosti, G.; Hochhaus, A.; Soverini, S.; Apperley, J.F.; Cervantes, F.; Clark, R.E.; Cortes, J.E.; Guilhot, F. European LeukemiaNet recommendations for the management of chronic myeloid leukemia: 2013. *Blood* **2013**, *122*, 872–884.
 77. Steegmann, J.L.; Baccarani, M.; Breccia, M.; Casado, L.F.; García-Gutiérrez, V.; Hochhaus, A.; Kim, D.W.; Kim, T.D.; Khoury, H.J.; Le Coutre, P. European LeukemiaNet recommendations for the management and avoidance of adverse events of treatment in chronic myeloid leukaemia. *Leukemia* **2016**, *30*, 1648–1671.
 78. Aghel, N.; Delgado, D.H.; Lipton, J.H. Cardiovascular events in chronic myeloid leukemia clinical trials. Is it time to reassess and report the events according to cardiology guidelines? *Leukemia* **2018**, *32*, 2095–2104.

79. Ross, D.M.; Pagani, I.S.; Shanmuganathan, N.; Kok, C.H.; Seymour, J.F.; Mills, A.K.; Filshie, R.J.; Arthur, C.K.; Dang, P.; Saunders, V.A. Long-term treatment-free remission of chronic myeloid leukemia with falling levels of residual leukemic cells. *Leukemia* **2018**, *32*, 2572–2579.
80. Saussele, S.; Richter, J.; Hochhaus, A.; Mahon, F.X. The concept of treatment-free remission in chronic myeloid leukemia. *Leukemia* **2016**, *30*, 1638–1647.
81. Houshmand, M.; Simonetti, G.; Circosta, P.; Gaidano, V.; Cignetti, A.; Martinelli, G.; Saglio, G.; Gale, R.P. Chronic myeloid leukemia stem cells. *Leukemia* **2019**, *33*, 1543–1556.
82. Schepers, K.; Campbell, T.B.; Passegué, E. Normal and leukemic stem cell niches: insights and therapeutic opportunities. *Cell Stem Cell* **2015**, *16*, 254–267.
83. Holyoake, T.; Jiang, X.; Eaves, C.; Eaves, A. Isolation of a highly quiescent subpopulation of primitive leukemic cells in chronic myeloid leukemia. *Blood, J. Am. Soc. Hematol.* **1999**, *94*, 2056–2064.
84. Eisterer, W.; Jiang, X.; Christ, O.; Glimm, H.; Lee, K.H.; Pang, E.; Lambie, K.; Shaw, G.; Holyoake, T.L.; Petzer, A.L. Different subsets of primary chronic myeloid leukemia stem cells engraft immunodeficient mice and produce a model of the human disease. *Leukemia* **2005**, *19*, 435–441.
85. Järås, M.; Johnels, P.; Hansen, N.; Ågerstam, H.; Tsapogas, P.; Rissler, M.; Lassen, C.; Olofsson, T.; Bjerrum, O.W.; Richter, J. Isolation and killing of candidate chronic myeloid leukemia stem cells by antibody targeting of IL-1 receptor accessory protein. *Proc. Natl. Acad. Sci.* **2010**, *107*, 16280–16285.
86. Ågerstam, H.; Karlsson, C.; Hansen, N.; Sandén, C.; Askmyr, M.; von Palffy, S.; Högborg, C.; Rissler, M.; Wunderlich, M.; Juliusson, G. Antibodies targeting human IL1RAP (IL1R3) show therapeutic effects in xenograft models of acute myeloid leukemia. *Proc. Natl. Acad. Sci.* **2015**, *112*, 10786–10791.
87. Barreyro, L.; Will, B.; Bartholdy, B.; Zhou, L.; Todorova, T.I.; Stanley, R.F.; Ben-Neriah, S.; Montagna, C.; Parekh, S.; Pellagatti, A. Overexpression of IL-1 receptor accessory protein in stem and progenitor cells and outcome correlation in AML and MDS. *Blood, J. Am. Soc. Hematol.* **2012**, *120*, 1290–1298.
88. Warda, W.; Larosa, F.; Da Rocha, M.N.; Trad, R.; Deconinck, E.; Fajloun, Z.; Faure, C.; Caillot, D.; Moldovan, M.; Valmary-Degano, S. CML Hematopoietic Stem Cells Expressing IL1RAP Can Be Targeted by Chimeric Antigen Receptor–Engineered T Cells. *Cancer Res.* **2019**, *79*, 663–675.
89. Amu, S.; Brisslert, M. Phenotype and function of CD25-expressing B lymphocytes isolated from human umbilical cord blood. *Clin. Dev. Immunol.* **2011**, *2011*.
90. Goudy, K.; Aydin, D.; Barzaghi, F.; Gambineri, E.; Vignoli, M.; Mannurita, S.C.; Doglioni, C.; Ponzoni, M.; Cicalese, M.P.; Assanelli, A. Human IL2RA null mutation mediates immunodeficiency with lymphoproliferation and autoimmunity. *Clin. Immunol.* **2013**, *146*, 248–261.

91. Terwijn, M.; Feller, N.; van Rhenen, A.; Kelder, A.; Westra, G.; Zweegman, S.; Ossenkoppele, G.; Schuurhuis, G.J. Interleukin-2 receptor alpha-chain (CD25) expression on leukaemic blasts is predictive for outcome and level of residual disease in AML. *Eur. J. Cancer* **2009**, *45*, 1692–1699.
92. Miltiades, P.; Lamprianidou, E.; Vassilakopoulos, T.P.; Papageorgiou, S.G.; Galanopoulos, A.G.; Vakalopoulou, S.; Garypidou, V.; Papaioannou, M.; Hadjiharissi, E.; Pappa, V. Expression of CD25 antigen on CD34+ cells is an independent predictor of outcome in late-stage MDS patients treated with azacitidine. *Blood Cancer J.* **2014**, *4*, e187–e187.
93. Paietta, E.; Racevskis, J.; Neuberg, D.; Rowe, J.M.; Goldstone, A.H.; Wiernik, P.H. Expression of CD25 (interleukin-2 receptor α chain) in adult acute lymphoblastic leukemia predicts for the presence of BCR/ABL fusion transcripts: results of a preliminary laboratory analysis of ECOG/MRC Intergroup Study E2993. *Leuk.* **1997**, *11*.
94. Nakase, K.; Kita, K.; Miwa, H.; Nishii, K.; Shikami, M.; Tanaka, I.; Tsutani, H.; Ueda, T.; Nasu, K.; Kyo, T. Clinical and prognostic significance of cytokine receptor expression in adult acute lymphoblastic leukemia: interleukin-2 receptor α -chain predicts a poor prognosis. *Leukemia* **2007**, *21*, 326–332.
95. Herrmann, H.; Sadovnik, I.; Cerny-Reiterer, S.; Rülcke, T.; Stefanzl, G.; Willmann, M.; Hoermann, G.; Bilban, M.; Blatt, K.; Herndlhofer, S. Dipeptidylpeptidase IV (CD26) defines leukemic stem cells (LSC) in chronic myeloid leukemia. *Blood* **2014**, *123*, 3951–3962.
96. Campbell, T.B.; Broxmeyer, H.E. CD26 inhibition and hematopoiesis: a novel approach to enhance transplantation. *Front Biosci* **2008**, *13*, 1795–1805.
97. Morimoto, C.; Schlossman, S.F. The structure and function of CD26 in the T-cell immune response. *Immunol. Rev.* **1998**, *161*, 55–70.
98. Bocchia, M.; Sicuranza, A.; Abruzzese, E.; Iurlo, A.; Sirianni, S.; Gozzini, A.; Galimberti, S.; Aprile, L.; Martino, B.; Pregno, P. Residual peripheral blood CD26+ leukemic stem cells in chronic myeloid leukemia patients during TKI therapy and during treatment-free remission. *Front. Oncol.* **2018**, *8*, 194.
99. Warfvinge, R.; Geirons, L.; Sommarin, M.N.E.; Lang, S.; Karlsson, C.; Roschupkina, T.; Stenke, L.; Stentoft, J.; Olsson-Strömberg, U.; Hjorth-Hansen, H. Single-cell molecular analysis defines therapy response and immunophenotype of stem cell subpopulations in CML. *Blood* **2017**, *129*, 2384–2394.
100. Cro, L.; Morabito, F.; Zucal, N.; Fabris, S.; Lionetti, M.; Cutrona, G.; Rossi, F.; Gentile, M.; Ferrario, A.; Ferrarini, M. CD26 expression in mature B-cell neoplasia: its possible role as a new prognostic marker in B-CLL. *Hematol. Oncol.* **2009**, *27*, 140–147.
101. Inamoto, T.; Yamochi, T.; Ohnuma, K.; Iwata, S.; Kina, S.; Inamoto, S.; Tachibana, M.; Katsuoka, Y.; Dang, N.H.; Morimoto, C. Anti-CD26 Monoclonal Antibody-Mediated G1-S Arrest of Human Renal Clear Cell Carcinoma Caki-2 Is Associated with Retinoblastoma Substrate Dephosphorylation, Cyclin-Dependent Kinase 2 Reduction, p27kip1 Enhancement, and Disruption of Binding to the Extracellular Matrix. *Clin. cancer Res.* **2006**, *12*, 3470–3477.

