UNIVERSIDADE DE SÃO PAULO
FACULDADE DE CIÊNCIAS FARMACÊUTICAS DE RIBEIRÃO PRETO

Deregulation of microRNAs expression and its role in Bcr-Abl^{+} cells resistance to TKI therapy

Expressão alterada de microRNAs e seu papel na resistência de células Bcr-Abl^{+} à terapia com TKI

Maria Gabriela Berzoti Coelho

Ribeirão Preto
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Doctoral thesis presented to the Graduate Program of School of Pharmaceutical Sciences of Ribeirão Preto/USP for the degree of Doctor in Sciences.

Concentration Area: Biosciences Applied to Pharmacy

Supervisor: Prof. Dr. Fabíola Attié de Castro
Co-supervisor: Dr. Eva Hernando-Monge

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Ribeirão Preto
2020

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Maria Gabriela Berzoti Coelho

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I dedicate this work...

To my beloved family, for always support my dreams and choices, for the trust, and for the unconditional love.

To the close friends that have always been by my side.

To all the researchers and scientists who have collaborated with my learning.

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"I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena, which impress him like a fairy tale."

Marie Curie

ABSTRACT

BERZOTI-COELHO, M. G. **Deregulation of microRNAs expression and its role in Bcr-Abl$^+$ cells resistance to TKI therapy**. 2020. 173p. Thesis (Doctoral) - de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, 2019.

Chronic myeloid leukemia (CML) is a myeloproliferative neoplasm characterized by stem cell deregulation and increased myeloid proliferation without impairment of differentiation capacity. The hematopoiesis deregulation arises from the formation of a Bcr-Abl oncoprotein with constitutive tyrosine kinase (TK) activity, responsible for the malignant cell transformation. The tyrosine kinase inhibitors (TKI) therapy changed dramatically the CML natural history, promoting high rates of disease remissions in patients. However, at least 25% of CML patients are resistant to TKI therapy. Despite all the knowledge about the pathogenesis and progression of CML, the cellular and molecular mechanisms underlying the TKI-resistance are not fully understood. Therefore, we supposed that microRNAs deregulated expression contributes to IM-resistance in Bcr-Abl$^+$ cells. MicroRNAs are small endogenous RNAs that regulate gene expression. The changes of microRNAs expression have been associated with the pathogenesis of different neoplasms, and the role of microRNAs in the development of resistance to drug-therapies was already demonstrated in a wide range of cancers. In this current investigation, we hypothesized that the inhibition of specific microRNAs could sensitize Bcr-Abl$^+$ resistant cells to the TKI imatinib mesylate (IM) therapy. The expression of miR-125a-5p, miR-125b, and miR-132 was assessed in 61 CML patients, of which 10 samples were from newly diagnosed patients, 15 from patients in the advanced stages of the disease (accelerated phase and blast crisis), and 36 samples from patients in the disease remission after IM or dasatinib (DAS) therapy. The miR-125b was less expressed in the patients in the disease remission after DAS treatment than in the newly diagnosed patients and patients in the advanced stages. Other five microRNAs up-regulated in Bcr-Abl$^+$ cells were selected using the NanoString's microRNA panel (miR-23a, miR-24, miR-155, miR-222, and miR-342). Then, the transient and stable inhibition of all the above mentioned microRNAs was performed in the IM-resistant Bcr-Abl$^+$ cell line, LAMA-84R. The inhibition of miR-125a-5p, miR-132, and miR-23a did not sensitize the LAMA-84R cells to IM treatment. The inhibition of miR-125b, miR-24, miR-155, miR-222, and miR-342 promoted a modest increase of LAMA-84R cells sensitivity to the IM treatment. Additionally, the miR-222 and miR-342 overexpression in LAMA-84S, the sensitive counterpart of LAMA-84R, didn't promote the cells' resistance to the IM treatment. A new approach was performed to select the microRNAs that are strongly associated with IM-resistance, the phenotypic screen based on the pLX-miR Library. After Next-Generation sequencing analysis, let-7e, miR-181a, miR-484, miR-616, and miR-96 were selected. These microRNAs expression was assessed in the patients groups. All the five microRNAs were less expressed in the patients in the disease remission after IM treatment than in the newly diagnosed patients. Currently, we are validating the results obtained from the phenotypic screen by the individual knockout of the five microRNAs through the CRISPR-Cas9 gene-editing system. The results will contribute to elucidate the mechanisms involved in CML resistance to imatinib mesylate and to describe new therapeutic targets that are independent of the Bcr-Abl oncoprotein.

Keywords: Chronic Myeloid Leukemia; microRNAs; Imatinib mesylate; IM-resistance; Tyrosine-kinase inhibitors.

RESUMO

BERZOTI-COELHO, M. G. **Expressão alterada de microRNAs e seu papel na resistência de células Bcr-Abl$^+$ à terapia com TKI.** 2020. 173f. Tese (Doutorado) – Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, 2019.

A leucemia mieloide crônica (LMC) é uma neoplasia mieloproliferativa caracterizada pela alteração de células-tronco hematopoéticas e aumento da proliferação de células mieloides, as quais mantém sua capacidade de diferenciação. A oncoproteína Bcr-Abl com atividade tirosina-quinase constitutiva (TK) é a responsável pela transformação maligna. A terapia com os inibidores de tirosina-quinase (TKI) é capaz de promover altas taxas de remissão da doença. No entanto, ao menos 25% dos pacientes com LMC são resistentes à terapia com TKI. Apesar de todo o conhecimento sobre a fisiopatologia e progressão da LMC, os mecanismos celulares e moleculares subjacentes à resistência aos TKI não estão completamente elucidados. Nesse contexto, acreditamos que os microRNAs podem contribuir para a resistência das células Bcr-Abl$^+$ ao IM. Os microRNAs são pequenos RNAs endógenos que regulam a expressão gênica. Alterações na expressão de microRNAs tem sido associadas à patogênese de diferentes neoplasias e seu papel no desenvolvimento de resistência à terapias medicamentosas já foi demonstrado em numerosos tipos de câncer. Dessa forma, partimos da hipótese de que a inibição de microRNAs específicos é capaz de sensibilizar células Bcr-Abl$^+$ resistentes à terapia com o IM. A expressão do miR-125a-5p, miR-125b e miR-132 foi avaliada em 61 pacientes com LMC (10 amostras de pacientes ao diagnóstico da LMC, 15 amostras de pacientes nas fases avançadas da doença - fase acelerada e crise blástica - e 36 amostras de pacientes em remissão após o tratamento com IM ou dasatinibe (DAS)). O miR-125b foi menos expresso no grupo de pacientes em remissão após tratamento com DAS que nos grupos de pacientes ao diagnóstico ou nas fases avançadas da doença. Outros cinco microRNAs *up-regulados* em células Bcr-Abl$^+$ foram selecionados através do painel NanoString^{TM} de microRNAs (miR-23a, miR-24, miR-155, miR-222 e miR-342). A inibição transiente e estável de todos os microRNAs acima citados foi realizada na linhagem celular Bcr-Abl$^+$ resistente ao IM, LAMA-84R. A inibição do miR-125a-5p, miR-132 e miR-23a não foi capaz de sensibilizar as células LAMA-84R ao IM, ao passo que a inibição do miR-125b, miR-24, miR-155, miR-222 e miR-342 promoveu um pequeno aumento de sensibilidade das células LAMA-84R ao IM. Além disso, a superexpressão do miR-222 e do miR-342 na linhagem celular Bcr-Abl+ sensível ao IM, LAMA-84S, não promoveu a resistência dessas células ao IM. Uma nova abordagem para selecionar microRNAs fortemente associados à resistência ao IM foi realizada, o *screen* fenotípico baseado na *pLX-miR library*. Após a análise de sequenciamento de última geração, os microRNAs let-7e, miR-181a, miR-484, miR-616 e miR-96 foram selecionados. A expressão de tais microRNAs foi avaliada nos grupos de pacientes e todos os cinco foram menos expressos nos pacientes em remissão após o tratamento com IM que nos pacientes ao diagnóstico da LMC. Atualmente, os resultados obtidos desta triagem fenotípica estão sendo validados através do nocaute individual dos cinco microRNAs por meio da técnica de edição gênica CRISPR-Cas9. Os resultados obtidos contribuirão para elucidar os mecanismos envolvidos na resistência da LMC ao IM e para a descrição de novos alvos terapêuticos independentes da oncoproteína Bcr-Abl.

Palavras-chave: Leucemia Mieloide Crônica; microRNAs; mesilato de imatinibe; resistência ao imatinibe; inibidores de tirosina-quinase.