102. Porwit, Anna, Jeffrey McCullough, and W.N.E. Blood and Bone Marrow Pathology. In *E-Book*; Elsevier Health Sciences, **2011**; p. Cap. 22.
103. Klcio, J.M.; Vij, R.; Kreisel, F.H.; Hassan, A.; Frater, J.L. Molecular pathology of myeloproliferative neoplasms. *Am. J. Clin. Pathol.* **2010**, *133*, 602–615.
104. Stein, B.L.; Moliterno, A.R.; Tiu, R. V Polycythemia vera disease burden: contributing factors, impact on quality of life, and emerging treatment options. *Ann. Hematol.* **2014**, *93*, 1965–1976.
105. Harrison, C.N.; Lee, J.S. Myeloproliferative neoplasms. *Medicine (Baltimore)*. **2017**, *45*, 275–279.
106. Mesa, R.A.; Li, C.-Y.; Ketterling, R.P.; Schroeder, G.S.; Knudson, R.A.; Tefferi, A. Leukemic transformation in myelofibrosis with myeloid metaplasia: a single-institution experience with 91 cases. *Blood* **2005**, *105*, 973–977.
107. Wilkins, B.S.; Erber, W.N.; Bareford, D.; Buck, G.; Wheatley, K.; East, C.L.; Paul, B.; Harrison, C.N.; Green, A.R.; Campbell, P.J. Bone marrow pathology in essential thrombocythemia: interobserver reliability and utility for identifying disease subtypes. *Blood, J. Am. Soc. Hematol.* **2008**, *111*, 60–70.
108. Sanchez, S.; Ewton, A. Essential thrombocythemia: a review of diagnostic and pathologic features. *Arch. Pathol. Lab. Med.* **2006**, *130*, 1144–1150.
109. Klampfl, T.; Gisslinger, H.; Harutyunyan, A.S.; Nivarthi, H.; Rumi, E.; Milosevic, J.D.; Them, N.C.C.; Berg, T.; Gisslinger, B.; Pietra, D. Somatic mutations of calreticulin in myeloproliferative neoplasms. *N. Engl. J. Med.* **2013**, *369*, 2379–2390.
110. Wolanskyj, A.P.; Schwager, S.M.; McClure, R.F.; Larson, D.R.; Tefferi, A. Essential thrombocythemia beyond the first decade: life expectancy, long-term complication rates, and prognostic factors. In *Proceedings of the Mayo Clinic Proceedings*; Elsevier, **2006**; Vol. 81, pp. 159–166.
111. Beer, P.A.; Erber, W.N.; Campbell, P.J.; Green, A.R. How I treat essential thrombocythemia. *Blood, J. Am. Soc. Hematol.* **2011**, *117*, 1472–1482.
112. Tefferi, A. Primary myelofibrosis: 2017 update on diagnosis, risk-stratification, and management. *Am. J. Hematol.* **2016**, *91*, 1262–1271.
113. Tefferi, A.; Guglielmelli, P.; Larson, D.R.; Finke, C.; Wassie, E.A.; Pieri, L.; Gangat, N.; Fjerza, R.; Belachew, A.A.; Lasho, T.L.; et al. Long-term survival and blast transformation in molecularly annotated essential thrombocythemia, polycythemia vera, and myelofibrosis. *Blood* **2014**, *124*, 2507–2513, doi:10.1182/blood-2014-05-579136.
114. Cervantes, F.; Dupriez, B.; Passamonti, F.; Vannucchi, A.M.; Morra, E.; Reilly, J.T.; Demory, J.-L.; Rumi, E.; Guglielmelli, P.; Roncoroni, E. Improving survival trends in primary myelofibrosis: an international study. *J. Clin. Oncol.* **2012**, *30*, 2981–2987.
115. Quintás-Cardama, A.; Kantarjian, H.; Pierce, S.; Cortes, J.; Verstovsek, S. Prognostic model to identify patients with myelofibrosis at the highest risk of transformation to acute myeloid leukemia. *Clin. Lymphoma Myeloma Leuk.* **2013**, *13*, 315–318.

116. Khwaja, A.; Bjorkholm, M.; Gale, R.E.; Levine, R.L.; Jordan, C.T.; Ehninger, G.; Bloomfield, C.D.; Estey, E.; Burnett, A.; Cornelissen, J.J. Acute myeloid leukaemia. *Nat. Rev. Dis. Prim.* **2016**, *2*, 1–22.
117. Juskevicius, R.; Thompson, M.A.; Shaver, A.; Head, D. Clinical Presentation, Diagnosis, and Classification of Acute Myeloid Leukemia. In; Springer, Cham, **2021**; pp. 11–55.
118. Sill, H.; Olipitz, W.; Zebisch, A.; Schulz, E.; Wölfler, A. Therapy-related myeloid neoplasms: Pathobiology and clinical characteristics. *Br. J. Pharmacol.* **2011**, *162*, 792–805.
119. Takahashi, S. Current findings for recurring mutations in acute myeloid leukemia. *J. Hematol. Oncol.* **2011**, *4*, 36.
120. Kelly, L.M.; Gilliland, D.G. Genetics of myeloid leukemias. *Annu. Rev. Genomics Hum. Genet.* **2002**, *3*, 179–198.
121. Panuzzo, C.; Signorino, E.; Calabrese, C.; Ali, M.S.; Petiti, J.; Bracco, E.; Cilloni, D. Landscape of Tumor Suppressor Mutations in Acute Myeloid Leukemia. *J. Clin. Med.* **2020**, *9*, 802, doi:10.3390/jcm9030802.
122. Szotkowski, T.; Rohon, P.; Zapletalová, J.; Sicova, K.; Hubacek, J.; Indrak, K. Secondary acute myeloid leukemia—a single center experience. *Neoplasma* **2010**, *57*, 170.
123. Shallis, R.M.; Wang, R.; Davidoff, A.; Ma, X.; Zeidan, A.M. Epidemiology of acute myeloid leukemia: Recent progress and enduring challenges. *Blood Rev.* **2019**, *36*, 70–87.
124. Deschler, B.; Lübbert, M. Acute myeloid leukemia: epidemiology and etiology. In *Acute Leukemias*; Springer, **2008**; pp. 47–56.
125. Albiero, E.; Madeo, D.; Bolli, N.; Giaretta, I.; Di Bona, E.; Martelli, M.F.; Nicoletti, I.; Rodeghiero, F.; Falini, B. Identification and functional characterization of a cytoplasmic nucleophosmin leukaemic mutant generated by a novel exon-11 NPM1 mutation. *Leukemia* **2007**, *21*, 1099–1103.
126. Grummitt, C.G.; Townsley, F.M.; Johnson, C.M.; Warren, A.J.; Bycroft, M. Structural consequences of nucleophosmin mutations in acute myeloid leukemia. *J. Biol. Chem.* **2008**, *283*, 23326–23332.
127. Yokota, S.; Kiyoi, H.; Nakao, M.; Iwai, T.; Misawa, S.; Okuda, T.; Sonoda, Y.; Abe, T.; Kahsima, K.; Matsuo, Y. Internal tandem duplication of the FLT3 gene is preferentially seen in acute myeloid leukemia and myelodysplastic syndrome among various hematological malignancies. A study on a large series of patients and cell lines. *Leukemia* **1997**, *11*, 1605–1609.
128. Iwai, T.; Yokota, S.; Nakao, M.; Okamoto, T.; Taniwaki, M.; Onodera, N.; Watanabe, A.; Kikuta, A.; Tanaka, A.; Asami, K. Internal tandem duplication of the FLT3 gene and clinical evaluation in childhood acute myeloid leukemia. *Leukemia* **1999**, *13*, 38–43.
129. Abu-Duhier, F.M.; Goodeve, A.C.; Wilson, G.A.; Care, R.S.; Peake, I.R.; Reilly, J.T. Identification of novel FLT-3 Asp835 mutations in adult acute myeloid leukaemia. *Br. J. Haematol.* **2001**, *113*, 983–988.

130. Yamamoto, Y.; Kiyoi, H.; Nakano, Y.; Suzuki, R.; Kodera, Y.; Miyawaki, S.; Asou, N.; Kuriyama, K.; Yagasaki, F.; Shimazaki, C. Activating mutation of D835 within the activation loop of FLT3 in human hematologic malignancies. *Blood, J. Am. Soc. Hematol.* **2001**, *97*, 2434–2439.
131. ERG, E. Incidence and prognostic influence of DNMT3A mutations in acute myeloid leukemia. *J Clin Oncol* **2011**, *29*, 2889–2896.
132. Challen, G.A.; Sun, D.; Jeong, M.; Luo, M.; Jelinek, J.; Berg, J.S.; Bock, C.; Vasanthakumar, A.; Gu, H.; Xi, Y. Dnmt3a is essential for hematopoietic stem cell differentiation. *Nat. Genet.* **2012**, *44*, 23–31.
133. Ward, P.S.; Patel, J.; Wise, D.R.; Abdel-Wahab, O.; Bennett, B.D.; Collier, H.A.; Cross, J.R.; Fantin, V.R.; Hedvat, C. V; Perl, A.E. The common feature of leukemia-associated IDH1 and IDH2 mutations is a neomorphic enzyme activity converting α -ketoglutarate to 2-hydroxyglutarate. *Cancer Cell* **2010**, *17*, 225–234.
134. Patel, J.L.; Schumacher, J.A.; Frizzell, K.; Sorrells, S.; Shen, W.; Clayton, A.; Jattani, R.; Kelley, T.W. Coexisting and cooperating mutations in NPM1-mutated acute myeloid leukemia. *Leuk. Res.* **2017**, *56*, 7–12.
135. Irish, J.M.; Ånensen, N.; Hovland, R.; Skavland, J.; Børresen-Dale, A.-L.; Bruserud, Ø.; Nolan, G.P.; Gjertsen, B.T. Flt3 Y591 duplication and Bcl-2 overexpression are detected in acute myeloid leukemia cells with high levels of phosphorylated wild-type p53. *Blood* **2007**, *109*, 2589–2596.
136. Kumar, C.C. Genetic abnormalities and challenges in the treatment of acute myeloid leukemia. *Genes Cancer* **2011**, *2*, 95–107.
137. Döhner, H.; Estey, E.; Grimwade, D.; Amadori, S.; Appelbaum, F.R.; Büchner, T.; Dombret, H.; Ebert, B.L.; Fenaux, P.; Larson, R.A. Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. *Blood* **2017**, *129*, 424–447.
138. Döhner, H.; Estey, E.H.; Amadori, S.; Appelbaum, F.R.; Büchner, T.; Burnett, A.K.; Dombret, H.; Fenaux, P.; Grimwade, D.; Larson, R.A. Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. *Blood* **2010**, *115*, 453–474.
139. Vardiman, J.W.; Harris, N.L.; Brunning, R.D. The World Health Organization (WHO) classification of the myeloid neoplasms. *Blood* **2002**, *100*, 2292–2302.
140. Bennett, J.M.; Catovsky, D.; Daniel, M.; Flandrin, G.; Galton, D.A.G.; Gralnick, H.R.; Sultan, C. Proposals for the classification of the acute leukaemias French-American-British (FAB) co-operative group. *Br. J. Haematol.* **1976**, *33*, 451–458.
141. Walter, R.B.; Othus, M.; Burnett, A.K.; Löwenberg, B.; Kantarjian, H.M.; Ossenkoppele, G.J.; Hills, R.K.; Van Montfort, K.G.M.; Ravandi, F.; Evans, A. Significance of FAB subclassification of “acute myeloid leukemia, NOS” in the 2008 WHO classification: analysis of 5848 newly diagnosed patients. *Blood* **2013**, *121*, 2424–2431.
142. Rowley, J.; Golomb, H.; Dougherty, C. 15/17 translocation, a consistent chromosomal