LIST OF ABBREVIATIONS AND ACRONYMS

A2780 – epithelial ovarian cancer cell line

A278/CP – cisplatin-resistant A2780 cell line

ABI – Applied Biosystems

ABL – ABL proto-oncogene 1, non-receptor tyrosine kinase (previous name: abelson1)

AGO2 – argonaute RISC catalytic component 2

AKT – AKT serine/threonine kinase 1 (previous name: protein kinase B)

ALL – acute lymphoblastic leukemia

AlloSCT – allogeneic stem cell transplantation

AML – acute myeloid leukemia

AP – accelerated phase

APL – acute promyelocytic leukemia

ATCC – American Type Culture Collection

ATP – adenosine triphosphate

ATRA – all-trans retinoic acid

BACH1 – BTB domain and CNC homolog 1

BAD – BCL2 associated agonist of cell death

BAK1 – BCL2 antagonist/killer 1

BAX – BCL2 associated X, apoptosis regulator

BC – blast crisis

BCL2 –BCL2 apoptosis regulator

BCL-X_L – BCL2 like 1 (current symbol: BCL2L1)

BCR – BCR activator of RhoGEF and GTPase (previous name: breakpoint cluster region)

BM – bone marrow

BIK – BCL2 interacting killer

Bim – Bcl2-like protein 11 (current symbol: BCL2 like 11)

bp – base pair

C5b-9 – membrane attack complex

Caspase-3 – caspase 3 (previous name: caspase 3, apoptosis-related cysteine protease)

Caspase-9 – caspase 9 (previous name: caspase 9, apoptosis-related cysteine protease)

CCR – complete cytogenetic response

CD46 – CD46 molecule (previous name: CD46 antigen, complement regulatory protein)

CD59 – CD59 molecule (previous name: CD59 antigen, complement regulatory protein)

CDKN1B – cyclin dependent kinase inhibitor 1B

c-FLIP – CASP8 and FADD like apoptosis regulator (current symbol: CFLAR)

chr – chromosome

CLL – chronic lymphocytic leukemia

CML – Chronic Myeloid Leukemia

CMR – complete molecular response

c-Myc – MYC proto-oncogene, bHLH transcription factor

CSC – cancer stem cells

CP – chronic phase

DAS – dasatinib

DASISION – dasatinib versus imatinib study in treatment-naïve CML patients

DDP – cisplatin

DGCR8 – DGCR8 microprocessor complex subunit

Dicer – dicer 1, ribonuclease III

DNA – deoxyribonucleic acid

Dox – doxorubicin

DOX – doxycycline

Drosha – drosha ribonuclease III

DSMZ – German collection of microorganisms and cell cultures GmbH

EGFR-TKIs – epidermal growth factor receptor-targeted tyrosine kinase inhibitors

ENESTnd – evaluating nilotinib efficacy and safety in clinical trials-newly diagnosed patients

ERK – mitogen-activated protein kinase 1 (current symbol: MAPK1)

EZH2 – enhancer of zeste 2 polycomb repressive complex 2 subunit

F: female

FAS – Fas cell surface death receptor

FASL – Fas ligand

FISH – fluorescence in situ hybridization

FOXO3 – forkhead box O3

GAS5 – growth arrest-specific 5

GFP – green fluorescent protein

GIST – gastrointestinal stromal tumors

GIST48 – gastrointestinal stromal tumor cell line resistant to the imatinib therapy

GIST882 – gastrointestinal stromal tumor cell line sensitive to the imatinib therapy

H2AFX – H2A histone family member X

HCT-8 – human colorectal cancer cell line

HCT-8/5FU – 5-fluorouracil-resistant HCT-8 cell line

HL-60 – acute promyelocytic leukemia cell line

HL-60.Bcr-Abl^{+} – acute promyelocytic cell line expressing the *BCR-ABL1* oncogene

HSC – hematopoietic stem cell

hsa – *Homo sapiens*

IAP-1 – baculoviral IAP repeat containing 3 (current symbol: BIRC3)

IFN-α – interferon-alfa

IM – imatinib mesylate

IS – international scale

JAK2 – janus-kinase 2

JAK5 – janus-kinase 5

JUN – Jun proto-oncogene, AP-1 transcription factor subunit (previous symbols: JNK, SAPK)

Jurkat – human acute T cell leukemia cell line

K562 – human chronic myeloid leukemia cell line

K562S – imatinib mesylate-sensitive K562 cell line

K562R – imatinib mesylate-resistant K562 cell line

kb – kilobase

KD – kinase domain

K-RAS – KRAS proto-oncogene, GTPase

LAMA-84 – human chronic myeloid leukemia cell line

LAMA-84S – imatinib mesylate-sensitive LAMA-84 cell line

LAMA-84R – imatinib mesylate-resistant LAMA-84 cell line

LAMP1 – lysosomal associated membrane protein 1

LB – Luria-Bertani

lncRNA – long non-coding RNA

LoVo – humam colon cancer cell line

LSCs – leukemic stem cells

M: male

MAPK – Mitogen-activated protein kinase-activated protein kinases

MAP2K1 – mitogen-activated protein kinase kinase 1 (previous name: MEK)

MCF7 –human breast cancer cell line

MCL1 – MCL1 apoptosis regulator, BCL2 family member

MCR – major cytogenetic response

MEK – mitogen-activated protein kinase kinase 1 (current name: MAP2K1)

MEKK – mitogen-activated protein kinase kinase kinase 1 (current symbol: MAP3K1)

Min – minutes

miR – microRNA

miRNA – microRNA

MR – major molecular response

MR^3: major molecular response ≥ 3-log reduction from the IRIS baseline

MR^4: major molecular response ≥ 4-log reduction from the IRIS baseline

$MR^{4.5}$: major molecular response ≥ 4.5-log reduction from the IRIS baseline

MR^5: major molecular response ≥ 5-log reduction from the IRIS baseline

MPRO – myeloid hematopoietic cell line

MRD – minimal residual disease

mRNA – messenger RNA

mTOR – mechanistic target of rapamycin kinase (current symbol: MTOR)

MVs – microvesicles

MYC – MYC proto-oncogene, bHLH transcription factor

n.a.: information not available

NB4 – acute promyelocytic leukemia cell line

NGS – next generation sequencing

NSCLC – non-small cell lung cancer

NTC – negative control cells

OCT-1 – POU class 2 homeobox 1 (current symbol: POU2F1)

$p27^{kip1}$ – cyclin-dependent kinase inhibitor 1B (current symbol: CDKN1B)

PB – peripheral blood

PBMC – peripheral blood mononuclear cells

PCR – polymerase chain reaction

Ph1 – philadelphia chromosome

PI3K – phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (current symbol: PIK3CA; previous name: phosphatidylinositol 3-kinase)

PIK3R3 – phosphatidylinositol 3-kinase regulatory subunit 3

PKA – protein kinase A

PTEN – phosphatase and tensin homolog

qPCR – quantitative PCR / real time PCR

R – resistant

RAF – Raf-1 proto-oncogene, serine/threonine kinase

RAS – Ras related GTP binding proteins

RLU – relative light units

RNA – ribonucleic acid

RT – room temperature

RT – reverse transcription

S – sensitive

Sec – seconds

SEK - SAPK/ERK kinase

sgRNA – single guide RNA

SH2 – Src homology 2

shRNA – short hairpin RNA

siRNA – small interfering RNA

SKOV3 – human ovary adenocarcinoma cell line

SOX7 – SRY-box transcription factor 7

SREBF2 – sterol regulatory element binding transcription factor 2

STAT – signal transducer and activator of transcription

STAT5 – signal transducer and activator of transcription 5

TCL1A – T-cell leukemia/lymphoma-1A

THP-1 – human acute monocytic leukemia cell line

TK – tyrosine kinase

TKI – tyrosine-kinase inhibitor

U87MG – human glioblastoma astrocytoma cell line

U87MG-res – temozolomide-resistant U87MG cell line

UTR – untranslated region

70Z/3 – mouse pre-B lymphoblast cell line

LIST OF SIMBOLS

+ positive

- negative

μ – micro

α – alfa

β – beta

γ – gama

Δ – delta

TM – trademark

® – registered

°C – degrees Celsius

< less than

≤ less than or equal to

> greater than

≥ greater than or equal to

= equal

SUMMARY

Introduction

I. INTRODUCTION

I.1 Chronic Myeloid Leukemia: general features and molecular basis

Chronic Myeloid Leukemia (CML) is a myeloproliferative neoplasm with a high incidence of 1 to 2 cases per 100,000 individuals, which accounts for approximately 15% of all the newly diagnosed leukemias in adults (JABBOUR; KANTARJIAN, 2018). Slightly more frequent in men, the CML comprehends a male/female ratio of 1.2–1.7 cases. The median age at the diagnosis is usually 57 to 60 years in the general population but seems to be variable according to the studied region, comprising 66 years in the United States, 59 years in the United Kingdom, 56 years in France, and 57 years in Germany, for instance (HÖGLUND; SANDIN; SIMONSSON, 2015). In Brazil, the median age at the diagnosis is at least 10 years younger in comparison with the international statistics, ranging from 40 to 47 years old (BORTOLHEIRO; CHIATTONE, 2008; NEVES et al., 2019).

The CML arises from a disorder in hematopoietic stem cells (HSC), consisted of the Philadelphia chromosome (Ph). Characterized as the first chromosomal abnormality linked to diseases pathogenesis, the Ph chromosome results from the reciprocal translocation between long arms of chromosomes 9 and 22, the t(9;22)(q34;q11) (QUINTÁS-CARDAMA; CORTES, 2006; NOWELL; HUNGERFORD, 1960). The translocation involves the 5' end of the *Breakpoint Cluster Region* (*BCR*) gene located on chromosome (chr) 22 and the 3' end of *Abelson1* region (*ABL1*) proto-oncogene located on chromosome 9. The juxtaposition of both *BCR* and *ABL1* regions results in the *BCR-ABL1* neo-oncogene formation (Figure 1) (CHEREDA; MELO, 2015; QUINTÁS-CARDAMA; CORTES, 2006; SCORE et al., 2010).

In general, the genomic breakpoint location involves the region between exons 1b and 2 of *ABL1*, and exons 13 or 14 of *BCR* (CHEREDA; MELO, 2015; SCORE et al., 2010). The splicing of the mRNA leads to transcripts with e13a2 or e14a2 junctions (exon 13 of *BCR* and exon 2 of *ABL1* or exon 14 of *BCR* and exon 2 of *ABL1*, respectively) (Figure 2). The e13a2 and e14a2 are the most frequent transcripts of the *BCR-ABL1* gene and both are translated into a Bcr-Abl fusion protein of 210-kDa (CHEREDA; MELO, 2015; MELO, 1996).

The Bcr-Abl is a tyrosine kinase (TK) protein constitutively activated that promotes the aberrant triggering of several signaling pathways, which gives rise to the cell's leukemic transformation characterized by high cell growth and proliferation, cytokine-independent cell cycle, and apoptosis blockade. As consequence, myeloid cells accumulate in the bone marrow

(BM) and peripheral blood (PB) (CHEREDA; MELO, 2015; JABBOUR; KANTARJIAN, 2014).

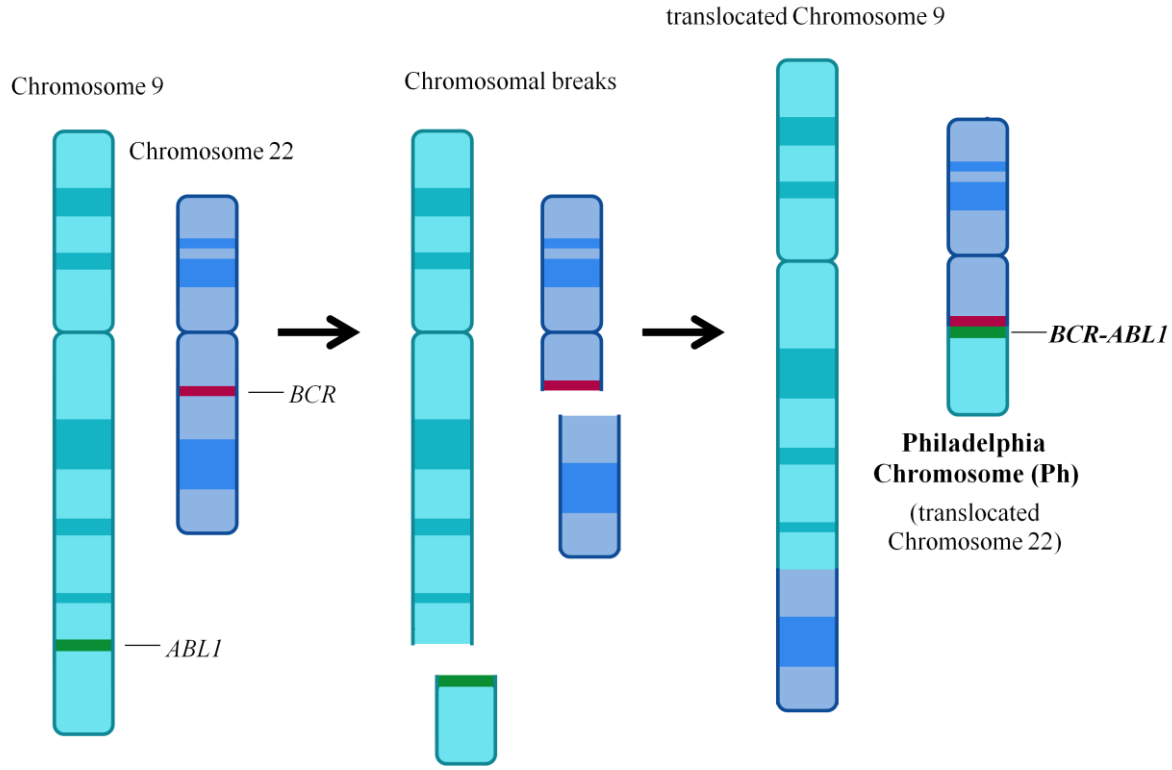

Figure 1. The t(9;22)(q34;q11) and BCR-ABL1 oncogene assembly. The fusion between the *ABL1* gene, located on chr9, and the *BCR* gene, located on chr22, gives rise to the *BCR-ABL1* oncogene. The *BCR-ABL1* encodes the protein responsible for the cell's leukemic transformation, the Bcr-Abl fusion protein. Adapted from (HAI et al., 2014).

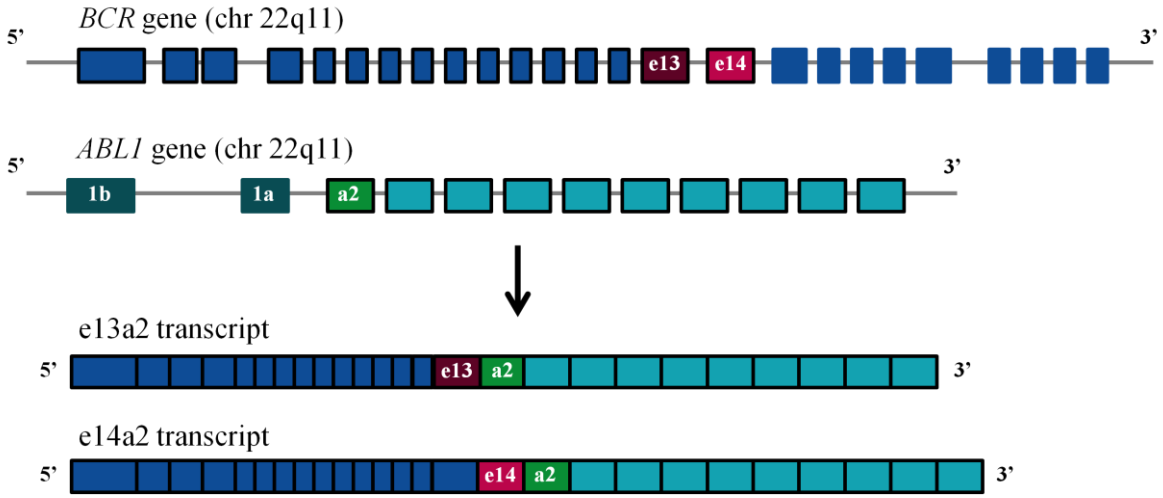

Figure 2. The e13a2 and e14a2 transcripts set-up. After chromosomal breaks, the fusion of exon e2 from BCR and exon 13 or 14 from the ABL1 region leads to e13a2 and e14a2 transcripts formation, respectively. Both of them are translated into the 210-kDa Bcr-Abl protein. Adapted from (REJALI et al., 2015).

The JAK/STAT, PI3K/AKT, RAS/MEK, JUN kinase, and MYC are the main signaling pathways affected by the Bcr-Abl tyrosine kinase activation (GOLDMAN; MELO, 2003; JABBOUR; KANTARJIAN, 2014).

The signal transducer and activator of transcription (STAT) proteins are transcription factors activated by the membrane receptor janus-kinase 2 (JAK2) and the aberrant signaling of the JAK/STAT pathway is implicated in leukemia pathogenesis (CHEREDA; MELO, 2015; LIN; MAHAJAN; FRANK, 2000). In CML, the JAK2/STAT signaling is triggered by the Bcr-Abl, contributing to cell growth and survival (WARSCH; WALZ; SEXL, 2013). Besides being regulated by the Bcr-Abl, JAK2 can also regulate the Bcr-Abl protein activity through the phosphorylation of the tyrosine-177 (SAMANTA et al., 2011). The depletion of STAT5 in impaired the development of a CML-like disease in the presence of the *BCR-ABL1* oncogene (WALZ et al., 2012). Furthermore, STAT5 can be activated directly by the Bcr-Abl, without the upstream activation of JAK2 (HANTSCHEL et al., 2012).

The phosphatidylinositol-3-kinase (PI3K) proteins transduce extracellular signals to the intracellular environment, regulating the activation of transcription factors that promote cell growth and survival (ZHAO et al., 2006). The AKT serine/threonine kinase 1 (AKT) plays a major role in the PI3K signaling since it is the first downstream effector of PI3K. In CML, the PI3K signaling is constitutively stimulated by the Bcr-Abl through adaptor proteins. The PI3K signaling is required for the malignant transformation of the hematopoietic cells and CML maintenance (CHEREDA; MELO, 2015; SKORSKI, 1997). The PI3K also stimulates the mechanistic target of rapamycin kinase (mTOR) pathway, responsible for protein synthesis and cell growth (MAYERHOFER et al., 2002).

The RAS related GTP binding proteins /mitogen-activated protein kinase (RAS/MEK) signaling is very frequently deregulated in cancer and promotes the transcription of several growth factor genes involved in cell proliferation (STEELMAN et al., 2004). The RAS/MEK signaling is activated by the Bcr-Abl and promotes cell growth in CML. The mechanism of RAS activation involves distinct adaptor proteins that complex with Bcr-Abl, which allows the link of Bcr-Abl and RAS through the recruitment of guanine nucleotide exchange factors (RAITANO; WHANG; SAWYERS, 1997). The NF-κB transcription factor is one of the most important effectors of RAS and it is required for Bcr-Abl leukemogenesis (HSIEH; VAN ETTEN, 2014).

The Jun kinase (JNK, also known by SAPK) signaling is also one of the well-characterized cascades triggered by the activation of the activation of the mitogen-activated protein kinase-activated protein kinases (MAPK). In CML, the MAPK/JNK signaling is one

of the major signaling pathways affected by the Bcr-Abl protein activity and is required for the cell's malignant transformation. In a RAS dependent manner, the Bcr-Abl protein activates the axis MEKK/SEK/JNK (serine/threonine mitogen-activated protein kinase, SAPK/ERK kinase, and Jun kinase, respectively) signaling, culminating in the activation of transcription factors (RAITANO et al., 1995; RAITANO; WHANG; SAWYERS, 1997).

By regulating gene expression and favoring cell growth and proliferation, the proto-oncogene *MYC* is one of the most implicated transcription factors in cancer (DANG, 2012). In CML, the Bcr-Abl protein controls the c-Myc expression and stability through PI3K, JAK2, and MEK proteins activation (CHEREDA; MELO, 2015). It is also well known that one of the Bcr-Abl domains, the Src Homology 2 (SH2), can enhance *MYC* expression by stimulating its promoter (RAITANO; WHANG; SAWYERS, 1997). The requirement of c-Myc for the CML malignant transformation has been demonstrated (SAWYERS; CALLAHAN; WITTE, 1992), as well as for the CML maintenance and progression to advanced stages (REAVIE et al., 2013).

As previously mentioned, the apoptosis resistance is a characteristic of the cells expressing the Bcr-Abl protein (Bcr-Abl$^+$ cells). The cell death by apoptosis is crucial for physiological processes including embryonic development, cell turnover for the tissues homeostasis and immune modulation. On the other side, the apoptosis deregulation is implicated in pathologic processes, including hematological malignancies (JACOBSON; WEIL; RAFF, 1997; KERR; WYLLIE; CURRIE, 1972).