- change in acute promyelocytic leukaemia. *Lancet (London, England)* **1977**, *1*, 549–550.
143. Lo-Coco, F.; Avvisati, G.; Vignetti, M.; Thiede, C.; Orlando, S.M.; Iacobelli, S.; Ferrara, F.; Fazi, P.; Cicconi, L.; Di Bona, E. Retinoic acid and arsenic trioxide for acute promyelocytic leukemia. *N Engl J Med* **2013**, *369*, 111–121.
 144. Pollyea, D.A.; Gutman, J.A.; Gore, L.; Smith, C.A.; Jordan, C.T. Targeting acute myeloid leukemia stem cells: a review and principles for the development of clinical trials. *Haematologica* **2014**, *99*, 1277–1284.
 145. Gong, Q.; Zhou, L.; Xu, S.; Li, X.; Zou, Y.; Chen, J. High doses of daunorubicin during induction therapy of newly diagnosed acute myeloid leukemia: a systematic review and meta-analysis of prospective clinical trials. *PLoS One* **2015**, *10*, e0125612.
 146. Li, X.; Xu, S.; Tan, Y.; Chen, J. The effects of idarubicin versus other anthracyclines for induction therapy of patients with newly diagnosed leukaemia. *Cochrane Database Syst. Rev.* **2015**.
 147. Schuurhuis, G.J.; Heuser, M.; Freeman, S.; Béné, M.-C.; Buccisano, F.; Cloos, J.; Grimwade, D.; Haferlach, T.; Hills, R.K.; Hourigan, C.S. Minimal/measurable residual disease in AML: a consensus document from the European LeukemiaNet MRD Working Party. *Blood* **2018**, *131*, 1275–1291.
 148. Visani, G.; Olivieri, A.; Malagola, M.; Brunori, M.; Piccaluga, P.P.; Capelli, D.; Pomponio, G.; Martinelli, G.; Isidori, A.; Sparaventi, G. Consolidation therapy for adult acute myeloid leukemia: a systematic analysis according to evidence based medicine. *Leuk. Lymphoma* **2006**, *47*, 1091–1102.
 149. Gooley, T.A.; Chien, J.W.; Pergam, S.A.; Hingorani, S.; Sorrow, M.L.; Boeckh, M.; Martin, P.J.; Sandmaier, B.M.; Marr, K.A.; Appelbaum, F.R. Reduced mortality after allogeneic hematopoietic-cell transplantation. *N. Engl. J. Med.* **2010**, *363*, 2091–2101.
 150. Gupta, V.; Tallman, M.S.; Weisdorf, D.J. Allogeneic hematopoietic cell transplantation for adults with acute myeloid leukemia: myths, controversies, and unknowns. *Blood, J. Am. Soc. Hematol.* **2011**, *117*, 2307–2318.
 151. Fiorentini, A.; Capelli, D.; Saraceni, F.; Menotti, D.; Poloni, A.; Olivieri, A. The time has come for targeted therapies for AML: lights and shadows. *Oncol. Ther.* **2020**, 1–20.
 152. Nielsen, C.P.; MacGurn, J.A. Coupling Conjugation and Deconjugation Activities to Achieve Cellular Ubiquitin Dynamics. *Trends Biochem. Sci.* **2020**, *45*, 427–439.
 153. Pergolizzi, B.; Bozzaro, S.; Bracco, E. Dictyostelium as model for studying ubiquitination and deubiquitination. *Int. J. Dev. Biol.* **2019**, *63*, 529–539, doi:10.1387/ijdb.190260eb.
 154. Deol, K.K.; Lorenz, S.; Strieter, E.R. Enzymatic logic of ubiquitin chain assembly. *Front. Physiol.* **2019**, *10*, 835.
 155. Zheng, N.; Shabek, N. Ubiquitin Ligases: Structure, Function, and Regulation. *Annu. Rev. Biochem.* **2017**, *86*, 129–157, doi:10.1146/annurev-biochem-060815-014922.
 156. Wang, D.; Ma, L.; Wang, B.; Liu, J.; Wei, W. E3 ubiquitin ligases in cancer and implications

- for therapies. *Cancer Metastasis Rev.* **2017**, 36, 683–702.
157. Kravtsova-Ivantsiv, Y.; Ciechanover, A. Non-canonical ubiquitin-based signals for proteasomal degradation. *J. Cell Sci.* **2012**, 125, 539–548.
 158. Yau, R.; Rape, M. The increasing complexity of the ubiquitin code. *Nat. Cell Biol.* **2016**, 18, 579–586.
 159. Komander, D.; Rape, M. The ubiquitin code. *Annu. Rev. Biochem.* **2012**, 81, 203–229.
 160. Liu, P.; Gan, W.; Su, S.; Hauenstein, A. V.; Fu, T.; Brasher, B.; Schwerdtfeger, C.; Liang, A.C.; Xu, M.; Wei, W. K63-linked polyubiquitin chains bind to DNA to facilitate DNA damage repair. *Sci. Signal.* **2018**, 11, eaar8133.
 161. Iwai, K.; Tokunaga, F. Linear polyubiquitination: a new regulator of NF- κ B activation. *EMBO Rep.* **2009**, 10, 706–713.
 162. Koyano, F.; Okatsu, K.; Kosako, H.; Tamura, Y.; Go, E.; Kimura, M.; Kimura, Y.; Tsuchiya, H.; Yoshihara, H.; Hirokawa, T. Ubiquitin is phosphorylated by PINK1 to activate parkin. *Nature* **2014**, 510, 162–166.
 163. Ohtake, F.; Saeki, Y.; Sakamoto, K.; Ohtake, K.; Nishikawa, H.; Tsuchiya, H.; Ohta, T.; Tanaka, K.; Kanno, J. Ubiquitin acetylation inhibits polyubiquitin chain elongation. *EMBO Rep.* **2015**, 16, 192–201.
 164. Clague, M.J.; Barsukov, I.; Coulson, J.M.; Liu, H.; Rigden, D.J.; Urbé, S. Deubiquitylases from genes to organism. *Physiol. Rev.* **2013**, 93, 1289–1315.
 165. Groothuis, T.A.M.; Dantuma, N.P.; Neefjes, J.; Salomons, F.A. Ubiquitin crosstalk connecting cellular processes. *Cell Div.* **2006**, 1, 21.
 166. Suresh, B.; Lee, J.; Kim, H.; Ramakrishna, S. Regulation of pluripotency and differentiation by deubiquitinating enzymes. *Cell Death Differ.* **2016**, 23, 1257–1264.
 167. Chaugule, V.K.; Walden, H. Specificity and disease in the ubiquitin system. *Biochem. Soc. Trans.* **2016**, 44, 212–227, doi:10.1042/BST20150209.
 168. Fajner, V.; Maspero, E.; Polo, S. Targeting HECT-type E3 ligases—insights from catalysis, regulation and inhibitors. *FEBS Lett.* **2017**, 591, 2636–2647.
 169. Marteiijn, J.A.F.; Jansen, J.H.; van der Reijden, B.A. Ubiquitylation in normal and malignant hematopoiesis: Novel therapeutic targets. *Leukemia* **2006**, 20, 1511–1518.
 170. Bueso-Ramos, C.E.; Yang, Y.; DeLeon, E.; McCown, P.; Stass, S.A.; Albitar, M. The human MDM-2 oncogene is overexpressed in leukemias. **1993**.
 171. Caligiuri, M.A.; Briesewitz, R.; Yu, J.; Wang, L.; Wei, M.; Arnoczky, K.J.; Marburger, T.B.; Wen, J.; Perrotti, D.; Bloomfield, C.D. Novel c-CBL and CBL-b ubiquitin ligase mutations in human acute myeloid leukemia. *Blood, J. Am. Soc. Hematol.* **2007**, 110, 1022–1024.
 172. Sala-Gaston, J.; Martinez-Martinez, A.; Pedrazza, L.; Lorenzo-Martín, L.F.; Caloto, R.; Bustelo, X.R.; Ventura, F.; Rosa, J.L. Herc ubiquitin ligases in cancer. *Cancers (Basel)*.

- 2020, 12, 1–13.
173. Garcia-Gonzalo, F.R.; Rosa, J.L. The HERC proteins: functional and evolutionary insights. *Cell. Mol. Life Sci.* **2005**, 62, 1826–1838, doi:10.1007/s00018-005-5119-y.
 174. Sánchez-Tena, S.; Cubillos-Rojas, M.; Schneider, T.; Rosa, J.L. Functional and pathological relevance of HERC family proteins: A decade later. *Cell. Mol. Life Sci.* **2016**, 73, 1955–1968.
 175. Marín, I. Animal HECT ubiquitin ligases: evolution and functional implications. *BMC Evol. Biol.* **2010**, 10, 56.
 176. García-Cano, J.; Martínez-Martínez, A.; Sala-Gaston, J.; Pedrazza, L.; Rosa, J.L. HERCing: structural and functional relevance of the large HERC ubiquitin ligases. *Front. Physiol.* **2019**, 10, 1014.
 177. Rosa, J.L.; Casaroli-Marano, R.P.; Buckler, A.J.; Vilaro, S.; Barbacid, M. p619, a giant protein related to the chromosome condensation regulator RCC1, stimulates guanine nucleotide exchange on ARF1 and Rab proteins. *EMBO J.* **1996**, 15, 4262–4273.
 178. Garcia-Gonzalo, F.R.; Muñoz, P.; González, E.; Casaroli-Marano, R.P.; Vilaró, S.; Bartrons, R.; Ventura, F.; Rosa, J.L. The giant protein HERC1 is recruited to aluminum fluoride-induced actin-rich surface protrusions in HeLa cells. *FEBS Lett.* **2004**, 559, 77–83, doi:10.1016/S0014-5793(04)00030-4.
 179. Rosa, J.L.; Barbacid, M. A giant protein that stimulates guanine nucleotide exchange on ARF1 and Rab proteins forms a cytosolic ternary complex with clathrin and Hsp70. *Oncogene* **1997**, 15, 1–6, doi:10.1038/sj.onc.1201170.
 180. Hadjebi, O.; Casas-Terradellas, E.; Garcia-Gonzalo, F.R.; Rosa, J.L. The RCC1 superfamily: from genes, to function, to disease. *Biochim. Biophys. Acta (BBA)-Molecular Cell Res.* **2008**, 1783, 1467–1479.
 181. Wang, Y.; Argiles-Castillo, D.; Kane, E.I.; Zhou, A.; Spratt, D.E. HECT E3 ubiquitin ligases - Emerging insights into their biological roles and disease relevance. *J. Cell Sci.* **2020**, 133.
 182. Holloway, A.; Simmonds, M.; Azad, A.; Fox, J.L.; Storey, A. Resistance to UV-induced apoptosis by β -HPV5 E6 involves targeting of activated BAK for proteolysis by recruitment of the HERC1 ubiquitin ligase. *Int. J. Cancer* **2015**, 136, 2831–2843, doi:10.1002/ijc.29350.
 183. Garcia-Gonzalo, F.R.; Cruz, C.; Muñoz, P.; Mazurek, S.; Eigenbrodt, E.; Ventura, F.; Bartrons, R.; Rosa, J.L. Interaction between HERC1 and M2-type pyruvate kinase. *FEBS Lett.* **2003**, 539, 78–84.
 184. Feng, S.; Chen, J.K.; Yu, H.; Simon, J.A.; Schreiber, S.L. Two binding orientations for peptides to the Src SH3 domain: development of a general model for SH3-ligand interactions. *Science (80-.).* **1994**, 266, 1241–1247.
 185. Yuasa, I.; Umetsu, K.; Nishimukai, H.; Fukumori, Y.; Harihara, S.; Saitou, N.; Jin, F.; Chattopadhyay, P.K.; Henke, L.; Henke, J. HERC1 polymorphisms: population-specific variations in haplotype composition. *Cell Biochem. Funct.* **2009**, 27, 402–405,

- doi:10.1002/cbf.1582.
186. Mashimo, T.; Hadjebi, O.; Amair-Pinedo, F.; Tsurumi, T.; Langa, F.; Serikawa, T.; Sotelo, C.; Guénet, J.-L.; Rosa, J.L. Progressive Purkinje Cell Degeneration in tambaleante Mutant Mice Is a Consequence of a Missense Mutation in HERC1 E3 Ubiquitin Ligase. *PLoS Genet.* **2009**, *5*, e1000784, doi:10.1371/journal.pgen.1000784.
 187. Ruiz, R.; Pérez-Villegas, E.M.; Bachiller, S.; Rosa, J.L.; Armengol, J.A. HERC 1 Ubiquitin Ligase Mutation Affects Neocortical, CA3 Hippocampal and Spinal Cord Projection Neurons: An Ultrastructural Study. *Front. Neuroanat.* **2016**, *10*, 42, doi:10.3389/fnana.2016.00042.
 188. Pérez-Villegas, E.M.; Negrete-Díaz, J. V.; Porras-García, M.E.; Ruiz, R.; Carrión, A.M.; Rodríguez-Moreno, A.; Armengol, J.A. Mutation of the HERC 1 Ubiquitin Ligase Impairs Associative Learning in the Lateral Amygdala. *Mol. Neurobiol.* **2018**, *55*, 1157–1168, doi:10.1007/s12035-016-0371-8.
 189. Chong-Kopera, H.; Inoki, K.; Li, Y.; Zhu, T.; Garcia-Gonzalo, F.R.; Rosa, J.L.; Guan, K.L. TSC1 stabilizes TSC2 by inhibiting the interaction between TSC2 and the HERC1 ubiquitin ligase. *J. Biol. Chem.* **2006**, *281*, 8313–8316, doi:10.1074/jbc.C500451200.
 190. Ortega-Recalde, O.; Beltrán, O.I.; Gálvez, J.M.; Palma-Montero, A.; Restrepo, C.M.; Mateus, H.E.; Laissue, P. Biallelic *HERC1* mutations in a syndromic form of overgrowth and intellectual disability. *Clin. Genet.* **2015**, *88*, e1–e3, doi:10.1111/cge.12634.
 191. Aggarwal, S.; Bhowmik, A. Das; Ramprasad, V.L.; Murugan, S.; Dalal, A. A splice site mutation in *HERC1* leads to syndromic intellectual disability with macrocephaly and facial dysmorphism: Further delineation of the phenotypic spectrum. *Am. J. Med. Genet. Part A* **2016**, *170*, 1868–1873, doi:10.1002/ajmg.a.37654.
 192. Voight, B.F.; Kudaravalli, S.; Wen, X.; Pritchard, J.K. A Map of Recent Positive Selection in the Human Genome. *PLoS Biol.* **2006**, *4*, e72, doi:10.1371/journal.pbio.0040072.
 193. Craig, D.W.; O’Shaughnessy, J.A.; Kiefer, J.A.; Aldrich, J.; Sinari, S.; Moses, T.M.; Wong, S.; Dinh, J.; Christoforides, A.; Blum, J.L.; et al. Genome and transcriptome sequencing in prospective metastatic triple-negative breast cancer uncovers therapeutic vulnerabilities. *Mol. Cancer Ther.* **2013**, *12*, 104–116, doi:10.1158/1535-7163.MCT-12-0781.
 194. Choi, J.; Busino, L. E3 ubiquitin ligases in B-cell malignancies. *Cell. Immunol.* **2019**, *340*, 103905.
 195. Diouf, B.; Cheng, Q.; Krynetskaia, N.F.; Yang, W.; Cheok, M.; Pei, D.; Fan, Y.; Cheng, C.; Krynetskiy, E.Y.; Geng, H.; et al. Somatic deletions of genes regulating MSH2 protein stability cause DNA mismatch repair deficiency and drug resistance in human leukemia cells. *Nat. Med.* **2011**, *17*, 1298–1303, doi:10.1038/nm.2430.
 196. Walz, C.; Grimwade, D.; Saussele, S.; Lengfelder, E.; Haferlach, C.; Schnittger, S.; Lafage-Pochitaloff, M.; Hochhaus, A.; Cross, N.C.P.; Reiter, A. Atypical mRNA fusions in *PML-RARA* positive, *RARA-PML* negative acute promyelocytic leukemia. *Genes, Chromosom. Cancer* **2010**, *49*, NA-NA, doi:10.1002/gcc.20757.
 197. Noble, C.; Mercer, K.; Hussain, J.; Carragher, L.; Giblett, S.; Hayward, R.; Patterson, C.;

- Marais, R.; Pritchard, C.A. CRAF Autophosphorylation of Serine 621 Is Required to Prevent Its Proteasome-Mediated Degradation. *Mol. Cell* **2008**, *31*, 862–872, doi:10.1016/j.molcel.2008.08.026.
198. Schneider, T.; Martinez-Martinez, A.; Cubillos-Rojas, M.; Bartrons, R.; Ventura, F.; Rosa, J.L. The E3 ubiquitin ligase HERC1 controls the ERK signaling pathway targeting C-RAF for degradation. *Oncotarget* **2018**, *9*, 31531–31548, doi:10.18632/oncotarget.25847.
 199. Rothman, J.E. Mechanisms of intracellular protein transport. *Nature* **1994**, *372*, 55–63.
 200. Nie, Z.; Hirsch, D.S.; Randazzo, P.A. Arf and its many interactors. *Curr. Opin. Cell Biol.* **2003**, *15*, 396–404.
 201. Pfeffer, S.R. Rab GTPases: master regulators of membrane trafficking. *Curr. Opin. Cell Biol.* **1994**, *6*, 522–526, doi:10.1016/0955-0674(94)90071-X.
 202. Garcia-Gonzalo, F.R.; Bartrons, R.; Ventura, F.; Rosa, J.L. Requirement of phosphatidylinositol-4, 5-bisphosphate for HERC1-mediated guanine nucleotide release from ARF proteins. *FEBS Lett.* **2005**, *579*, 343–348.
 203. Skippen, A.; Jones, D.H.; Morgan, C.P.; Li, M.; Cockcroft, S. Mechanism of ADP ribosylation factor-stimulated phosphatidylinositol 4,5-bisphosphate synthesis in HL60 cells. *J. Biol. Chem.* **2002**, *277*, 5823–5831, doi:10.1074/jbc.M110274200.
 204. Honda, A.; Nogami, M.; Yokozeki, T.; Yamazaki, M.; Nakamura, H.; Watanabe, H.; Kawamoto, K.; Nakayama, K.; Morris, A.J.; Frohman, M.A.; et al. Phosphatidylinositol 4-phosphate 5-kinase α is a downstream effector of the small G protein ARF6 in membrane ruffle formation. *Cell* **1999**, *99*, 521–532, doi:10.1016/S0092-8674(00)81540-8.
 205. Jacquemet, G.; Humphries, M.J. IQGAP1 is a key node within the small GTPase network. *Small GTPases* **2013**, *4*, 199–207, doi:10.4161/sgtp.27451.
 206. Pedrazza, L.; Schneider, T.; Bartrons, R.; Ventura, F.; Rosa, J.L. The ubiquitin ligase HERC1 regulates cell migration via RAF-dependent regulation of MKK3/p38 signaling. *Sci. Rep.* **2020**, *10*, 1–14, doi:10.1038/s41598-020-57756-7.
 207. Schaller, M.D. Paxillin: A focal adhesion-associated adaptor protein. *Oncogene* **2001**, *20*, 6459–6472.
 208. Galligan, J.T.; Martinez-Noël, G.; Arndt, V.; Hayes, S.; Chittenden, T.W.; Harper, J.W.; Howley, P.M. Proteomic analysis and identification of cellular interactors of the giant ubiquitin ligase HERC2. *J. Proteome Res.* **2015**, *14*, 953–966, doi:10.1021/pr501005v.
 209. Millius, A.; Weiner, O.D. Manipulation of neutrophil-like HL-60 cells for the study of directed cell migration. In *Live Cell Imaging*; Springer, **2010**; pp. 147–158.
 210. Collins, S.J.; Ruscetti, F.W.; Gallagher, R.E.; Gallo, R.C. Terminal differentiation of human promyelocytic leukemia cells induced by dimethyl sulfoxide and other polar compounds. *Proc. Natl. Acad. Sci.* **1978**, *75*, 2458–2462.
 211. Kitanaka, A.; Waki, M.; Kamano, H.; Tanaka, T.; Kubota, Y.; Ohnishi, H.; Takahara, J.; Irino, S. Antisense src expression inhibits proliferation and erythropoietin-induced