The interaction between the cell death receptor FAS with its ligand molecule FASL is a common stimulus for apoptosis (BERGANTINI et al., 2005). In CML, the Bcr-Abl protein seems to protect cells from the FAS induced apoptosis (MCGAHON et al., 1995). The Bcr-Abl protein can also promote apoptosis resistance by inducing the expression of BCL-2 and BCL-X_L antiapoptotic proteins (SALOMONI et al., 2000). The up-regulation of antiapoptotic genes *c-FLIP*, *IAP-1* e *MCL-1* and the down-regulation of pro-apoptotic genes *BAK*, *BAX*, and *BIK* in CML patients resistant to imatinib mesylate therapy were also demonstrated (FERREIRA et al., 2015).

The PI3K and STAT5 signaling are important mediators of the Bcr-Abl anti-apoptotic action. The activation of STAT5 via Bcr-Abl phosphorylation promotes increased expression of BCL-X_L anti-apoptotic protein (DE GROOT et al., 2000; HORITA et al., 2000). The phosphorylation of the pro-apoptotic protein BAD by the PI3K/AKT pathway favors the interaction between BAD and the 14-3-3 chaperone protein, promoting the restriction of BAD into the cytoplasm (NESHAT et al., 2000). This prevents the migration of BAD to the

mitochondria and its action against BCL2 and BCL-XL anti-apoptotic effects (CHEREDA; MELO, 2015).

Furthermore, the Bcr-Abl kinase activity seems to play also a substantial role in the apoptosis resistance phenotype. The sole expression of Bcr-Abl is capable of blocking the mitochondrial permeability and consequently prevent the cytochrome-c release, which inhibits the apoptosis cascade (AMARANTE-MENDES et al., 1998b; BUENO-DA-SILVA et al., 2003; DEININGER et al., 2000).

Despite all the knowledge about CML, the mechanisms involved in disease progression and resistance to the inhibitors of the Bcr-Abl protein need to be further elucidated. Therefore, the description of new therapeutic targets and novel prognostic biomarkers beyond *BCR-ABL1* are of great importance.

I.2 Chronic Myeloid Leukemia: progression, diagnosis and treatment

The CML presents three distinct phases: chronic phase (CP), accelerated phase (AP), and blast crisis (BC) (JABBOUR; KANTARJIAN, 2018).

About 90% to 95% of CML patients are diagnosed in CP after casual blood cells count for routine exams. The CML-CP patients can remain asymptomatic for prolonged periods, but when the symptoms appear, they are related to the expansion of Bcr-Abl^{+} cells, anemia and splenomegaly. The most frequent signs and symptoms are fatigue, loss of weight, easy satiety, malaise, and left superior quadrant fullness or pain (JABBOUR; KANTARJIAN, 2018; KANTARJIAN et al., 1985).

The CP is characterized by leukocytosis with a remarkably left shift in peripheral blood (PB). The white blood cells count is usually greater than 100 x 10^9 cells/L (QUINTÁS-CARDAMA; CORTES, 2006). All the stages of myeloid maturation are observed in PB and the myelocytes are the most predominant cells in the bone marrow. The amount of immature forms, myeloblasts and promyelocytes, doesn't exceed 10% of the total cells in the bone marrow. In peripheral blood, basophilia and eosinophilia are common and platelets count can be either low or high (CRAMER et al., 1977; GORDON et al., 1987).

After 3 to 5 years from diagnosis, the CML patients frequently progress from CP to BC. The majority of patients evolve first to the accelerated phase, but about 20% of patients evolve directly to BC (JABBOUR; KANTARJIAN, 2014). The accelerated phase is characterized by blast cell count (myeloblasts and promyelocytes) between 10% and 20% in

bone marrow. The AP can be insidious or present as severe anemia, massive basophilia in PB, organ infiltration, and splenomegaly (KANTARJIAN et al., 1987).

In the blast crisis, blast cells represent more than 20% of the total cells in the bone marrow or peripheral blood. The constitutive worsening of the symptoms observed, including bleeding, fever, infections, headaches, bone pain, and arthralgias. The patients mortality is almost invariably with the median survival between three and six months (JABBOUR; KANTARJIAN, 2014, 2018; KANTARJIAN et al., 1987; QUINTÁS-CARDAMA; CORTES, 2006). Characterized as acute leukemia, the BC phenotype can be myeloid or lymphoid, but the myeloid phenotype is predominantly observed (JABBOUR; KANTARJIAN, 2018; KANTARJIAN et al., 1987).

In the advanced stages (AP and BC), the leukemic cells miss the ability of maturation, and the expansion of undifferentiated cells rather than granulocytes is the consequence. The mechanisms related to CML progression are not clear, but is largely accepted for the scientific community that a second hit is needed additionally to Bcr-Abl activity (CHEREDA; MELO, 2015).

The CML diagnosis is confirmed by the detection of the Philadelphia chromosome (the t(9;22)(q34q11)) by cytogenetic exam, or the *BCR-ABL1* detection by fluorescence in situ hybridization (FISH) or molecular studies such as the qualitative polymerase chain reaction (PCR) technique. (JABBOUR; CORTES; KANTARJIAN, 2008; JABBOUR; KANTARJIAN, 2018; KANTARJIAN et al., 2008; SCHOCH et al., 2002; WANG et al., 2016).

The Ph chromosome is usually the only cytogenetic abnormality and frequently found in 100% of the metaphases from the cytogenetics. However, 10% to 15% of patients present more chromosomal abnormalities, for instance, the trisomy of chromosome 8, additional loss of material from 22q, isochromosome 17, and double Ph. Regarding the molecular analysis, about 2% to 5% of patients have the e13a3 or e14a3 transcripts variations rather than e13a2 or e14a2, but all of them are translated into the 210 kDa Bcr-Abl (JABBOUR; KANTARJIAN, 2018).

Before the introduction of the imatinib mesylate, almost two decades ago, the drug therapy for CML was carried out with nonspecific agents like busulfan, hydroxyurea, and interferon-alfa (IFN-α) (JABBOUR; KANTARJIAN, 2014). The chemotherapy with busulfan and hydroxyurea was used as a palliative treatment to control the white blood cells count since these agents were unable to promote the disease remission and the substantial extension of the patients' survival. Moreover, the use of busulfan and, to a less extent the use of

hydroxyurea, was largely related to severe and very often lethal myelosuppression (ALLAN, 1989).

The use of IFN-α was capable of extending the patients' survival longer than the hydroxyurea and busulfan (CHRONIC MYELOID LEUKEMIA TRIALISTS' COLLABORATIVE GROUP, 1997). Major cytogenetic responses (MCR; presence of less than 35% of Ph chromosome-positive cells) and complete cytogenetic responses (CCR; absence of Ph chromosome-positive cells) were reported for up to 40% and 25% of patients treated with IFN-α, respectively (KANTARJIAN et al., 1995). The combination of IFN-α with cytarabine improved the therapy outcomes, with at least 35% of patients achieving the CCR (QUINTÁS-CARDAMA; CORTES, 2006), which was related to an extension of the patients' survival in approximately 10 years (KANTARJIAN et al., 2003). The greater success of the interferon-alfa in comparison with the use of chemotherapy has been attributed to IFN-α-induced immune modulation (MOLLDREM et al., 2000). Despite the better outcomes, IFN-α therapy was hindered due to a wide range of side effects (JABBOUR; KANTARJIAN, 2018).

Allogeneic stem cell transplantation (AlloSCT) is still the only curative intervention of CML and remains an important alternative for eligible patients. However, the inclusion criteria are very rigorous, which makes difficult the AlloSCT performing in a large percentage of the patients. To be eligible for the transplantation, the patients must be younger than 40 years old, have been diagnosed in CP for less than a year ago, have an HLA-matched sibling and demonstrate adequate organ function (HANSEN et al., 1998; JABBOUR; KANTARJIAN, 2018; QUINTÁS-CARDAMA; CORTES, 2006).

In addition, the AlloSCT is associated with a high risk of morbidity, mortality, and other complications such as graft-versus-host disease, life-threatening infections, veno-occlusive disease, and risk of secondary malignancy (BAKER et al., 2004; JABBOUR; KANTARJIAN, 2018). The transplant is also a challenge in the CML advanced stages, showing poorer outcomes in comparison with the transplantations performed in CP (QUINTÁS-CARDAMA; CORTES, 2006).

The scenario of the CML treatment changed drastically when the tyrosine-kinase inhibitors (TKI) were developed. Considered as targeted-therapy, the TKI are small molecules that interfere in the interaction of the Bcr-Abl protein with the adenosine triphosphate (ATP), resulting in the blockade of the Bcr-Abl activity and phosphorylation of the proteins involved in the signal transduction, with consequent proliferation inhibition of the malignant clones (DRUKER; LYDON, 2000; JABBOUR; KANTARJIAN, 2018). .

The imatinib mesylate (IM) was the first TKI approved by the Food and Drug Administration for the treatment of CML patients in the chronic phase (DRUKER; LYDON, 2000). The Randomized Study of Interferon and STI571 (IRIS) is a clinical trial that randomized 1,106 CML patients who received IM 400mg/day or IFN-α plus cytarabine at low-dose. After the 19 months of follow-up, the patients receiving IM showed better outcomes than the patients receiving IFN-α plus cytarabine, in which the rate of complete cytogenetic response (CCR) was 74% versus 9%, respectively. The response to IM was also durable, as demonstrated in the 8-year follow-up of the IRIS study (DEININGER et al., 2009).

Despite the quite impressive results obtained with the IM therapy, new TKI were developed to the treatment of patients who failed or were intolerant to the IM. Dasatinib (DAS) and nilotinib (NIL) are second-generation TKI, 350 times and 50 times more potent than the IM in vitro, respectively (LOMBARDO et al., 2004; O'HARE et al., 2005; TOKARSKI et al., 2006; WEISBERG et al., 2005).