- erythroid differentiation of K562 human leukemia cells. *Biochem. Biophys. Res. Commun.* **1994**, *201*, 1534–1540.
212. Kong, X.-Z.; Yin, R.-H.; Ning, H.-M.; Zheng, W.-W.; Dong, X.-M.; Yang, Y.; Xu, F.-F.; Li, J.-J.; Zhan, Y.-Q.; Yu, M. Effects of THAP11 on erythroid differentiation and megakaryocytic differentiation of K562 cells. *PLoS One* **2014**, *9*, e91557.
 213. Neumann, M.; Vosberg, S.; Schlee, C.; Heesch, S.; Schwartz, S.; Gökbuget, N.; Hoelzer, D.; Graf, A.; Krebs, S.; Bartram, I.; et al. Mutational spectrum of adult T-ALL. *Oncotarget* **2015**, *6*, 2754–2766, doi:10.18632/oncotarget.2218.
 214. Johansson, P.; Klein-Hitpass, L.; Choidas, A.; Habenberger, P.; Mahboubi, B.; Kim, B.; Bergmann, A.; Scholtysik, R.; Brauser, M.; Lollies, A.; et al. SAMHD1 is recurrently mutated in T-cell prolymphocytic leukemia. *Blood Cancer J.* **2018**, *8*, doi:10.1038/s41408-017-0036-5.
 215. Döhner, H.; Weisdorf, D.J.; Bloomfield, C.D. Acute Myeloid Leukemia. *N. Engl. J. Med.* **2015**, *373*, 1136–1152, doi:10.1056/NEJMra1406184.
 216. Spivak, J.L. Myeloproliferative neoplasms. *N. Engl. J. Med.* **2017**, *376*, 2168–2181.
 217. Ren, R. Mechanisms of BCR-ABL in the pathogenesis of chronic myelogenous leukaemia. *Nat. Rev. Cancer* **2005**, *5*, 172–183.
 218. Preisinger, C.; Schwarz, J.P.; Bleijerveld, O.B.; Corradini, E.; Müller, P.J.; Anderson, K.I.; Kolch, W.; Scholten, A.; Heck, A.J.R. Imatinib-dependent tyrosine phosphorylation profiling of Bcr-Abl-positive chronic myeloid leukemia cells. *Leukemia* **2013**, *27*, 743–746.
 219. Hoffbrand, A.V.; Steensma, D.P. *Hoffbrand's essential haematology*; John Wiley & Sons, **2019**; ISBN 1119495903.
 220. Jacquél, A.; Herrant, M.; Legros, L.; Belhacene, N.; Luciano, F.; Pages, G.; Hofman, P.; Auberger, P. Imatinib induces mitochondria-dependent apoptosis of the Bcr-Abl-positive K562 cell line and its differentiation toward the erythroid lineage. *FASEB J.* **2003**, *17*, 2160–2162.
 221. Ponting, C.; Schultz, J.; Bork, P. SPRY domains in ryanodine receptors (Ca (2+)-release channels). *Trends Biochem. Sci.* **1997**, *22*, 193–194.
 222. Neer, E.J.; Schmidt, C.J.; Nambudripad, R.; Smith, T.F. The ancient regulatory-protein family of WD-repeat proteins. *Nature* **1994**, *371*, 297–300.
 223. Pergolizzi, B.; Bracco, E.; Bozzaro, S. A new HECT ubiquitin ligase regulating chemotaxis and development in Dictyostelium discoideum. *J. Cell Sci.* **2017**, *130*, 551–562, doi:10.1242/jcs.194225.
 224. Park, C.W.; Ryu, K.Y. Cellular ubiquitin pool dynamics and homeostasis. *BMB Rep.* **2014**, *47*, 475–482.
 225. Bernassola, F.; Chillemi, G.; Melino, G. HECT-Type E3 Ubiquitin Ligases in Cancer. *Trends Biochem. Sci.* **2019**, *44*, 1057–1075.
 226. Senft, D.; Qi, J.; Ronai, A. Ubiquitin ligases in oncogenic transformation and cancer

- therapy. *Nat. Publ. Gr.* **2017**, *18*, doi:10.1038/nrc.2017.105.
227. Popovic, D.; Vucic, D.; Dikic, I. Ubiquitination in disease pathogenesis and treatment. *Nat. Med.* **2014**, *20*, 1242–1253.
 228. Weber, J.; Polo, S.; Maspero, E. HECT E3 ligases: a tale with multiple facets. *Front. Physiol.* **2019**, *10*, 370.
 229. Molenaar, R.J.; Maciejewski, J.P.; Wilmink, J.W.; Van Noorden, C.J.F. Wild-type and mutated IDH1/2 enzymes and therapy responses. *Oncogene* **2018**, *37*, 1949–1960.
 230. Uvarov, A. V.; Mesaeli, N. Enhanced ubiquitin-proteasome activity in calreticulin deficient cells: A compensatory mechanism for cell survival. *Biochim. Biophys. Acta - Mol. Cell Res.* **2008**, *1783*, 1237–1247, doi:10.1016/j.bbamcr.2008.03.004.
 231. Deegan, S.; Koryga, I.; Glynn, S.A.; Gupta, S.; Gorman, A.M.; Samali, A. A close connection between the PERK and IRE arms of the UPR and the transcriptional regulation of autophagy. *Biochem. Biophys. Res. Commun.* **2015**, *456*, 305–311, doi:10.1016/j.bbrc.2014.11.076.
 232. Reckel, S.; Hamelin, R.; Georgeon, S.; Armand, F.; Jolliet, Q.; Chiappe, D.; Moniatte, M.; Hantschel, O. Differential signaling networks of Bcr-Abl p210 and p190 kinases in leukemia cells defined by functional proteomics. *Leukemia* **2017**, *31*, 1502–1512, doi:10.1038/leu.2017.36.
 233. Antonenko, S. V.; Telegeev, G.D. Inhibition of USP1, a new partner of Bcr-Abl, results in decrease of Bcr-Abl level in K562 cells. *Exp. Oncol.* **2020**, *42*, 109–114, doi:10.32471/exp-oncology.2312-8852.vol-42-no-2.14533.
 234. Mao, J.H.; Sun, X.Y.; Liu, J.X.; Zhang, Q.Y.; Liu, P.; Huang, Q.H.; Li, K.K.; Chen, Q.; Chen, Z.; Chen, S.J. As4S4 targets RING-type E3 ligase c-CBL to induce degradation of BCR-ABL in chronic myelogenous leukemia. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107*, 21683–21688, doi:10.1073/pnas.1016311108.
 235. Persaud, A.; Alberts, P.; Mari, S.; Tong, J.; Murchie, R.; Maspero, E.; Safi, F.; Moran, M.F.; Polo, S.; Rotin, D. Tyrosine phosphorylation of NEDD4 activates its ubiquitin ligase activity. *Sci. Signal.* **2014**, *7*, ra95, doi:10.1126/scisignal.2005290.
 236. Angeles Montes-Fernández, M.; M^a Pérez-Villegas, E.; Garcia-Gonzalo, F.R.; Pedrazza, L.; Luis Rosa, J.; Alvarez de Toledo, G.; Armengol, J.A. The HERC1 ubiquitin ligase regulates presynaptic membrane dynamics of central synapses. **2020**, *10*, 12057, doi:10.1038/s41598-020-68970-8.
 237. Moran-Crusio, K.; Reavie, L.B.; Aifantis, I. Regulation of hematopoietic stem cell fate by the ubiquitin proteasome system. *Trends Immunol.* **2012**, *33*, 357–363.
 238. Sanarico, A.G.; Ronchini, C.; Croce, A.; Memmi, E.M.; Cammarata, U.A.; De Antoni, A.; Lavorgna, S.; DiVona, M.; Giacò, L.; Melloni, G.E.M.; et al. The E3 ubiquitin ligase WWP1 sustains the growth of acute myeloid leukaemia. *Leukemia* **2018**, *32*, 911–919, doi:10.1038/leu.2017.342.
 239. Wang, H.; Bei, L.; Shah, C.A.; Hu, L.; Eklund, E.A. HoxA10 terminates emergency

- granulopoiesis by increasing expression of Triad1. *J. Immunol.* **2015**, *194*, 5375–5387.
240. Wang, H.; Bei, L.; Shah, C.A.; Huang, W.; Platanias, L.C.; Eklund, E.A. The E3 ubiquitin ligase Triad1 influences development of Mll-Ell-induced acute myeloid leukemia. *Oncogene* **2018**, *37*, 2532–2544, doi:10.1038/s41388-018-0131-5.
 241. Wang, B.; Jie, Z.; Joo, D.; Ordureau, A.; Liu, P.; Gan, W.; Guo, J.; Zhang, J.; North, B.J.; Dai, X.; et al. TRAF2 and OTUD7B govern a ubiquitin-dependent switch that regulates mTORC2 signalling. *Nature* **2017**, *545*, 365–369, doi:10.1038/nature22344.
 242. Thomas, C.C.; Deak, M.; Alessi, D.R.; van Aalten, D.M.F. High-resolution structure of the pleckstrin homology domain of protein kinase b/akt bound to phosphatidylinositol (3, 4, 5)-trisphosphate. *Curr. Biol.* **2002**, *12*, 1256–1262.
 243. Fu, W.; Hall, M.N. Regulation of mTORC2 Signaling., doi:10.3390/genes11091045.
 244. Kamal, A.; Boehm, M.F.; Burrows, F.J. Therapeutic and diagnostic implications of Hsp90 activation. *Trends Mol. Med.* **2004**, *10*, 283–290.

ANNEX

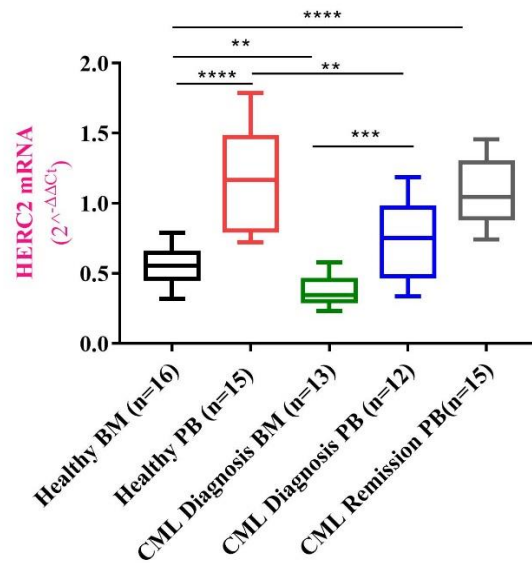


Figure A1. *HERC2* gene expression at different phases of CML patients. *HERC2* gene expression was significantly down-regulated at diagnosis, in CML patients, when compared to control specimens. While the patients when achieved the complete remission the *HERC2* gene expression reached levels comparatively similar to those of the healthy control.