The DASISION (dasatinib versus imatinib study in treatment-naïve CML patients) clinical trial compared newly diagnosed CML patients receiving IM 400 mg/day with patients receiving DAS 100 mg/day. The achievement of CCR at 12 months was more frequently in the DAS-treated patients (77%) than in the IM-treated patients (66%). The three-year follow-up demonstrated that DAS induces faster deeper responses at early time points than IM therapy (JABBOUR et al., 2014; KANTARJIAN et al., 2010). However, the use of DAS was associated with the development of pleural effusion, myelosuppression, and pulmonary hypertension in 19%, 20%, and 1-2% of the DAS-treated patients, respectively (JABBOUR; KANTARJIAN, 2018).

Similarly to DAS, NIL also shows the ability to induce a cytogenetic response in the CML patients who failed to IM therapy (JABBOUR; KANTARJIAN, 2018). The ENESTnd (evaluating nilotinib efficacy and safety in clinical trials-newly diagnosed patients) clinical trial compared patients receiving NIL (300 or 400 mg twice a day) with patients receiving IM 400 mg/day (SAGLIO et al., 2010). The major molecular response (MR) at 12 months was achieved at higher rates for the patients treated with both doses of NIL (44% and 43%) than in the IM-treated patients (22%). The follow-up of 36 months further demonstrated deep molecular responses more frequently in NIL-treated patients than IM-treated patients (LARSON et al., 2012).

Given the excellent results obtained with the use of TKI, either IM, DAS or NIL are currently recommended as first-line therapy for CML patients in the chronic phase. Thereby,

the chemotherapeutic agents and IFN-α are no longer used (JABBOUR; KANTARJIAN, 2018; O'BRIEN et al., 2013).

The therapy with TKI has promoted in CML patients a life expectancy close to the life expectancy of the general population (BOWER et al., 2016). For this reason, treatment monitoring is of great importance. Less invasive tests have been used rather than traditional bone marrow examinations. Although, the bone marrow examination is mandatory at the diagnosis for cytogenetic analysis to confirm the presence of the Ph chromosome (JABBOUR; KANTARJIAN, 2018; WANG et al., 2016).

The monitoring of the response to TKI demands cytogenetics for the Ph chromosome detection (cytogenetic response) and molecular testing for the detection of *BCR-ABL1* transcripts levels (molecular response) (BACCARANI et al., 2009). Currently, the bone marrow cytogenetics is recommended for a follow-up of 3, 6, and 12 months after starting the therapy. After this period, if the patient is responding optimally, FISH or PCR in the peripheral blood can be applied (JABBOUR; KANTARJIAN, 2018).

The major cytogenetic response is achieved when the number of Ph chromosome in cellular metaphases is < 35% after 3 months of therapy. The major molecular response (MR) is achieved when the percentage of *BCR-ABL1* transcripts are ≤ 10% after 3 months, < 1% after 6 months, and ≤ 0.1% after 12 months of therapy and at any time later. Complete cytogenetic and molecular responses (CCR and CMR) are achieved when negative values are found (JABBOUR; KANTARJIAN, 2018).

The molecular monitoring by the reverse transcriptase quantitative PCR (RT-qPCR) technique is estimated by the quantity of *BCR-ABL1* transcripts relative to an internal reference gene, most commonly ABL1, GUSB or BCR. To avoid variability between laboratories or within the same laboratory, an International Scale (IS) is used to express the results in percentage, in which the IRIS study standardized baseline value is taken as 100%. To be considered in major molecular response, the patient must show *BCR-ABL1* values lower than 0.1% of the baseline value (CROSS et al., 2015).

The precise definition of deep molecular response is important for patient management. The deep molecular response can be stratified according to the *BCR-ABL1* transcripts' levels: MR^3, MR^4, $MR^{4.5}$, and MR^5, in which the *BCR-ABL1* transcripts' levels correspond to ≥ 3-log, ≥ 4-log, ≥ 4.5-log, and ≥ 5-log reduction from the IRIS baseline, respectively (or 0.1%, 0.01%, 0.032%, and 0.001% of the baseline value, respectively) (CROSS et al., 2015).

The TKI therapy discontinuation is the most recent approach for CML patients who achieved a deep and stable major molecular response after treatment. Some studies have demonstrated that the TKI discontinuation has been successful in patients with the complete molecular response (MR^5) (MAHON, 2017; REA et al., 2017; ROSS et al., 2013). A high percentage of these patients do not present sign of disease after TKI discontinuation, but some of them quickly relapse, which has been attributed to the presence of CML quiescent stem-cells partially resistant to the TKI therapy (JABBOUR; KANTARJIAN, 2018).

Although the TKI therapy has drastically changed the CML therapy, about 25% of patients develop TKI-resistance at some point over the treatment. The resistance to targeted therapies can be primary or acquired (TALATI; PINILLA-IBARZ, 2018). The lack of response to the TKI treatment is defined as primary resistance, whereas the CML progression after an initial response to the TKI treatment is defined as acquired resistance (HOCHHAUS, 2006). The development of acquired resistance implies that the malignant cells have developed a mechanism to scape the continual inhibition of the target (LOVLY; SHAW, 2014), consisting of the Bcr-Abl protein in CML.

Regardless of being primary or acquired, the mechanisms of resistance to TKI therapy are classified into *BCR-ABL1* dependent or *BCR-ABL1* independent (PATEL; O'HARE; DEININGER, 2017) (Figure 3).

Considered as a *BCR-ABL1* dependent, mutations in the kinase domain (KD) of *BCR-ABL1* are the most prevalent mechanism of acquired imatinib resistance in patients with chronic myeloid leukemia (CML). This mechanism consists of point mutations leading to the alteration of amino acids or conformational alterations that impair the interaction between the TKI and the Bcr-Abl protein (PATEL; O'HARE; DEININGER, 2017; SHAH et al., 2002). The KD mutations are more frequent in CML patients in accelerated phase and blast crisis than in the chronic phase (BRANFORD et al., 2003).

More than 50 different mutations that confer imatinib-resistance were described (APPERLEY, 2007; O'HARE et al., 2012), but DAS and NIL are active against the majority of them, except for the T315I mutation (EIDE; O'HARE, 2015). Like DAS and NIL, a third-generation TKI, bosutinib, can be used against a wide range of mutations that cause IM-failure, but not against the T315I. Ponatinib, another third-generation TKI, is the drug of choice for patients with T315I because it's the only TKI effective against this mutation that is currently approved (JABBOUR; KANTARJIAN, 2018).

Although to a less extent, other *BCR-ABL1* dependent mechanisms of resistance were described. The increased *BCR-ABL1* expression, for instance, is a consequence of the *BCR-*

ABL1 gene amplification or the Ph chromosome duplication (BARNES et al., 2005). Drug influx/efflux pumps and drug sequestration are also associated with the development of resistance because they interfere in the intracellular drug availability. The low activity of the influx pump OCT-1 (POU class 2 homeobox 1) (WHITE et al., 2007, 2010) and the overexpression of the efflux pump P-glycoprotein are good examples (DOHSE et al., 2010).

The *BCR-ABL1* independent mechanisms of resistance in CML represent 40% of the clinical failure to the TKI therapy (WAGLE et al., 2016). These mechanisms are associated with alternative survival pathways that operate even in the context of the Bcr-Abl inhibition by the TKI treatment. The survival promoted by these alternative pathways can be intrinsic to the leukemic cells or provided by microenvironmental factors of the bone marrow niche (EIRING et al., 2015; PATEL; O'HARE; DEININGER, 2017). The STAT3, PI3K, and RAF/MEK/ERK pathways, for instance, have been demonstrated as contributors for the Bcr-Abl$^+$ cells resistance to the TKI therapy. The STAT3, particularly, seems to contribute for the leukemic cells survival via intrinsic signaling and via bone marrow stromal cells modulation (BEWRY et al., 2008; PATEL; O'HARE; DEININGER, 2017; TRAER et al., 2012).

The persistence of leukemic stem cells (LSCs) has emerged as an important *BCR-ABL1* independent mechanism of resistance. The CML-LSCs are found in the bone marrow in a quiescent state and have been described as the cause of the minimal residual disease (MRD) during the TKI treatment, which can lead to the TKI-resistance overtime and relapse upon the therapy discontinuation, even in patients with the disease undetectable (LOSCOCCO et al., 2019).

It is unclear whether CML-LSCs result from the persistence of a pre-existing population in bone marrow, or if they emerge as a result of the TKI therapeutic selection process (GIUSTACCHINI et al., 2017). Either way, it is well-characterized that TKI therapy is unable of eliminating the CML-LSCs, and CML-LSCs activity is regulated by alternative aberrant pathways in a Bcr-Abl independent way (NEVIANI et al., 2013).

The epigenetic mechanisms have been notably associated with the function of the CML-LSCs. The EZH2, a histone methyltransferase, was reported overexpressed in CML-LSCs, which was associated with the protection of LSCs from apoptosis and the establishment of TKI-resistance (SCOTT et al., 2016; XIE et al., 2016). The SIRT1 deacetylase was found overexpressed in CML CD34$^+$ cells and this enzyme seems to be important for the CML-LSCs survival as well as for the acquisition of genetic mutations in CML cells that promote the TKI-resistance (LI et al., 2012; WANG et al., 2013).

Therefore, given the relevance of the *BCR-ABL1* independent mechanisms of resistance in CML, it is of great importance to further investigate the mechanisms underlying this process. The new knowledge may contribute to the design of new therapies capable of circumvent the TKI resistance and eliminate the CML-LSCs (JABBOUR; KANTARJIAN, 2018). In this context, we decide to investigate the relationship between microRNAs and TKI-resistance in CML.