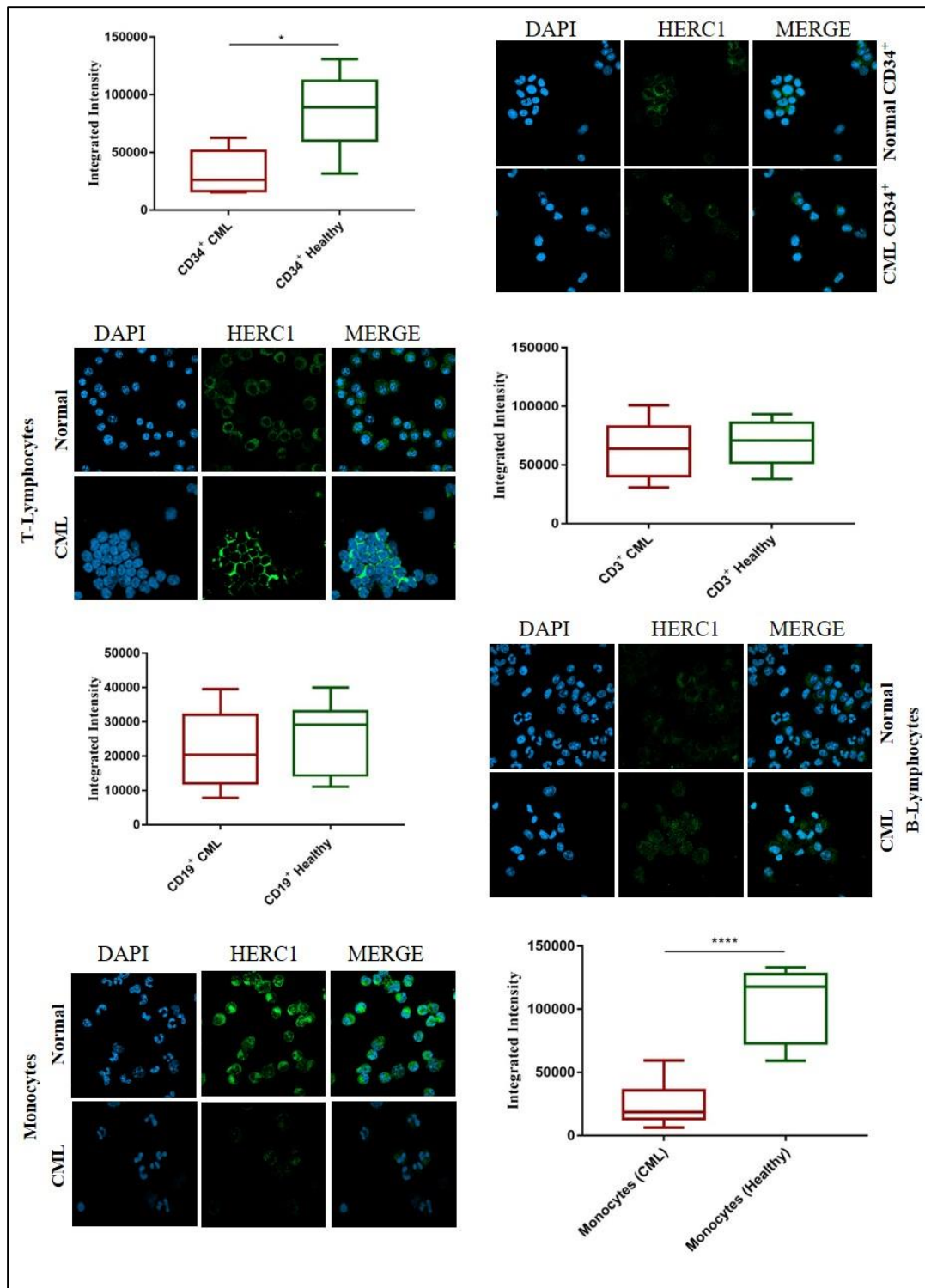


Figure A2. Quantification of HERC1 signal of immunofluorescence by measuring the integrated intensities of each sample. Immunofluorescence staining assay on different cell population after magnetic isolation from healthy donor and of CML bone marrow (BM) samples.

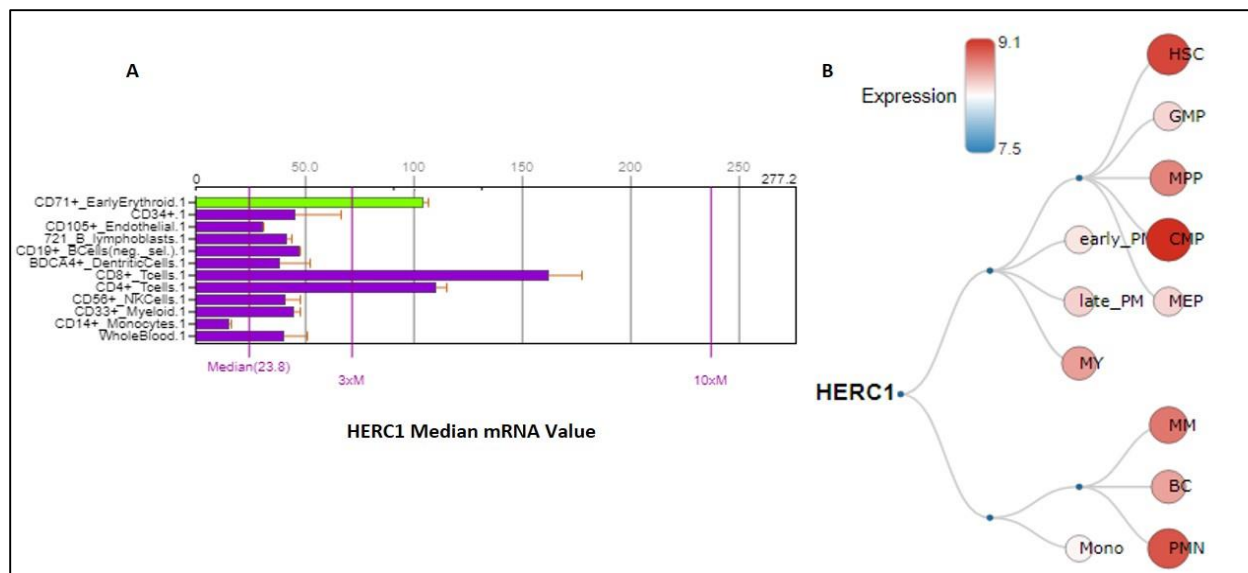


Figure A3. HERC1 transcript level in normal human blood cells population according to the Human Differentiation Map Portal databases. **A)** biogps.org, and **B)** BloodSpot.org database.

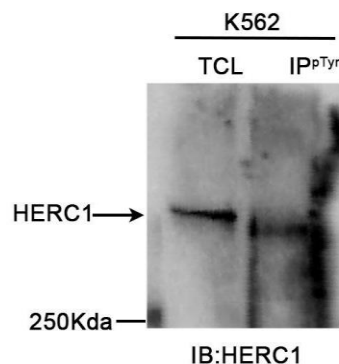


Figure A4. HERC1 is tyrosine phosphorylated in chronic myeloid leukemia K562 cells. K562 lysates were immunoprecipitated by using anti phosphotyrosine (p-Tyr) antibody. Immunocomplex was subsequently resolved by SDS-PAGE, transferred onto PVDF membranes and eventually probed with HERC1 antibody. The presence of HERC1 band indicated that in K562 cells HERC1 is tyrosine-phosphorylated.

Table A2.1: Chronic Myeloid Leukemia (CML) patient's characteristics.

ID	SEX	Age	Disease	Type of Sample	BCR-ABL1 (p210) Copy No (IS)
1	M	36	CML (Diagnosis)	Bone Marrow	61.203
2	F	35	CML (Diagnosis)	Bone Marrow	50.439
3	M	46	CML (Diagnosis)	Bone Marrow	74.213
4	M	82	CML (Diagnosis)	Bone Marrow	70.517
5	F	74	CML (Diagnosis)	Bone Marrow	40.876
6	M	65	CML (Diagnosis)	Bone Marrow	48.540
7	F	54	CML (Diagnosis)	Bone Marrow	55.957
8	F	76	CML (Diagnosis)	Bone Marrow	82.666
9	M	57	CML (Diagnosis)	Bone Marrow	79.062
10	M	55	CML (Diagnosis)	Bone Marrow	40.000
11	M	79	CML (Diagnosis)	Bone Marrow	119.340
12	M	52	CML (Diagnosis)	Bone Marrow	94.910
13	M	61	CML (Diagnosis)	Bone Marrow	88.418
14	M	61	CML (Diagnosis)	Bone Marrow	76.007
15	F	53	CML (Diagnosis)	Bone Marrow	54.700
16	F	66	CML (Diagnosis)	Bone Marrow	104.836
17	M	54	CML (Diagnosis)	Bone Marrow	157.280
18	M	11	CML (Diagnosis)	Bone Marrow	53.013
19	F	70	CML (Diagnosis)	Bone Marrow	94.800
20	F	52	CML (Diagnosis)	Bone Marrow	49.856
21	F	67	CML (Diagnosis)	Bone Marrow	100.477
22	M	79	CML (Diagnosis)	Bone Marrow	148.000
23	F	48	CML (Diagnosis)	Bone Marrow	100.980
24	F	63	CML (Diagnosis)	Bone Marrow	55.010
25	M	57	CML (Diagnosis)	Bone Marrow	68.500
26	F	53	CML (Diagnosis)	Bone Marrow	37.700
27	M	77	CML (Diagnosis)	Bone Marrow	40.061
28	F	56	CML (Diagnosis)	Bone Marrow	39.000
29	M	54	CML (Diagnosis)	Bone Marrow	59.020
30	F	61	CML (Diagnosis)	Bone Marrow	46.500
31	F	10	CML (Diagnosis)	Bone Marrow	15.600
32	F	68	CML (Diagnosis)	Bone Marrow	43.400
33	F	75	CML (Diagnosis)	Bone Marrow	87.000
34	F	72	CML (Diagnosis)	Bone Marrow	63.500
35	M	72	CML (Diagnosis)	Bone Marrow	68.630
36	M	63	CML (Diagnosis)	Peripheral Blood	39.223
37	F	58	CML (Diagnosis)	Peripheral Blood	52.139
38	M	71	CML (Diagnosis)	Peripheral Blood	65.898