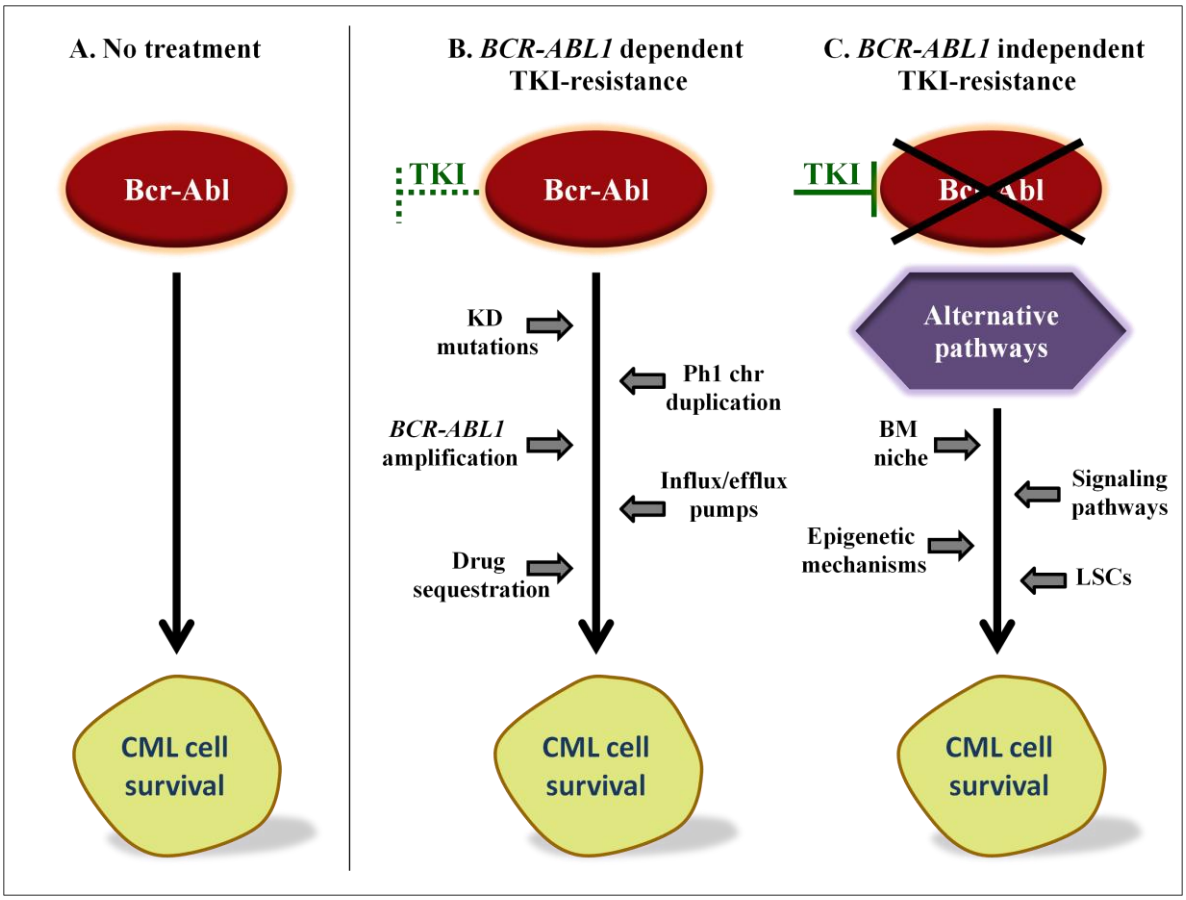

Figure 3. *BCR-ABL1* dependent and independent mechanisms of resistance. A. *BCR-ABL1* signaling promoting the CML leukemogenesis**. B.** *BCR-ABL1* signaling promoting the CML leukemogenesis**. B.** The *BCR-ABL1* dependent mechanisms of resistance either prevent the TKI from binding to the Bcr-Abl protein or interfere in the intracellular TKI availability. The *BCR-ABL1* dependent mechanisms of resistance are the Bcr-Abl kinase domain (KD) mutations, Ph chromosome duplication, *BCR-ABL1 gene* amplification, influx/efflux pumps deregulation and high drug sequestration. **C.** The *BCR-ABL1* independent mechanisms are associated with alternative survival pathways that operate even in the context of the Bcr-Abl inhibition by the TKI treatment, including mediators from bone marrow (BM) niche, signaling pathways, epigenetic mechanisms, and the persistence of leukemic stem cells (LSCs). Adapted from (PATEL; O'HARE; DEININGER, 2017).

I.3 MicroRNAs: biogenesis, functions and the role in cancer and drug-resistance

MicroRNAs (miR or miRNA) are small endogenous non-coding RNAs composed of 20 to 22 nucleotides. Characterized as an epigenetic mechanism at the post-transcriptional level, the microRNA's function is preventing the translation of messenger RNAs (mRNA) into proteins. (KIM; NAM, 2006; YAO; CHEN; ZHOU, 2019). The first description about microRNAs was done in *Caenorhabditis elegans* model, in 1993. Currently, it is known that the human genome has approximately 2,000 mature microRNAs genes (LITWIŃSKA; MACHALIŃSKI, 2017).

The majority of the microRNAs genes are located in intergenic regions of the genome. However, about 30% of the microRNAs genes are located in the introns of protein-coding genes (DI LEVA; GAROFALO; CROCE, 2014). The microRNAs' biogenesis starts with the microRNA gene transcription by polymerase II. The transcript, termed as primary microRNA (pri-miRNA), has a stem-loop structure with a 5'cap and poly(A) tail. The pri-miRNA is cleaved by the nuclear complex composed by the RNA-ribonuclease Drosha and its binding protein, DGCR8. The cleavage removes the 5'cap and poly(A) tail originating the precursor microRNA (pre-miRNA), composed of approximately 60 to 100 nucleotides (KIM; NAM, 2006; YAO; CHEN; ZHOU, 2019).

The pre-miRNA is transported from the nucleus to the cytoplasm by the exportin-5 transporter protein. Then, the pre-miRNA is processed by the RNase III endonuclease Dicer, originating double-strand microRNA composed of 18 to 25 nucleotides. The duplex is incorporated into the RNA-induced silencing complex (RISC). During the RISC assembly, one strand of the duplex is degraded and the other strand remains as a mature microRNA, which will guide RISC to the target mRNA (KIM; NAM, 2006; YAO; CHEN; ZHOU, 2019) (Figure 4).

Once in the RISC, the microRNA binds to complementary sequences in the 3'UTR of the mRNA. The perfect microRNA-mRNA complementarity promotes the mRNA degradation by argonaute proteins (AGO2) that are incorporated into the RISC. On the other hand, the partial microRNA-mRNA pairing promotes the repression of the mRNA translation (DI STEFANO et al., 2016; LITWIŃSKA; MACHALIŃSKI, 2017). Because of their role in the gene expression via translational impairment, the microRNAs are involved in the regulation of important cellular processes such as development, differentiation, proliferation, cell fate, and apoptosis (CALIN; CROCE, 2006; LITWIŃSKA; MACHALIŃSKI, 2017).

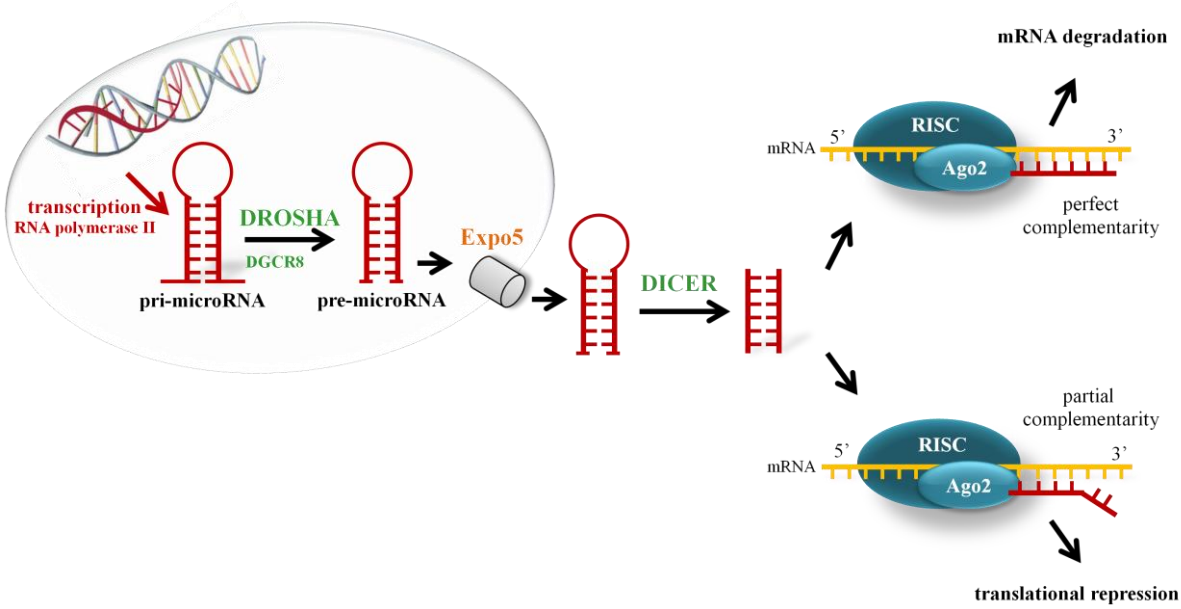

Figure 4. microRNA biogenesis. The pri-microRNA is transcribed by RNA polymerase II. The processing by Drosha and its binding protein, DGCR8, gives rise to the pre-microRNA. Transported from the nucleus to the cytoplasm by exportin-5, the pre-microRNA is processed by Dicer. Then the RNA duplex is conjugated with the RNA-induced silencing complex (RISC), where one of the strands is degraded and the other strand remains as mature microRNA, which will guide RISC to the mRNA target. The mRNA-microRNA perfect complementarity leads to mRNA degradation, whereas the mRNA-microRNA partial complementarity promotes translational repression. Adapted from (RYAN; JOILIN; WILLIAMS, 2015).