39	F	85	CML (Diagnosis)	Peripheral Blood	30.821
40	M	48	CML (Diagnosis)	Peripheral Blood	74.544
41	M	82	CML (Diagnosis)	Peripheral Blood	97.660
42	F	74	CML (Diagnosis)	Peripheral Blood	51.951
43	F	76	CML (Diagnosis)	Peripheral Blood	80.899
44	M	20	CML (Diagnosis)	Peripheral Blood	26.395
45	M	15	CML (Diagnosis)	Peripheral Blood	135.310
46	M	56	CML (Diagnosis)	Peripheral Blood	85.735
47	M	57	CML (Diagnosis)	Peripheral Blood	80.013
48	F	57	CML (Diagnosis)	Peripheral Blood	42.000
49	F	26	CML (Diagnosis)	Peripheral Blood	44.000
50	M	66	CML (Diagnosis)	Peripheral Blood	59.972
51	M	79	CML (Diagnosis)	Peripheral Blood	158.590
52	M	52	CML (Diagnosis)	Peripheral Blood	112.800
53	M	61	CML (Diagnosis)	Peripheral Blood	100.000
54	M	61	CML (Diagnosis)	Peripheral Blood	72.772
55	M	54	CML (Diagnosis)	Peripheral Blood	137.000
56	M	11	CML (Diagnosis)	Peripheral Blood	57.729
57	F	70	CML (Diagnosis)	Peripheral Blood	193.500
58	M	45	CML (Diagnosis)	Peripheral Blood	58.001
59	F	52	CML (Diagnosis)	Peripheral Blood	48.335
60	M	37	CML (Diagnosis)	Peripheral Blood	53.736
61	M	67	CML (Diagnosis)	Peripheral Blood	98.500
62	M	79	CML (Diagnosis)	Peripheral Blood	93.350
63	F	48	CML (Diagnosis)	Peripheral Blood	102.350
64	M	54	CML (Diagnosis)	Peripheral Blood	57.200
65	F	74	CML (Diagnosis)	Peripheral Blood	48.100
66	M	71	CML (Diagnosis)	Peripheral Blood	70.500
67	F	72	CML (Diagnosis)	Peripheral Blood	82.970
68	M	50	CML (Diagnosis)	Peripheral Blood	93.900
69	M	36	CML (Diagnosis)	Peripheral Blood	38.200
70	f	83	CML (Diagnosis)	Peripheral Blood	79.000
71	M	54	CML (Diagnosis)	Peripheral Blood	86.200
72	M	77	CML (Diagnosis)	Peripheral Blood	74.130
73	F	40	CML (relapse)	Bone Marrow	12.979
74	M	76	CML (Resistant)	Bone Marrow	18.032
75	F	44	CML (relapse)	Bone Marrow	28.094
76	F	42	CML (Resistant)	Bone Marrow	25.597
77	F	31	CML (relapse)	Bone Marrow	14.383
78	F	79	CML (relapse)	Bone Marrow	58.691
79	F	83	CML (relapse)	Peripheral Blood	21.246

80	M	89	CML (relapse)	Peripheral Blood	47.862
81	M	79	CML Remission	Peripheral Blood	0.500
82	F	68	CML Remission	Peripheral Blood	0.030
83	M	38	CML Remission	Peripheral Blood	0.005
84	M	76	CML Remission	Peripheral Blood	0.022
85	M	69	CML Remission	Peripheral Blood	0.524
86	F	79	CML Remission	Peripheral Blood	0.144
87	F	61	CML Remission	Peripheral Blood	0.392
88	M	34	CML Remission	Peripheral Blood	0.015
89	M	35	CML Remission	Peripheral Blood	0.004
90	M	62	CML Remission	Peripheral Blood	0.000
91	M	18	CML Remission	Peripheral Blood	0.289
92	F	61	CML Remission	Peripheral Blood	0.056
93	F	32	CML Remission	Peripheral Blood	0.934
94	F	54	CML Remission	Peripheral Blood	0.035
95	M	64	CML Remission	Peripheral Blood	0.000
96	M	73	CML Remission	Peripheral Blood	0.008
97	M	38	CML Remission	Peripheral Blood	0.090
98	F	68	CML Remission	Peripheral Blood	0.002
99	F	54	CML Remission	Peripheral Blood	2.900
100	M	48	CML Remission	Peripheral Blood	0.070
101	F	36	CML Remission	Peripheral Blood	0.000
102	F	70	CML Remission	Peripheral Blood	0.280
103	F	54	CML Remission	Peripheral Blood	0.035
104	F	44	CML Remission	Peripheral Blood	0.025
105	M	57	CML Remission	Peripheral Blood	0.980
106	F	65	CML Remission	Peripheral Blood	0.006
107	M	80	CML Remission	Peripheral Blood	0.080
108	M	12	CML Remission	Peripheral Blood	0.010
109	F	63	CML Remission	Peripheral Blood	1.000
110	F	66	CML Remission	Peripheral Blood	0.700
111	F	60	CML Remission	Peripheral Blood	0.000
112	F	39	CML Remission	Bone Marrow	1.072
113	M	49	CML Remission	Bone Marrow	0.060
114	F	54	CML Remission	Peripheral Blood	0.020
115	M	62	CML Remission	Peripheral Blood	0.060
116	F	58	CML Remission	Peripheral Blood	0.081
117	M	57	CML Remission	Peripheral Blood	0.008
118	F	20	CML Remission	Peripheral Blood	0.100
119	F	75	CML Remission	Peripheral Blood	0.000
120	F	74	CML Remission	Peripheral Blood	0.057

121	M	39	CML Remission	Peripheral Blood	1.250
122	f	87	CML Remission	Peripheral Blood	0.000
123	M	56	CML Remission	Peripheral Blood	0.093
124	M	61	CML Remission	Peripheral Blood	0.000
125	F	54	CML Remission	Peripheral Blood	0.280

Table A2.2: Characteristics of Acute Myeloid Leukemia (AML) patients's at diagnosis.

ID	Sex	Age	Disease	Type of Sample	Genetic Alteration
1	F	58	AML (Diagnosis)	Bone Marrow	<i>inv16</i>
2	M	63	AML (Diagnosis)	Bone Marrow	<i>inv16</i>
3	m	38	AML (Diagnosis)	Bone Marrow	<i>inv16</i>
4	M	6	AML (Diagnosis)	Bone Marrow	<i>inv16</i>
5	M	71	AML (Diagnosis)	Bone Marrow	<i>inv16</i>
6	M	42	AML (Diagnosis)	Bone Marrow	<i>inv16</i>
7	F	57	AML (Diagnosis)	Bone Marrow	<i>inv16</i>
8	M	64	AML (Diagnosis)	Bone Marrow	<i>inv16</i>
9	F	58	AML (Diagnosis)	Bone Marrow	<i>inv16</i>
10	M	64	AML (Diagnosis)	Bone Marrow	<i>t (8;21)</i>
11	M	51	AML (Diagnosis)	Bone Marrow	<i>t (8;21)</i>
12	M	38	AML (Diagnosis)	Bone Marrow	<i>t (8;21)</i>
13	M	33	AML (Diagnosis)	Bone Marrow	<i>t (8;21)</i>
14	M	29	AML (Diagnosis)	Bone Marrow	<i>t (8;21)</i>
15	M	63	AML (Diagnosis)	Bone Marrow	<i>t (8;21)</i>
16	M	34	AML (Diagnosis)	Bone Marrow	<i>t(8;21)</i>
17	M	75	AML (Diagnosis)	Bone Marrow	<i>t (8;21)</i>
18	M	31	AML (Diagnosis)	Bone Marrow	<i>t (8;21)</i>
19	M	73	AML (Diagnosis)	Bone Marrow	<i>IDH2 R140Q</i>
20	M	55	AML (Diagnosis)	Bone Marrow	<i>IDH2 R140Q</i>
21	m	84	AML (Diagnosis)	Bone Marrow	<i>IDH2 R140Q</i>
22	F	82	AML (Diagnosis)	Bone Marrow	<i>IDH2 R140Q</i>
23	M	79	AML (Diagnosis)	Bone Marrow	<i>IDH2 R140Q</i>
24	M	57	AML (Diagnosis)	Bone Marrow	<i>NPM1</i>
25	F	78	AML (Diagnosis)	Bone Marrow	<i>NPM1</i>
26	M	64	AML (Diagnosis)	Bone Marrow	<i>NPM1</i>
27	M	74	AML (Diagnosis)	Bone Marrow	<i>NPM1</i>
28	F	76	AML (Diagnosis)	Bone Marrow	<i>NPM1</i>
29	F	22	AML (Diagnosis)	Bone Marrow	<i>NPM1</i>
30	F	69	AML (Diagnosis)	Bone Marrow	<i>NPM1</i>
31	F	51	AML (Diagnosis)	Bone Marrow	<i>NPM1</i>
32	M	91	AML (Diagnosis)	Bone Marrow	<i>FLT3 ITD</i>
33	M	75	AML (Diagnosis)	Bone Marrow	<i>FLT3 ITD</i>
34	F	67	AML (Diagnosis)	Bone Marrow	<i>FLT-3 15% COD. 835</i>
35	F	77	AML (Diagnosis)	Bone Marrow	<i>FLT-3 ITD</i>
36	F	53	AML (Diagnosis)	Bone Marrow	<i>FLT-3 ITD</i>
37	M	36	AML (Diagnosis)	Bone Marrow	<i>FLT-3 ITD</i>
38	M	48	AML (Diagnosis)	Bone Marrow	<i>FLT-3 ITD</i>
39	M	41	AML (Diagnosis)	Bone Marrow	<i>FLT-3 ITD</i>