A single microRNA may present complementarity to as many as 200 gene targets. It has been estimated that at least 10% to 40% of the human mRNAs are target for microRNAs. Thus, the microRNAs potentially control about one-third of the human transcriptome (ESQUELA-KERSCHER; SLACK, 2006; TSAI; YU, 2010).

The role of microRNAs in biological processes can be illustrated in the hematopoiesis. The miR-181 seems to favor the hematopoietic differentiation of stem cells into B-lymphoid cells in mice (CHEN et al., 2004), whereas miR-155 and miR-451 are involved in the human erythropoiesis and are crucial for the normal erythroid differentiation (MASAKI et al., 2007). The microRNAs can also participate in apoptosis by regulating anti-apoptotic or pro-apoptotic genes and contributing to this cellular process regulation. The anti-apoptotic *MCL-1* gene, for instance, is regulated by the miR-29 and miR-101, and the pro-apoptotic gene *PTEN* is regulated by miR-21 (SUBRAMANIAN; STEER, 2010).

Despite being considered an epigenetic mechanism, the microRNA expression is also under epigenetic regulation. The hypermethylation of microRNAs' promoter CpG islands was already described for the miR-424, miR-196b-5p, and miR-10 (HE et al., 2015; KIGA et al.,

2014; SHAO et al., 2018). Similarly, the histones acetylation affecting the miR-124 expression was also reported (LIU et al., 2016).

In compliance with their important function of biological processes, the deregulated expression of microRNAs has been associated with a wide range of pathological conditions, especially cancer (CALIN; CROCE, 2006; LITWIŃSKA; MACHALIŃSKI, 2017). In general, the microRNAs down-regulation is associated with a tumor-suppressor role while, the microRNAs overexpression is associated with an oncogenic role by inhibiting the expression of tumor-suppressor genes (SVORONOS; ENGELMAN; SLACK, 2016; ZHANG et al., 2007).

In leukemia, the deregulation of microRNAs expression is associated with disease pathogenesis and progression. A range of microRNAs act as tumor-suppressor (for example miR-15, miR-16, let-7, and miR-127), or oncogene (for example miR-155, miR-17-92, miR-21, miR-125b, miR-93, miR-142-3p, miR-196b, and miR-223) (MARDANI et al., 2019).

The tumor-suppressor role of miR-15a and miR-16 was described in chronic lymphocytic leukemia (CLL) by the regulation of the anti-apoptotic gene *BCL-2*. The levels of miR-15a and miR-16 were reduced in CLL and negatively correlated with *BCL-2* levels (CALIN et al., 2004; CIMMINO et al., 2005). On the other hand, in acute lymphoblastic leukemia (ALL), the miR-142-3p shows an oncogenic function by down-regulating the tumor-suppressor protein kinase A (PKA) and consequently excluding the inhibitory effect of PKA over proliferation (LV et al., 2012).

In CML, miR-29b and miR-320a down-regulate the *BCR-ABL1* expression and for this reason they were considered as tumor-suppressors. These microRNAs also decrease the CML cell-lines invasion and proliferation. Additionally, the low levels of miR-320a were associated with CML poor prognosis (LI et al., 2013; XISHAN et al., 2015). In contrast, it was also described that *BCR-ABL1* up-regulates the miR-17-92, a well-characterized oncogenic cluster, facilitating the disease progression (LITWIŃSKA; MACHALIŃSKI, 2017; VENTURINI et al., 2007).

In addition, the miR-17-92 is regulated by the proto-oncogene *MYC*, which cooperates with the *BCR-ABL1* for the cells' malignant transformation. The miR-17-92 expression is up-regulated in CML $CD34^+$ cells from patients at chronic phase, but not in patients at blast crisis. Thus, a potential contribution of the *BCR-ABL1*-MYC-miR-17-92 pathway was suggested for the CML initiation (VENTURINI et al., 2007).

The microRNAs also contribute for drug-resistance in several types of cancer, including gastric cancer, lung-cancer, breast cancer, colorectal carcinoma, gastrointestinal

stromal cancer, ovarian cancer and prostate cancer (CAO et al., 2012; HUANG et al., 2018; MA et al., 2011; ROSCIGNO et al., 2017; VECCHIONE et al., 2013; XU et al., 2012; ZHANG et al., 2017). The overexpression of mR-125b, for instance, is capable of inducing resistance to Daunorubicin in different leukemia cells lines, K562, THP-1 and Jurkat cells, by reducing apoptosis (ZHOU et al., 2014).

In CML, San José-Enériz et al. reported the microRNAs differential expression between CML patients who achieved remission and IM-resistant patients. Eighteen microRNAs were found down-regulated in the IM-resistant patients (miR-7, miR-23a, miR-26a, miR-29a, miR-29c, miR-30b, miR-30c, miR-100, miR-126, miR-134, miR-141, miR-183, miR-196b, hmiR-199a, miR-224, miR-326, miR-422b, and miR-520a), whereas only one microRNA, miR-191, was found up-regulated (SAN JOSÉ-ENÉRIZ et al., 2009).

In the Bcr-Abl^{+} cell line K562, a microarray analysis demonstrated that miR-221, miR-379, miR-548, miR-603, and miR-648 are associated with NIL sensitivity (YOU et al., 2013). Another microarray analysis performed with samples of IM-resistant and IM-responder CML patients, detected the miR-181c down-regulated in the IM-resistant patients (MOSAKHANI et al., 2013). Additionally, it was recently proposed that the differential expression of the miR-29 cluster, miR-23, and miR-451 can be used to discriminate imatinib responders and non-responders CML patients (DULUCQ et al., 2008).

Most recently, the possibility of microRNAs introduction as diagnostic, prognostic and therapeutic biomarkers in leukemia has been considered. Different types of leukemia show different microRNAs' profile, which can be helpful to the differential diagnosis, previous assessment of relapse and prediction of the overall survival (MARDANI et al., 2019; MI et al., 2007). The identification of microRNAs in body fluids has strengthened, and the circulating microRNAs may emerge as a new class of effective biomarkers (WANG et al., 2014b).

The TKI-resistance is still a challenge in the CML therapy scenario. The mechanisms involved in the LSCs persistence after TKI therapy and the *BCR-ABL1* independent mechanism of resistance to TKI require further investigation. The microRNAs are important regulators of biological cellular processes and their role in the pathogenesis of several types of cancer (including leukemia), as well as in the development of drug-resistance, was already demonstrated. Thus, in the present study, we hypothesized that microRNAs can be one of the mechanisms contributing to the IM-resistance in Bcr-Abl^{+} cells and that the inhibition of specific microRNAs is capable of sensitizing these cells to IM therapy.

Conclusions

VI. CONCLUSIONS

Taken together, our results show that the inhibition of miR-125a-5p, miR-125b, miR-132, miR-23a, miR-24-3p, miR-155-5p, miR-222-3p, and miR-342-3p is not sufficient to substantially promote the sensitivity of LAMA-84R cells to the IM treatment. However, more investigation is necessary in order to describe potential microRNAs targets and its involvement in the cell cycle, proliferation and apoptosis.

In Bcr-Abl^{+} cells, the microRNAs let-7e, miR-181a, miR-484, miR-616, and miR-96 were associated with IM resistance, as shown by the phenotypic screen. Currently, these results are being validated using CRISPR-Cas9 gene-editing technology. This will contribute to elucidate the mechanisms involved in CML resistance to the TKI imatinib mesylate and to describe new therapeutic targets independent of the Bcr-Abl oncoprotein.

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Attachments

VIII. ATTACHMENTS

Attachment I. Approval letter from the School of Pharmaceutical Sciences of Ribeirão Preto – USP

UNIVERSIDADE DE SÃO PAULO
Faculdade de Ciências Farmacêuticas de Ribeirão Preto
Comitê de Ética em Pesquisa

Of. CEP/FCFRP nº. 029/2016
kms

Ribeirão Preto, 14 de setembro de 2016.

À Pós-graduanda
Maria Gabriela Berzoti Coelho
Orientadora: Profª. Drª. Fabíola Attié de Castro
FCFRP/USP

Prezada Pesquisadora,

Informamos que o projeto de pesquisa intitulado "**EXPRESSÃO DE MICRORNAS RELACIONADOS À VIA HIPPO/LATS EM LEUCEMIA MIELÓIDE CRÔNICA**", apresentado por Vossa Senhoria a este Comitê, Protocolo **CEP/FCFRP nº. 398 – CAAE nº 52501415.0.0000.5403,** foi aprovado pelo do Comitê de Ética em Pesquisa da FCFRP/USP em sua 155ª reunião ordinária realizada em 12 de agosto de 2016.

Lembramos que, de acordo com a Resolução 466/2012, item IV.5, letra d, o TCLE deverá "ser elaborado em duas vias, rubricadas em todas as suas páginas e assinadas, ao seu término, pelo convidado a participar da pesquisa, ou por seu representante legal, assim como pelo pesquisador responsável, ou pela(s) pessoa(s) por ele delegada(s), devendo as páginas de assinaturas estar na mesma folha. Em ambas as vias deverão constar o endereço e contato telefônico ou outro, dos responsáveis pela pesquisa e do CEP local".

Informamos que deverá ser encaminhado ao CEP o relatório final da pesquisa em formulário próprio deste Comitê, bem como comunicada qualquer alteração, intercorrência ou interrupção do mesmo, tais como eventos adversos e eventuais modificações no protocolo ou nos membros da equipe, através da interposição de emenda na Plataforma Brasil.

Atenciosamente,

PROFª. DRª. CLENI MARA MARZOCCHI MACHADO
Coordenadora do CEP/FCFRP

Avenida do Café S/Nº - Monte Alegre – CEP 14040-903 – Ribeirão Preto – SP
Comitê de Ética em Pesquisa – cep@fcfrp.usp.br
Fone: (16) 3315-4213 ou 3315-4216 – Fax: (16) 3315-4892

Attachment II. Approval letter from the Ethics Committee of the Clinical Hospital of School of Medicine of Ribeirão Preto – USP

HOSPITAL DAS CLÍNICAS DA FACULDADE DE MEDICINA
DE RIBEIRÃO PRETO DA UNIVERSIDADE DE SÃO PAULO

FMRP-USP
RIBEIRÃO PRETO

Ribeirão Preto, 26 de agosto de 2016

Projeto de pesquisa: "Expressão de MicroRNAs relacionados à via HIPPO/LATS em Leucemia Mielóide Crônica"

Pesquisador responsável: Maria Gabriela Berzoti Coelho e Profª Drª Fabíola Attié de Castro

Instituição Proponente: Faculdade de Ciências Farmacêuticas de Ribeirão Preto – USP

"O CEP do HC e da FMRP-USP concorda com o parecer ético emitido pelo CEP da Instituição Proponente, que cumpre as Resoluções Éticas Brasileiras, em especial a Resolução CNS 466/12. Diante disso, o HCFMRP-USP, como instituição co-participante do referido projeto de pesquisa, está ciente de suas co-responsabilidades e de seu compromisso no resguardo da segurança e bem-estar dos sujeitos desta pesquisa, dispondo de infra-estrutura necessária para a garantia de tal segurança e bem-estar".

Ciente e de acordo:

Drª Marcia Guimarães Villanova
Coordenadora do Comitê de Ética em Pesquisa - HCFMRP-USP

Prof. Dr. Eduardo Barbosa Coelho
Coordenador Técnico Científico da Unidade de Pesquisa Clínica – HCFMRP-USP

Campus Universitário – Monte Alegre
14048-900 Ribeirão Preto SP

Comitê de Ética em Pesquisa do HCRP e FMRP-USP
FWA-00002733; IRB-00002186 e
Registro Plataforma Brasil/CONEP nº 5440
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Attachment III. Informed consent form for patients' enrollment in the study

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO
(Participante de Pesquisa: Paciente)

Nome do Paciente: _____Idade:_____
Responsável Legal (se necessários):_____
RG do paciente:_____

Título do Projeto de pesquisa: "Expressão de microRNAs relacionados à via HiPPO/LATS em Leucemia Mielóide Crônica".
Responsável Clínico pelo Projeto: Profa. Dra. Belinda Pinto Simões, Docente de Hematologia da FMRP-USP e responsável pelo ambulatório de Hematologia.
Pesquisadoras responsáveis:
Doutoranda: Maria Gabriela Berzoti Coelho (telefone: 016-981665909)
Profa. Dra. Fabíola Attié de Castro - Departamento de Análises Clínicas, Toxicológicas e Bromatológicas da FCFRP-USP (telefone: 016-33150657 e 016-981779222).

Prezado (a),

Convidamos você a participar de nossa pesquisa.

A Leucemia Mielóide Crônica leva a um aumento do número de células brancas e vermelhas do sangue. Esse aumento de células deixa o sangue mais espesso (grosso, viscoso), de modo que esse passa a circular nas veias do nosso corpo com maior dificuldade, prejudicando sua função adequada. As causas do aparecimento desta doença são desconhecidas. Por isso, essa pesquisa quer estudar quais são as alterações nas células do sangue que causam esse aumento do número de células e que fazem com que algumas pessoas desenvolvam essa doença.

Você foi admitido no Hospital das Clínicas de Ribeirão Preto para tratamento da Leucemia Mielóide Crônica e, por esse motivo, o convidamos para participar de nossa pesquisa, pois o maior conhecimento sobre essa doença poderá ajudar os pesquisadores a desenvolver melhores formas de tratamento, beneficiando, no futuro, pessoas que também possam estar doentes.

Caso você aceite, você doará 30 ml de sangue (o que corresponde a duas colheres de sopa) que será colhido das veias do seu braço. As células do sangue serão usadas para estudar alguns de seus componentes chamados de proteínas e genes, que controlam a vida e o aumento dessas células. A colheita de seu sangue será realizada por profissionais de saúde com experiência, como enfermeiros, biomédicos ou farmacêuticos e com material descartável para que não haja riscos de contaminação. Os riscos dessa colheita serão mínimos, existindo uma pequena chance de você sentir um pouco de dor durante o procedimento, de aparecer inchaços roxos ou apenas inchaços em seu braço no local da colheita de sangue.

Você tem a liberdade de aceitar ou não que sua amostra seja empregada nesse estudo, sem que lhe cause qualquer prejuízo ou punição durante o seu tratamento no Hospital das Clínicas. Somente os resultados da pesquisa serão divulgados, sem que seu nome apareça, ele ficará em segredo. As amostras e componentes de seu sangue serão identificados por números codificados e somente as pesquisadoras terão acesso. Se você quiser, podemos lhe informar sobre os resultados das pesquisas sempre que desejar, tendo a garantia de receber a resposta a qualquer pergunta ou esclarecimento de quaisquer dúvidas acerca dos procedimentos, riscos, benefícios e outros relacionados com a pesquisa.

Você não terá nenhuma despesa a mais por participar dessa pesquisa e você não será pago por doar o sangue para a pesquisa. Sua doação será voluntária. No momento, a pesquisa não lhe trará nenhum benefício, mas poderá trazer benefícios no futuro, para outras pessoas que também tenham essa doença.

Caso aceite participar desse estudo e em algum momento quiser desistir, você também terá total liberdade para isso. Você poderá recorrer às leis vigentes no Brasil para indenização de qualquer dano que lhe seja causado pelo estudo. Para a sua segurança, você receberá segunda via desse termo.

Eu, _____, RG: _____, declaro que concordo em ser voluntário e autorizo a retirada de 30mL do meu sangue para a realização da pesquisa. Fui devidamente informado em detalhes pelos responsáveis do projeto sobre os meus direitos, que o volume de sangue retirado não causará danos, que esta pesquisa ajudará a entender o que causa o aumento do número de células do sangue nos pacientes com Leucemia Mielóide Crônica e que terei minha identidade mantida em sigilo.

_____

Assinatura do doador

Ribeirão Preto, _____de _____de _____.

Eu, Maria Gabriela Berzoti Coelho, RG: 46.280.524-4, declaro que tudo o que foi exposto anteriormente é verdade e será devidamente cumprido.
Nome do pesquisador responsável pelo estudo: Maria Gabriela Berzoti Coelho.

_____

Assinatura do pesquisador

Ribeirão Preto,_____de _____de _____.

Se o paciente não puder assinar:

Nome do representante:_____
Assinatura:_____
Ribeirão Preto, _____de _____de _____.

Informações importantes:
Telefone do Comitê de ética da FCFRP-USP: Fone: (16) 3315-4213. Fax: (16) 3602-4892

Endereço: FCFRP-USP, Av. do Café, s/n – Campus Universitário – Monte Alegre – Ribeirão Preto, CEP: 14049-903

Telefone do Comitê de ética do Hospital das Clínicas de Ribeirão Preto: Fone: (16) 3315-2228. Fax: (16) 3633-1144

Endereço: HCFMRP-USP, Av. Bandeirantes, 3.900 - Campus Universitário - Monte Alegre – Ribeirão Preto, CEP: 14.048-900.

Attachment IV. Informed consent form for peripheral blood samples' storage in the biorepository

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO
Armazenamento de Amostras Biológicas

O(a) Senhor(a) está sendo convidado a doar uma amostra de sangue periférico e/ou medula óssea para futuras pesquisas científicas. A cada nova pesquisa com esse material, o (a) Senhor (a) será procurado para receber explicações e autorizar ou não a utilização da sua amostra já armazenada.

Caso concorde, o(a) Senhor(a) doará uma amostra de sangue periférico ou de medula óssea. Esta sua amostra e os seus dados coletados serão identificados por números sequenciais (ex: SPP113), de modo que garanta seu sigilo. Após coletada, a amostra será guardada por 10 anos em um BIORREPOSITÓRIO (banco de amostras biológicas), intitulado "Biorrepositório do Laboratório de Hematologia da FCFRP", localizado no laboratório de hematologia, sala multiusuários 46A-M, segundo andar do bloco M da Faculdade de Ciências Farmacêuticas USP-RP em condições adequeadas de armazenamento, sob responsabilidade da Profª. Drª Fabíola Attié de Castro. Ao final deste tempo de armazenamento, a amostra será jogada fora.

O(a) Senhor(a) poderá desistir de participar da pesquisa a qualquer momento. Para isso, basta entrar em contato comigo nos telefones citados no final deste documento, para que eu possa suspender o armazenamento da sua amostra, que poderá ser entregue ao/à Senhor(a), se assim desejar, ou jogada fora após seu consentimento por escrito.

Caso não concorde em doar sua amostra ou desista de permitir que ela seja guardada, essa decisão não lhe trará qualquer penalização ou prejuízo **do atendimento que lhe é prestado no Hospital das Clínicas**.

A coleta deste material biológico nesse momento não trará nenhum benefício imediato para o(a) Senhor(a). Entretanto, no futuro, os dados obtidos com novas pesquisas poderão ajudar outras pessoas.

Coloco-me à disposição para lhe fornecer o resultado da pesquisa realizada com sua amostra.

Declaro que toda nova pesquisa, utilizando sua amostra biológica, será realizada somente quando o projeto for aprovado no Comitê de Ética em Pesquisa da FCFRP-USP.

_____

Prof.ª Drª. Fabiola Attié de Castro
(16)3602-4163/ (16)98177-9222
gabrielaberzoti@usp.br

Consentimento do Sujeito de Pesquisa

Eu,_____, RG:_____, residente na Rua _____ (número, bairro, cidade, telefone / celular), aceito que minha amostra biológica de sangue (DNA, cDNA, RNA) seja armazenada pela Prof.ª Drª. Fabiola Attié de Castro na Faculdade de Ciências Farmacêuticas de Ribeirão Preto da USP para fins de pesquisa científica.
Declaro estar ciente de que a previsão de guarda do material é de no máximo 10 anos.
Ribeirão Preto, _____ de_____ de _____.

_____

Assinatura do Doador ou responsável legal