40	M	59	AML (Diagnosis)	Bone Marrow	<i>FLT-3 ITD</i>
41	F	58	AML (Diagnosis)	Bone Marrow	<i>FLT-3 ITD/inv16</i>
42	M	41	AML (Diagnosis)	Bone Marrow	<i>FLT-3 ITD/t(15;17)</i>
43	F	81	AML (Diagnosis)	Bone Marrow	<i>NPM1-FLT3 ITD</i>
44	F	88	AML (Diagnosis)	Bone Marrow	<i>NPM1-FLT3 ITD</i>
45	F	52	AML (Diagnosis)	Bone Marrow	<i>FLT3 ITD</i>
46	M	46	AML (Diagnosis)	Bone Marrow	<i>FLT3 ITD</i>
47	M	47	AML (Diagnosis)	Bone Marrow	<i>FLT3 ITD</i>
48	F	38	AML (Diagnosis)	Bone Marrow	No
49	M	56	AML (Diagnosis)	Bone Marrow	No
50	M	18	AML (Diagnosis)	Bone Marrow	No
51	F	66	AML (Diagnosis)	Bone Marrow	No
52	M	73	AML (Diagnosis)	Bone Marrow	No
53	F	82	AML (Diagnosis)	Bone Marrow	No
54	F	78	AML (Diagnosis)	Bone Marrow	No
55	M	30	AML (Diagnosis)	Bone Marrow	No
56	M	72	AML (Diagnosis)	Bone Marrow	No
57	M	57	AML (Diagnosis)	Bone Marrow	No
58	M	35	AML (Diagnosis)	Bone Marrow	No
59	M	65	AML (Diagnosis)	Bone Marrow	No
60	M	40	AML (Diagnosis)	Bone Marrow	No
61	F	61	AML (Diagnosis)	Bone Marrow	No
62	M	76	AML (Diagnosis)	Bone Marrow	No
63	F	68	AML (Diagnosis)	Bone Marrow	<i>t(15;17)PML/RARA</i>
64	F	64	AML (Diagnosis)	Bone Marrow	<i>t(15;17)PML/RARA</i>
65	M	68	AML (Diagnosis)	Bone Marrow	<i>t(15;17)PML/RARA</i>
66	F	79	AML (Diagnosis)	Bone Marrow	<i>t(15;17)PML/RARA</i>
67	M	41	AML (Diagnosis)	Bone Marrow	<i>FLT-3 ITD/t(15;17)</i>
68	M	41	AML (Diagnosis)	Bone Marrow	<i>t(15;17)PML/RARA</i>
69	F	79	AML (Diagnosis)	Bone Marrow	<i>t(15;17)PML/RARA</i>
70	F	34	AML (Diagnosis)	Bone Marrow	<i>t(15;17)PML/RARA</i>
71	M	85	AML (Diagnosis)	Bone Marrow	<i>t(15;17)PML/RARA</i>
72	F	59	AML (Diagnosis)	Bone Marrow	<i>t(15;17)PML/RARA</i>
73	M	56	AML (Diagnosis)	Bone Marrow	<i>t(15;17)PML/RARA</i>
74	M	38	AML (Diagnosis)	Bone Marrow	<i>t(15;17)PML/RARA</i>
75	M	74	AML (Diagnosis)	Bone Marrow	<i>t(15;17)PML/RARA</i>
76	F	40	AML (Diagnosis)	Bone Marrow	<i>t(15;17)PML/RARA</i>
77	F	79	AML (Diagnosis)	Bone Marrow	<i>NPM1-FLT3 835</i>
78	F	70	AML (Diagnosis)	Bone Marrow	<i>NPM1-FLT3 835</i>
79	M	68	AML (Diagnosis)	Bone Marrow	<i>NPM1-FLT3 835</i>
80	M	76	AML (Diagnosis)	Bone Marrow	<i>NPM1-FLT3 835</i>
81	M	79	AML (Diagnosis)	Peripheral Blood	<i>inv16</i>
82	M	51	AML (Diagnosis)	Peripheral Blood	<i>inv16</i>
83	m	57	AML (Diagnosis)	Peripheral Blood	<i>inv16</i>
84	F	34	AML (Diagnosis)	Peripheral Blood	<i>inv16</i>
85	F	87	AML (Diagnosis)	Peripheral Blood	<i>t(8;21)</i>
86	F	51	AML (Diagnosis)	Peripheral Blood	<i>t(8;21)</i>
87	M	55	AML (Diagnosis)	Peripheral Blood	<i>IDH2 R140Q</i>
88	F	51	AML (Diagnosis)	Peripheral Blood	<i>NPM1</i>

89	F	24	AML (Diagnosis)	Peripheral Blood	<i>NPM1</i>
90	F	51	AML (Diagnosis)	Peripheral Blood	<i>FLT-3 ITD/t(15;17)</i>
91	F	80	AML (Diagnosis)	Peripheral Blood	<i>FLT-3 ITD</i>
92	F	51	AML (Diagnosis)	Peripheral Blood	<i>FLT-3 ITD/t(15;17)</i>
93	M	55	AML (Diagnosis)	Peripheral Blood	<i>IDH2 R140Q</i>
94	F	88	AML (Diagnosis)	Peripheral Blood	<i>NPM1-FLT3 835</i>
95	F	51	AML (Diagnosis)	Peripheral Blood	<i>FLT-3 ITD</i>
96	F	75	AML (Diagnosis)	Peripheral Blood	<i>IDH2 & IDH1</i>
97	M	37	AML (Diagnosis)	Peripheral Blood	<i>t(8;21)</i>

Table A2.3: Details of MyeloProliferative Neoplasms (MPNs) patients.

ID	Sex	Age (aa)	Disease	BM/PB	Mutation
1	M	87	ET (Diagnosis)	PB	<i>CALR-type 2</i>
2	F	53	ET (Diagnosis)	PB	<i>CALR-type 1</i>
3	F	68	ET (Diagnosis)	PB	<i>CALR -type 1</i>
4	M	57	ET (Diagnosis)	PB	<i>CALR-type 1</i>
5	M	55	ET (Diagnosis)	PB	<i>CALR-type 1</i>
6	M	53	ET (Diagnosis)	PB	<i>CALR-type 1</i>
7	F	70	ET (Diagnosis)	PB	<i>CALR-type 1</i>
8	M	59	ET (Diagnosis)	PB	<i>CALR-type 1</i>
9	M	48	ET (Diagnosis)	PB	<i>CALR-type 9</i>
10	M	64	ET (Diagnosis)	PB	<i>CALR-type 1</i>
11	M	47	ET (Diagnosis)	PB	<i>CALR-type 2</i>
12	M	50	ET (Diagnosis)	PB	<i>CALR-type 4</i>
13	M	83	ET (Diagnosis)	PB	<i>CALR-type 1</i>
14	M	62	ET (Diagnosis)	PB	<i>CALR-type 2</i>
15	F	54	ET (Diagnosis)	PB	<i>CALR-type 2</i>
16	F	57	ET (Diagnosis)	PB	<i>CALR-type 1</i>
17	F	82	ET (Diagnosis)	PB	<i>JAK2^{V617F}</i>
18	M	47	ET (Diagnosis)	PB	<i>JAK2^{V617F}</i>
19	M	78	ET (Diagnosis)	PB	<i>JAK2^{V617F}</i>
20	F	49	ET (Diagnosis)	PB	<i>JAK2^{V617F}</i>
21	M	63	ET (Diagnosis)	PB	<i>JAK2^{V617F}</i>
22	M	19	ET (Diagnosis)	PB	<i>JAK2^{V617F}</i>
23	F	65	ET (Diagnosis)	PB	<i>JAK2^{V617F}</i>
24	F	59	ET (Diagnosis)	PB	w.t.
25	F	42	ET (Diagnosis)	PB	NR
26	F	82	ET (Diagnosis)	PB	w.t.
27	M	59	ET (Diagnosis)	PB	w.t.
28	F	70	ET (Diagnosis)	PB	w.t.
29	F	75	ET (Diagnosis)	PB	w.t.
30	M	57	ET (Diagnosis)	PB	w.t.
31	M	64	ET (Diagnosis)	PB	w.t.

32	M	53	ET (Diagnosis)	PB	w.t.
33	M	75	ET (Diagnosis)	PB	w.t.
34	M	51	ET (Diagnosis)	PB	w.t.
35	F	52	ET (Diagnosis)	PB	NR
36	F	70	ET (Diagnosis)	PB	w.t.
37	F	27	ET (Diagnosis)	PB	w.t.
38	F	55	ET (Diagnosis)	PB	NR
39	M	92	ET (Diagnosis)	PB	w.t.
40	M	77	PV (Diagnosis)	PB	JAK2 ^{V617F}
41	M	49	PV (Diagnosis)	PB	JAK2 ^{V617F}
42	M	84	PV (Diagnosis)	PB	JAK2 ^{V617F}
43	F	37	PV (Diagnosis)	PB	JAK2 ^{V617F}
44	F	77	PV (Diagnosis)	PB	JAK2 ^{V617F}
45	M	56	PV (Diagnosis)	PB	JAK2 ^{V617F}
46	F	89	PV (Diagnosis)	PB	JAK2 ^{V617F}
47	F	60	PV (Diagnosis)	PB	JAK2 ^{V617F}
48	F	84	PV (Diagnosis)	PB	JAK2 ^{V617F}
49	S	38	PV (Diagnosis)	PB	JAK2 ^{V617F}
50	M	69	PV (Diagnosis)	PB	JAK2 ^{V617F}
51	M	69	PV (Diagnosis)	PB	w.t.
52	M	64	PV (Diagnosis)	PB	w.t.
53	M	45	PV (Diagnosis)	PB	w.t.
54	M	58	PV (Diagnosis)	PB	w.t.
55	M	66	PV (Diagnosis)	PB	w.t.
56	M	28	PV (Diagnosis)	PB	w.t.
57	M	72	PV (Diagnosis)	PB	w.t.
58	M	74	PV (Diagnosis)	PB	w.t.
59	M	65	PV (Diagnosis)	PB	w.t.
60	M	47	PV (Diagnosis)	PB	w.t.
61	M	80	PMF (Diagnosis)	PB	JAK2 ^{V617F}
62	M	69	PMF (Diagnosis)	PB	JAK2 ^{V617F}
63	F	85	PMF (Diagnosis)	PB	JAK2 ^{V617F}
64	M	78	PMF (Diagnosis)	PB	JAK2 ^{V617F}
65	M	60	PMF (Diagnosis)	PB	JAK2 ^{V617F}
66	F	76	PMF (Diagnosis)	PB	JAK2 ^{V617F}
67	F	67	PMF (Diagnosis)	PB	JAK2 ^{V617F}
68	M	77	PMF (Diagnosis)	PB	w.t.
69	F	84	PMF (Diagnosis)	PB	NR
70	M	42	PMF (Diagnosis)	PB	w.t.
71	M	39	PMF (Diagnosis)	PB	w.t.
72	M	47	PMF (Diagnosis)	PB	w.t.
73	F	60	PMF (Diagnosis)	PB	w.t.
74	M	40	PMF (Diagnosis)	PB	w.t.
75	F	73	PMF (Diagnosis)	PB	w.t.

76	F	78	PMF (Diagnosis)	PB	w.t.
77	F	78	PMF (Diagnosis)	PB	w.t.
78	F	68	PMF (Diagnosis)	PB	w.t.